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VALIDATION OF THE GAS CHROMATOGRAPHIC METHOD FOR THC, CBD AND CBN DETERMINATION

Master`s thesis

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Abbreviations

THC	Δ^9-tetrahydrocannabinol
CBD	Cannabidiol
CBN	Cannabinol
THCA	Δ^9-tetrahydrocannabinolcarboxylic acid
GC	Gas chromatography
TP	Temperature program
MS	Mass spectrometry
SIM	Selected ion monitoring
FID	Flame ionisation detector
ECN	Effective carbon number
LC	Liquid chromatography
HPLC	High-performance liquid chromatography
UV(DAD)	Ultraviolet diode array detector
SPME	Solid phase micro extraction
PDMS	Polydimethylsiloxane
TLC	Thin layer chromatography
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
QC	Quality control sample
EFSI	Estonian Forensic Science Institute
EU	European Union
ARIB	Estonian Agricultural Registers and Information Board
NMR	Nuclear Magnetic Resonance
IRMS	Stable isotope ratio-mass spectrometry
CRM	Certified reference material
LoD	Limit of detection
LoQ	Limit of quantification

1. Introduction

The aim of this work is to validate the analytical method of quantifying cannabinoids (THC, CBD and CBN) using the GC-FID system. The method was to be accredited and will be used daily in the chemistry department of the Estonian Forensic Science Institute`s.

THC, CBD and CBN are three out of 70 cannabinoids unique to cannabis. As THC is the main psychoactive agent in cannabis plant, it is carefully monitored.

In Estonia as in most European Union countries it is legal to grow cannabis in which the THC concentration does not exceed 0.2%. Determining the THC concentration is one of the routine analyses in EFSI.

Cannabis is mainly used in two ways – agricultural cannabis, which is grown for its fibre, energy, seeds and oil; and illegal cannabis, which is used as narcotic substance or the source of narcotic substances. In EFSI both kind of cannabis samples must have been analysed. 0.2% of THC is the level critical for both sample groups.

In illegal cannabis and its products the THC concentration reaches up to almost 30% whereas the THC concentration in agricultural cannabis is well below the limit.

During the validation procedure many problems and discussions have cropped up. It is known that THC is not a stable substance; hence it is not widely used as a reference standard for calibration. Instead, CBD or CBN can be used since their molecule construction and effective carbon numbers are similar to that of THC. Also the preparation and storage of the stable QCs are problematic because of the instability of the THC.

Another bottleneck of the method is related to the matrices of the samples. Cannabis contains over 400 substances and the matrix may vary considerably. It is almost impossible to get cannabis samples containing no THC, CBN or CBD for the selectivity estimation.

During the validation process several analyses and calculations were done to test the suitability of CBD for calibration graph of THC and CBN, to evaluate the linearity of the calibration graph, to estimate the limit of detection, limit of quantification, reproducibility, repeatability, selectivity, trueness and finally the uncertainty.

This work consists of two main parts. The first, theoretical part gives a short overview of cannabis, legislation related to cannabis and cannabinoids and also of the chemical methods that are used for analysing cannabinoids. The second part focuses on the particular GC-FID method and to the analyses used to achieve the validation.

This thesis would not have come about without the help of many wonderful persons whom I would like to thank from the bottom of my heart: first of all my teachers from the University of Tartu, specially the head of the Institute of Chemistry Mr Ivo Leito and my supervisors Ms Anneli Kruve from University of Tartu and Mr Peep Rausberg from EFSI. Also I would like to thank the board of EFSI for the help and support, my colleagues from the EFSI chemistry department, my friends, my family and my course mates.

2. Literature overview

2.1 Cannabis plant and Δ^9-tetrahydocannabinol

2.1.1 Cannabis plant and its products

Cannabis plant is considered to be a monospecific (*Cannabis sativa* L.) which is divided into several subspecies (*C. sativa* subsp. *sativa*, *C. sativa* subsp. *indica*, *C. sativa* subsp. *ruderalis*, *C. sativa* subsp. *spontanea*, *C. sativa* subsp. *kafiristanca*). [1]

The scientific classification of cannabis plant [2]:

Kingdom: *Plantae*

Phylum: *Angiospermae*

Family: *Cannabaceae*

Genus: *Cannabis L.*

Species: *C. sativa*

Cannabis is an annual or biennial herbaceous plant growing usually 90 to 500 cm high. It is a dioecious anemophil. The leaves are long, thin and finger-like with cogged edge. The flower heads comprise of small green flowers. Cannabis originates from Middle-Asia, but it also grows in Europe. Nowadays cannabis is cultivated on the large areas with the mild and tropical climate for the cannabis oil and fibre. In Middle-Asia and Africa, especially in Morocco, cannabis is cultivated as a narcotic substance or a source of narcotic substances like hashish, cannabis oil etc. [3]

There are four main illegal cannabis products known. Dried leaves and flowers of the cannabis plant are known as “marijuana”. The fruiting and flowering tops and leaves next to the flowering tops contain the highest amount of Δ^9-tetrahydrocannabinol (THC), but illicitly consumed herbal cannabis also includes bigger leaves located at greater distance from the flowering tops. Seeds can also be present and sometimes marijuana can be compressed into hard blocks to reduce volume for transport. The resinous secretions of the plant produced in the glandular trichomes can be collected, thus obtaining a higher THC-containing product from which most recognizable plant material is removed – cannabis resin (hashish). It appears as loose or compressed sticky brown powder, depending on the method of production. The material is usually compressed into hard blocks. Liquid cannabis (hashish oil) is a concentrated liquid extract of either herbal cannabis material or of cannabis resin. The reason for producing liquid cannabis is to concentrate the psychoactive ingredient. Cannabis seeds are potent source

of Ω-3-fatty-acids and their oil is a clear yellow liquid. Seeds contain approximately 29 to 34% oil by weight. The essential oil of cannabis is a clear and slightly yellow-coloured liquid. It is obtained by steam distillation of the freshly cut cannabis plants. It is rather a side product from seed oil or hashish oil production. [1;4]

Cannabis products are by far the most abused drugs on the illicit drug market. Production of herbal cannabis (marijuana) is widely spread, existing in almost every country in the world. Cannabis resin (hashish) is produced in about 65 countries, with main sources being North Africa and countries in South-West Asia, particularly Afghanistan and Pakistan. [1]

Morocco, the largest known cannabis cultivation area, is also the leading producer of cannabis resin. Most of the herbal cannabis is produced for domestic markets and for export to neighbouring countries. [1]

Limited time-series data on cannabis potency suggest that the mean THC concentration in home-produced herbal cannabis seizures increased from the 1.5% in the 1980s to around 4% in the late 1990s and around 10% in the last five years. Recent reports from some European countries suggest mean THC concentrations of up to 15% to 20% in certain herbal materials, but there is significant variation between samples even within a given year [1]. Analyses carried out in Estonian Forensic Science Institute (EFSI) during the years 2005 to 2010 indicates, that the concentration of THC is between 0.030% and 28% [17].

Industrial cannabis is grown for their seeds and fibres. Industrial cannabis is characterised by low THC content and high cannabidiol (CBD) content. [8]

2.1.2 Cannabinoids and Δ^9-tetrahydrocannabinol

According to various references cannabis plant contains more than 400 compounds of which about 60 to 70 are called cannabinoids [3;9;10].

Cannabinoids are a group of terpenophenolic compounds unique to cannabis. The highest cannabinoid concentrations are found in the resin secreted by the plant`s flowering buds. [11;12]

Original cannabinoids seem to be cannabinoid acids that are formed in the plant but are later decarboxylated (possibly in part in the plant itself) to yield the better known neutral cannabinoids. [6]

Most of the major terpenoids were not isolated until the end of the 19th century or even much later, and in many cases their purity was doubtful. The reason is that alkaloids are relatively easy to separate and crystallized as salts, whereas terpenoids are usually present in mixtures

whose separation is tedious and was in many cases impossible with the techniques available to the chemists 100 years ago. [6]

Five different numbering systems have been used for cannabinoids. Nowadays 70 cannabinoids are known and they may be classified as follows: cannabigerol (CBG) type (7 known); cannabichromene (CBC) type (5 known); cannabidiol (CBD) type (7 known); (-)-Δ^9-trans-tetrahydrocannabinol (Δ^9-THC) type (9 known); (-)-Δ^8-trans-tetrahydrocannabinol (Δ^8-THC) type (2 known); cannabicyclol (CBL) type (3 known); cannabielsoin (CBE) type (5 known); cannainol (CBN) type (7 known); cannabinodiol (CBND) type (2 known); cannabitriol (CBT) type (9 known) and miscellaneous types (14 known). [10]

Cannabinol (CBN) represents the first natural cannabinoid to be obtained in pure form. It was isolated and named by W. R. Dunstan, T. A. Henry (1898) and T. B. Wood (1899) from high boiling, viscous oil first obtained by a group in Cambridge at the turn of the 19th century. [6]

CBN does not exist in freshly and carefully dried marihuana. If it is present, the sample is understood to have started to degrade. It is feasible to estimate the age of given marihuana sample on the basis of its THC and CBN content, assuming storage was carried out at room temperature. THC appears to degrade at a higher rate for the first year than for subsequent years. [1]

The first isolation in a pure form of a psychoactive cannabis principle, THC, was reported finally in 1964. A hexane extract of hashish was separated into acidic and neutral fractions. Repeated chromatography of the neutral fraction on Florisil, acid-washed alumina, and alumina containing 12% silver nitrate eluted the following compounds (in order of increasing polarity): a mixture of waxy, non cannabinoid materials, cannabicyclol, CBD, THC, CBN, cannabichromene, cannabigerol, and polar constituents and polymers. CBD had already been obtained in the early 1940`s, but its structure and stereochemistry were determined only in 1963. The structure of THC as well as the structure of CBD was elucidated mainly on the basis of the then novel nuclear magnetic resonance (NMR) method. The final proof of the structure was made by the conversion of CBD into THC by a mild acid treatment. The absolute configuration of THC was established in 1967. [6]

The absolute configuration of THC was determined to be *trans*-(6aR, 10aR) by comparison with D-(+)-glyceraldehyde and (-)-CBD. Nine THC-type cannabinoids are known; although it is not certain if the C_4- and C_1-acids are the A and/or B acids. (Table 1) [10]

Table 1. Δ^9-trans-THC-type cannabinoids [10]

Compound	R^1	R^2	R^3
Tetrahydrocannabinolic acid A (Δ^9-THCA-C_5 A)	COOH	n-C_5H_{11}	H
Tetrahydrocannabinolic acid B (Δ^9-THCA-C_5 B)	H	n-C_5H_{11}	COOH
Tetrahydrocannabinol (Δ^9-THC-C_5)	H	n-C_5H_{11}	H
Tetrahydrocannabinolic acid-C_4 (Δ^9-THCA-C_4 A and/or B)	COOH or H	n-C_4H_9	H or COOH
Tetrahydrocannabinol-C_4 (Δ^9-THC-C_4)	H	n-C_4H_9	H
Tetrahydrocannabivarinic acid A (Δ^9-THCVA-C_3 A)	COOH	n-C_3H_7	H
Tetrahydrocannabivarin (Δ^9-THCV-C_3)	H	n-C_3H_7	H
Tetrahydrocannabiorcolic acid (Δ^9-THCOA-C_1 A and/or B)	COOH or H	CH_3	H or COOH
Tetrahydrocannabiorcol (Δ^9-THCO-C_1)	H	CH_3	H

Besides free THC the cannabis plant material mainly contains Δ^9-tetrahydrocannabinolcarboxylic acid (THCA). THCA is the most important precursor acid, which converts into the psychotropically active THC when heated. It occurs by a simple decarboxylation when cannabis products are smoked. [13;14]

THC is the main psychoactive agent in cannabis. The activity of THC was initially established by evaluation in rhesus monkeys. Since then thousands of papers have been published on the activity of THC, in vitro and in vivo, including human trials. But considerable evidence has emerged suggesting that the effects of cannabis are not solely due to THC, CBD was found to cause pharmacological effects. Some researches show that CBD and possibly other cannabis components achieve synergy with THC causing potentiation of benefits, antagonism of adverse effects, summation, pharmacokinetic advantages, and metabolism. [6;9;15]

As THC is thermo-labile and photo-labile, the storage of cannabis leads to a cumulative decrease in THC content through oxidation of THC to CBN. [9]

Besides cannabinoids the following chemical classes (number known) has been identified in marijuana (the crude drug derived from the cannabis plant): nitrogenous compounds (27), amino acids (18), proteins (3), enzymes (6), glycoproteins (2), sugars and related compounds (34), hydrocarbons (50), simple alcohols (7), simple aldehyds (12), simple ketones (13), simple acids (20), fatty acids (23), simple esters (12), lactones (1), steroids (11), terpenes (120), non-cannabinoid phenols (25), flavonoids (23), vitamins (1), pigments (2), elements (9). [10]

2.1.3 Legal regulations related to cannabis and Δ^9-tetrahydrocannabinol

In terms of analytical approach, it is a choice whether THCA and THC are measured separately or whether "total-THC" (i.e. the combined amount of THC and THCA) is measured. This

choice is sometimes made by national legislation. If there are no legal requirements for either approach (e.g. in Estonia), it is common practice to measure total-THC as it represents the pharmacological activity of the substance in the best way. [1] Henceforth in this work the total-THC is meant by THC content.

In Estonia, as well as in other European countries, the limit of THC concentration in cannabis plant and its products is set on the level 0.2% [5;16]. If THC concentration is higher than 0.2% of the dried material, the cannabis product is considered a narcotic substance, whereas cannabis with less than 0.2% THC is considered agricultural cannabis, growing and possession of which is legal.

There are more than fifty different strains of cannabis listed in the European Union (EU) directive that are legal to grow in the EU and also in Estonia. Estonian Agricultural Registers and Information Board (ARIB) is the government agency that coordinates the EU agricultural support payments in Estonia and carries out routine checking and sampling of the agricultural cannabis plants. [18; 19]

Sampling of the cannabis plants is also regulated in the EU directives. The psychoactive potency of hemp cultivars is expressed in the THC content of a sample prepared by collecting the upper part of cannabis plants, drying and removing stems and seeds, and grinding. [8;16]

2.2 Analytical methods of determining and quantifying Δ^9-tetrahydrocannabinol

Two main methods are most commonly used in laboratories to determine the THC concentration – liquid chromatography (LC) and gas chromatography (GC) with different detectors. Depending on the goal of the analysis, both of these methods have advantages as well as faults.

According to the EU legislation, the GC with flame ionisation detector (FID) is the recommended method for determining the THC concentration in the agricultural cannabis samples. [20]

2.2.1 Liquid chromatography

LC is the unrivalled method when it comes to analytes sensitive to temperature. Usually the temperature is higher than 150^{o}C in the injectors of GC. At this temperature cannabinoid acids will convert into free cannabinoids as they are thermo-sensitive compounds [13]. If it is critical

to determine the concentration of cannabinoid acids such as CBD-acids and THC-acids in addition to that of cannabinoids, the LC method is most commonly used.

When high-performance liquid chromatography (HPLC) is used for total THC content determination, it must be kept in mind that the result can be too low. Because using HPLC no decarboxylation, as with the GC method by the high temperatures of the injector and column, occurs. [13]

In all the researched references the C18 LC-columns were used. Of detectors, the ion-trap-mass-spectrometry [9] and ultraviolet diode array detector (UV(DAD)) [13;21] seemed to give good results. Several studies demonstrate the feasibility of LC with mass spectrometry (MS) and LC-MS-MS for the determination of cannabinoids in biological fluids, but the use of LC-MS for the determination of cannabinoids in cannabis products can be very effective as well. It combines the advantages of LC-UV(DAD) and GC-MS [9]. LC method has been used in a large number of studies [9;13;21].

2.2.2. Gas chromatography-mass spectrometry

GC is used quite often for cannabinoid separation. Different temperature programs and injector temperatures (will be disserted later) have also been used. However, as stated in the previous section, if the goal is to determine the concentration of carboxyl acids as well, the derivatisation process is necessary for its higher temperatures.

For quantification with mass spectrometry (MS) detector, the two or three chosen characteristic mass fragments were monitored in the selected ion monitoring (SIM) mode; m/z 382, 367, 310 [11], 314, 299 [8], 299, 231, 314 [22] for THC; m/z 458, 390, 337 [11], 314, 231 [8], 231, 246, 314 [22] for CBD; m/z 382, 367, 310 [11], 295, 296, 310 [22] for CBN have been used.

2.2.3 Other methods

There is also a number of thin layer chromatography (TLC) methods for the qualitative and semi-quantitative analysis of cannabis, which use a variety of different stationary phases (TLC plates) and solvent systems as well as slightly different sample preparation and spot visualisation techniques. [1]

Stable isotope ratio-mass spectrometry (IRMS) has been used for sourcing the geographical origin of plant materials. As cannabis is not chemically processed for illicit supply, it maintains its original elemental and isotopic profiles, which can be used as an indication of geographic origin. [1]

2.3 Gas chromatography with flame ionisation detector for quantification of Δ^9-tetrahydrocannabinol

As the GC analysis decarboxylates THCA and produces the total THC content of a cannabis sample, the usage of GC-FID systems is widely used. [1] According to references [13], it must be noted that when the THC content is measured using only the GC, the results are smaller, for the decarboxylation process is not complete. The most precise results are achieved when the THCA and THC concentrations are measured separately (using HPLC) and then summed up. [13]

2.3.1 Gas chromatography

GC is a separation method in which the components of a sample partition between two phases. One phase is a stationary bed with a large surface area, and the other is the gas that passes through the stationary bed. The sample is vaporised and carried by the mobile gas (the carrier gas) through the column. Samples partition into the stationary liquid phase by their solubility at a given temperature. The components of the sample separate from one another on the basis of their relative vapour pressure and affinity to the stationary bed. [23]

Classically, qualitative analysis with gas chromatography involves the comparison of retention data (retention time) of an unknown sample with that of a known one. Retention time is the time from the injection of the sample component until the recording of the peak maximum. [24]

2.3.1.1 Temperature program

The column temperature should be high enough for the sample components to pass through it at a reasonable speed. It need not be higher than the boiling point of the sample, but at higher temperatures the retention time decreases and the time of the analysis will shorten. [23]

The most common aim of using temperature program (TP) is to shorten the time of an analysis. The trade-off of such time-saving is that it takes longer to cool the oven down to starting conditions prior to the next injection. A little-used advantage of TP is the optimised separation of closely eluting compounds. [24]

2.3.1.2 Parameters for gas chromatograph for Δ^9-tetrahydrocannabinol quantification

As there are many manufacturers, who produce the analytical equipment, there exists a variety of non-polar columns used to analyse cannabinoids. For example, there are HP-5MS with stationary phase 5%phenyl-95%methylsiloxane, DB-5MS with stationary phase phenyl arylene polymer and HP Ultra-1 with stationary phase 100% cross-linked methylsiloxane by Agilent

Technologies; BP5 with stationary phase 5%phenyl-95%dimethylpolysiloxane by SGE. The parameters of the columns used for cannabinoid analysis are the following: length is from 15 m to 30 m, diameter is from 0.25 mm to 0.35 mm and the thickness of the stationary phase ranges from 0.25 to 0.32 μm. TPs that have been used vary on a rather large scale – the initial temperature can be from 100^{o} C up to 230^{o} C and final temperature from 280^{o} C to 300^{o} C. The properties of the column must be taken into account when setting the TP because different columns have different maximum temperature tolerance. For example, lower temperatures must be used with Agilent columns (except HP-Ultra-1 with maximum working temperature over 300^{o} C), SGE columns are suitable when higher temperatures are required. In the mobile phase, helium and nitrogen are commonly used for their flow rate from 0.76 ml/min to 2 ml/min. This parameter, too, depends highly on the purpose of the specific analyseis. [8;13;25;26;27;28]

2.3.1.3 Split/splitless inlets

The most popular capillary-column inlet is the split/splitless inlet. It can be used in a split-mode to reduce the amount of sample reaching the column and to produce very narrow initial bandwidths. It can also be used in a splitless-mode to maximise sensitivity. Split inlets are vaporising inlets – the sample, vaporised in the inlet, flows down the liner and is split between the column and the split vent. [24]

Split ratio is an important parameter to notice and record when using a split inlet. It is the ratio of the split vent flow to the column flow. For example, split ratio 100:1 means that for every sample injected, 100 parts are vented and one part enters the column. Split ratio can be changed, measured and documented. High split ratio is appropriate for analyses of major components and when using small-bore capillary columns. [24]

Split ratios from 25:1 to 50:1 are mentioned in the references 8, 13 and 26 for cannabinoid analyses.

2.3.1.4 On-column inlet

The cool on-column inlet is a capillary column inlet that allows direct deposition of liquid sample into the column. [24]

The solvent containing the sample is introduced to the retention gap (a piece of a deactivated, uncoated capillary column) at a temperature below the solvent's boiling point. The liquid spreads to the retention gap, forming a flooded zone with the solutes distributed throughout the sample layer. The solvent starts evaporating at the rear end of the flooded zone. Other volatile compounds also evaporate, but they are trapped again in the liquid layer ahead. Less volatile

compounds do not evaporate but spread out over the surface of the retention gap. As the last portion of the solvent has evaporated, the solutes start the chromatographic process when the oven heats. [29]

As the sample is deposited directly to the column without prior evaporation, the cool on-column inlets have the highest reproducibility and lowest discrimination and decomposition of any inlet. The entire sample is deposited into the column with cool on-column injection, due to which the analytical sensitivity is very high and detection limits are at least as good as with splitless injection, if not better. [24]

Since the condensed sample is injected into the column, cool on-column injection can suffer from solvent overload, peak splitting, premature degradation of the stationary phase, and contamination from non-volatile sample components. [24]

2.3.2 Flame ionisation detector and effective carbon number

FID is the most widely used GC detector. The column effluent is burned in a small oxygen-hydrogen flame producing some ions in the process. These ions are collected and form a small current that becomes the signal. The FID is a specific property-type detector with characteristically high sensitivity. The FID responds to all organic compounds that burn in oxygen-hydrogen flame. The FID is mass flow sensitive. Its response (peak area) for a compound does not change with minor changes in carrier flow like those in temperature-programmed operation. The units for its response factors are coulombs per gram of carbon. The signal is approximately proportional to the carbon content, giving rise to the so-called “equal per carbon” rule. All hydrocarbons should exhibit the same response per carbon atom. However, in the presence of heteroatoms like oxygen and nitrogen the factor decreases. Relative response values are often tabulated as “effective carbon numbers (ECN)”. [23;24]

The concept of ECN was introduced to estimate the relative response for any compound. Particular groups of atoms are given a value relative to a reference material, usually n-paraffin, for which the ECN is simply its carbon number. The set of parameters used to calculate the ECN is given in Table 2. One obvious use of ECN is in determining the relative response factors for compounds that cannot be secured in sufficient purity for experimental determination. [24]

Table 2: Contribution of various types of atoms to the FID response (expressed as ECN).

Atom	Type	ECN contribution [31]	ECN contribution [25]
C	Aliphatic	1.00	1.0
C	Aromatic	1.00	1.0
C	Olefinic	0.95	0.95
O	Ether	-0.78	-1.0
O	Esters	-1.27	-0.25
O	Ketones	-0.80	-
O	Alcohols and phenols	-0.64	-0.60
O	Secondary alcohols	-	-0.75
O	Tertiary alcohols	-	-0.25
N	Amine	-0.58	Similar to O in corresponding alcohols
S	IN methylthio ether	0	-

2.3.3 Indirect reference standards

Various difficulties arose when attempting to acquire certified reference materials for all the analytes, e.g. the compound is not available at all, its purity is not guaranteed with a certificate, the reference compound available for purchase is highly diluted (typically 1mg/ml) and its stability is questionable. Those difficulties led to questioning whether and why the reference compound should always consist of the compound to be determined as is customary practice or even the *de facto* standard. In theory, however, another compound could also serve as "indirect" reference standard provided that the relation between the analyte and the indirect reference is well defined and stable. Such indirect approach has been described in drug analysis where scopolamine is used as an indirect reference standard for the determination of cocaine. Another example is of THC determination. For over a decade, German state forensic laboratories have used CBN instead of THC as the reference standard because variations in their THC reference solutions urged them to look for a more reliable reference standard. Based on the similarity of the structures, a response ratio 1:1 was assumed. [21] It has also been stated that both cannabinoids CBN and CBD can be used as the reference standard for the determination of the THC due to their structural relationship. [26] Some of the THC`s, CBN`s and CBD`s ECNs and response ratios are given in the tables 3, 4 and 5.

The prerequisite for the successful use of an indirect reference standard in gas-chromatography is the fixed ratio between the flame ionisation detector responses of two compounds.

The GC-FID systems are mostly calibrated with CBN [1;13], the properties of CBD are also acceptable for use in the calibration procedure.

Table 3: Effective carbon numbers of cannabinoids according to 2 different methods. [26]

Compound	ECN (1)	ECN (2)
THC	19.15	19.48
CBD	19.30	19.52
CBN	19.25	19.58

Table 4: Calculated response ratios for cannabinoids, according to theoretical concepts. [26]

Response ratio	With method 1	With method 2
THC/CBN	0.982	0.982
THC/CBD	0.992	0.998
CBN/CBD	1.010	1.016

Table 5: Response ratios of cannabinoids reported in literature. [26]

Reference	THC/CBD	THC/CBN	CBN/CBD
1	0.95	0.97	0.98
2	0.89	1.11	0.81
3	0.98	1.08	0.91

2.3.4 Internal standard method

Standardisation procedure involves two important steps – chromatographic peak measurement and quantitative analysis in order to convert the size of the peak into a measure of the quantity of a particular material of interest. In some fashion this involves chromatographing the known amounts of materials and measuring their peak size. Depending on the technique used, the composition of the unknown is determined by relating the unknown peaks to the known amounts through peak size. [23]

The internal standard method does not require precise or consistent sample volumes for response factors since the latter is built into the method. The standard chosen for this method can never be a component in a sample and it cannot overlap any sample peaks. Prior to any chemical derivatisation or other reactions, a known amount of this standard is added to each sample in approximately the same concentration as the analyte of interest. The calibration curve is made from three or more calibration mixtures of pure samples of the analyte. [23]

The weight of an analyte in the solution (W_A) and then the concentration of the analyte (C%) can be calculated using following formulas:

$$W_A = \frac{A_A}{A_{ST}} \times \frac{1}{R} \times W_{ST} \qquad \text{and} \qquad C\% = \frac{W_A}{W_S} \times 100\%,$$

where the weights of sample (W_S) and internal standard (W_{ST}), also slope of the calibration graph (R) are known and the peak areas of standard (A_{ST}) and analyte (A_A) have been detected. [24]

2.4 Sample preparation

Majority of descriptions of the extraction procedures in cannabinoids analysis are quite simple. For example, a solution, which may contain an internal standard, is added directly to the solid sample and the mixture is processed with the ultrasound for 10 min to 30 min. Finally the sample is centrifuged. [8;11;13;21]

With derivatisation the sample preparation procedure can be much more time consuming as several extractions must be carried out. The solid sample is first processed with an internal standard solution and then extracted several times with different organic dissolving agents. Finally, the organic layer can be evaporated to dryness and derivatised with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), for example. MSTFA is an effective trimethylsilyl donor. It reacts to replace labile hydrogens on a wide range of polar compounds with a -$Si(CH_3)_3$ group. Therefore, it is used to prepare volatile and thermally stable derivatives for GC-MS. [11;34]

The choice of dissolving agent may become an issue in the process of refining a method. Cannabinoids dissolve easily in most organic solvents. Methanol, petroleum ether, n-hexane, toluene, chloroform, ethyl acetate and solvent combinations such as methanol/chloroform (9:1) are equally suitable for their extraction. A range of solvents have been assessed in order to determine which one extracts the most cannabinoids and other compounds, i.e. is best suited for proofing. Ethyl acetate and n-hexane were found to extract the most compounds. It should, however, be noted that non-polar solvents such as n-hexane and petroleum ether give a relatively clean extract, but only extract the neutral/free cannabinoids quantitatively, while other solvents and their combinations give quantitatively extractions of the cannabinoid acids as well. It must be kept in mind, that only the minimum content of cannabinoids is determined – the recovery is not 100% because of the not complete decarboxylation process or the dissolving power of the solvent. Recoveries from 84,2% to 86,2% for THC, 80,5% to 83,7% for CBD and 80,2% to 83,3% for CBN have been detected when extracting cannabis grass samples with methanol. [1;8;11;12;13;21]

The amounts of sample and volumes of the solvent used in cannabinoid analysis varies from 50 mg to 100 mg of sample in 2 ml of solvent up to 200 mg of sample and 20 ml of solvent. [8;13;21]

Solid phase micro extraction (SPME) is a solvent-free sample preparation technique using fibres, which are, for example, coated with 30 μm of polydimethylsiloxane (PDMS). The latter can be used for the sampling and analysis of volatile chemical markers in the headspace over solutions, directly over the suspected material, or it can be used for the analysis of aqueous solutions containing the target analytes. For cannabis products, especially for the liquid matrices (mostly in analyses of hemp food products like hemp beer and hemp oil), the SPME analyses of both, the volatile constituents and the cannabinoids, have been reported. Headspace-SPME has also been performed in hemp food using alkaline hydrolysis (NaOH) and on-fibre derivatisation (MSTFA) followed by GC-MS detection. This method provides the same reproducibility, sensitivity and robust for the analysis of the THC, CBN and CBD. Compared to the liquid-liquid extraction, it is substantially faster. [1;14;22]

2.5 Validation

2.5.1 Validation parameters

All methods used for routine analyses have to be validated in order to prove that the method is fit for the purpose. In the process of validation accuracy, precision, linearity range, limit of detection (LoD) and limit of quantification (LoQ) are usually determined. For total validation specificity, ruggedness, robustness, stability of samples, reagents, instruments and system suitability criteria have to be included as well.

The accuracy criterion is defined as closeness of the measured value to the “true value”. Accuracy is usually presented and determined as recovery. Recovery describes the efficiency of extracting the analyte from the sample.

Precision can be measured by means of repeatability, intermediate precision and/or reproducibility. Repeatability is the precision of the method under the same operating conditions over a short period of time. Intermediate precision is the agreement of complete measurements when the same method is applied many times within the same laboratory. Reproducibility is precision between laboratories and is often determined in collaborative studies or method transfer experiments.

The linearity of a method is the measure of how well a calibration plot (response vs. concentration) approximates a straight line. The data at several concentrations is processed using linear least squares regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity. The working range of a method is defined as the lowest and highest concentrations for which the analytical method has adequate

accuracy, precision and where the change in concentration produces adequate change of signal intensity.

The limit of detection is the smallest level of the analyte that gives a measurable response. It is recommended that the signal to noise ratio for the analyte concentration at the limit of detection should be at least 3.

The limit of quantification is the smallest concentration of the analyte giving a response that can be accurately quantified. It is recommended that the signal to noise ratio higher than 10 should be used as the limit of quantification. [31] It can also say that the limit of quantification is the lowest calibration point in the calibration graph. [32]

2.5.2 Quality control system

The quality control system is established as a means to control errors and generate reproducible results for laboratory analyses. There are various quality control checks designed for this purpose and they are implemented in various stages of analysis: blank samples for discovering contaminations, control samples that are fortified with known levels of target compounds, etc. Periodically collected data arranged in chronological order and expressed in graphs – control charts – are an extremely useful tools for the evaluation of method proficiency for analytes, verification of results obtained for method quality control indicators, and the identification of trends or biases that may indicate potential problems with the analysis. [24]

Quality control samples (QC) containing selections of cannabinoids or cannabinoid acids of a known amount can be used to determine repeatability, reproducibility and accuracy of the method. The QCs can be prepared using cannabis or certified reference materials (CRM). The QCs can be solutions, for example in methanol, or can be plant material like hop pellets with cannabis or cannabinoids. [9]

The storage of the QCs is critical, because the content of the sample must not change in time. The QCs containing cannabinoids can be stored at less than -18º C [9] to -20º C [11] for 5 years maximum. [9] For a short period of time (less than 4 weeks) the samples can also be stored at room temperature. [9]

3. Experimental

3.1 Instruments and reagents

As certified reference material the cannabinoid reference standards with Lipomed certificates were used – CBN (99.65% pure), CBD (99.337% pure) and THC solution in ethanol at concentration 1.0 mg/ml (with purity 98.517%). All other chemicals must have the purity level "for analyses" or higher: tetracosane (Merck, Germany) as internal standard; heptane (Merck, Germany) for extraction and for internal standard solution; toluene (Merck, Germany) and ethyl acetate (Merck, Germany) for syringe wash solutions in autosampler; ethanol for test samples during the validation.

In GC helium with purity 6.0 was used as the mobile phase. Hydrogen with purity 4.5 and nitrogen (make up gas) with purity 5.0 together with compressed air made by Zero Air Generator (Agilent 5182-0807) were used in FID.

For reference standard solutions and internal standard solution preparation the class A volumetric flasks with volume 5 ml, 10 ml and 1000 ml were used, also digital pipettes with different adjustable volumes (Thermo Scientific Finnpipette, USA) and glass vials (Agilent Technologies, USA) with stoppers.

In sample preparation, mortar and sieve were used for sample homogenisation. Plastic tubes (10 mm x 130 mm) with stoppers, digital pipettes with different adjustable volumes (Thermo Scientific Finnpipette, USA), Pestaur pipettes, syringe filters (Phenex RC, 0.45μm) for pre-injection filtration and glass vials (Agilent Technologies, USA) with stoppers were used for sample solution preparation.

For all kinds of weighing a digital scale with 0.01 mg accuracy (Sartorius BP 211D) was used. Ultrasonic bath (Bransonic, USA) and centrifuge (Jouan BB VVV, USA) were used for sample extraction and sedimentation.

3.2 Gas chromatography with flame ionisation detector

Samples were analysed using GC Agilent 6890N with split/splitless injector, FID, autosampler 7683 (for 100 samples) with 10.0 ml syringe, capillary column HP-5 (5% phenyl-methylsiloxane) 15 m long and 0.25 mm in diameter and phase thickness 0.25 μm (Agilent nr 19091J-431). The acquired data was reprocessed using Agilent ChemStation version A.10.01 software.

3.3 The methods

3.3.1 Parameters for gas chromatograph

Auto sampler parameters: injection volume 1.00 µl, preinjection solvent A (toluene) and solvent B (ethyl acetate) were used for washing 3 times; post injection solvent A and solvent B were used for washing 2 times.

Inlet parameters: injector temperature 250º C; split mode (split ratio 50:1; split flow 49.6 ml/min); pressure 7.75 psi; total flow 53.8 ml/min.

Column TP: initial temperature 80º C for 1.00 minute, first ramp (rate 30º C/min, final temperature 230º C for 0.00 min), second ramp (rate 10º C/min, final temperature 280º C for 2.00 min). Total run time is 13 min.

Detector parameters: temperature 250º C, hydrogen flow 40.0 ml/min; air flow 450 ml/min.

Compared to the method previously used in our laboratory, the TP was changed. On-capillary type of injection was used now.

Cooling down the column oven takes longer due to large temperature difference between initial and final temperatures. Hence, the entire time for one sample between the injections of the samples is now longer, reaching up to around 20 minutes.

3.3.2 Sample preparation

Upon the delivery of cannabis plants or its parts to the laboratory, the sample must be first dried and then homogenised manually using pestle and mortar. Agricultural cannabis samples from the ARIB are already homogenised before they arrive to the laboratory.

20 mg to 40 mg of homogenised sample was weighed into a plastic tube with a stopper. 1.00 ml to 3.00 ml of internal standard solution at concentration 0.50 mg/ml was added with digital pipette and the solution was sonicated for 30 minutes. Then the plastic tube with sample solution was centrifuged for 5 min (2500 rot/min) and the supernatant was transferred into the vial. If the supernatant is not clear enough it must be filtered with syringe filter.

4. Results and discussion

4.1 Chromatographic separation and memory effect

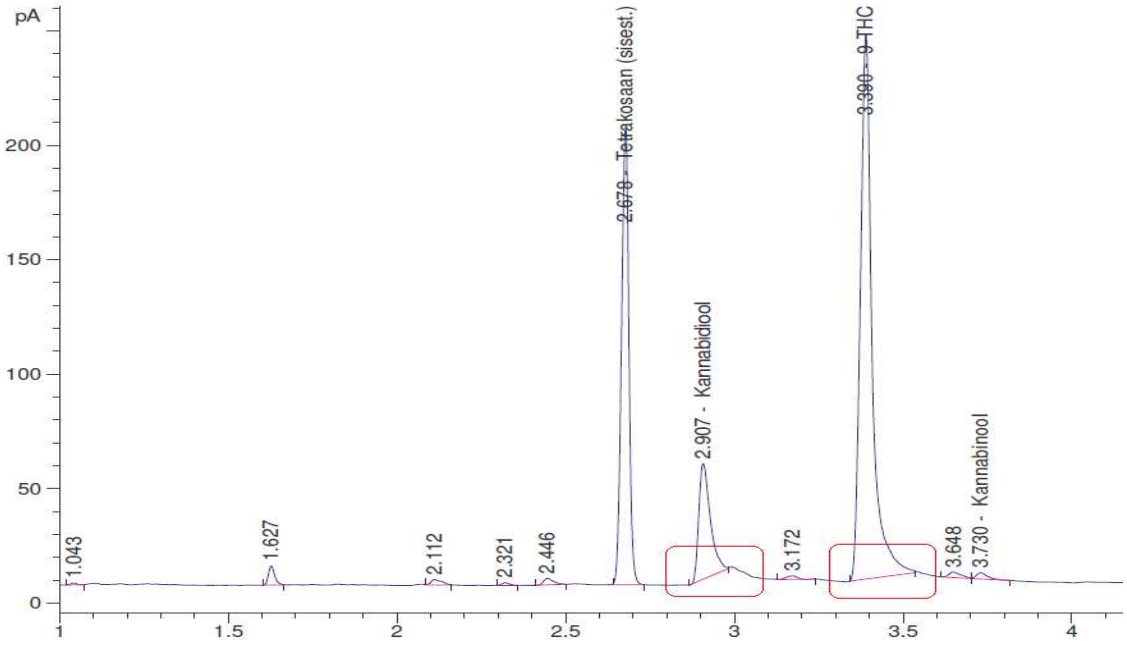
The previous GC-FID method of quantifying THC, CBN and CBD was created in EFSI in spring 2006. It was developed mainly for monitoring illegal cannabis and confirming the low THC concentrations (less than 0.2%) in agricultural cannabis samples. This method has many weaknesses, which are mainly related to the validation process: the calibration graph was developed using only two points and the quality control system was deficient. Also, the uncertainty estimation was too general and mostly based on the data given in literature. The process of accrediting methods was intensified with the effect of the new law of measurement. To achieve accreditation, the method had to be improved and validated properly.

The chromatograms achieved with the gas chromatographic parameters of the old method weren't acceptable mostly because of the shape of peaks. On-column injection type was tested with the main intention to increase the tailing of the peaks. Retention gap was not used in our GC system. As retention gap helps to limit many disadvantages such as solvent overload, peak splitting and contamination of the column, we acknowledged the possibility of such occurrences in our case. The cool on-column injection needs different injection equipment like small diameter needles to deposit the sample directly to the column without being evaporated first, there was a question whether we could use this technique without any particular equipment. As the same instrument is also used for other analyses and installing new equipment is time consuming and expensive, we decided to use the same split/splitless injector where the sample is evaporated first and then condensed into the beginning of the analytical column. Since in our case the analytes are not sensitive to temperature evaporation does not affect the results. Moreover, higher temperature facilitates the THCA decarboxylation process.

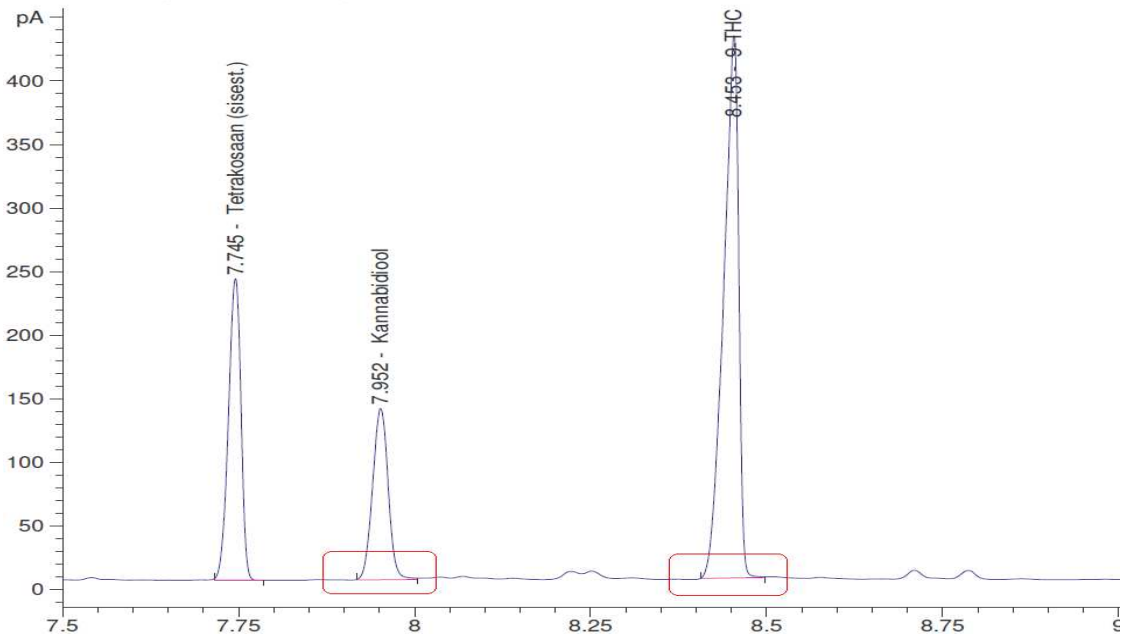
In the old method the initial temperature of the column was 230º C for 1.00 min after which the temperature was raised by 10.00º C/min to 270º C for 4 min. The total run time was 9.00 min. Now the column temperature was lowered below the solvent's boiling point (boiling point of heptane is 98.42º C). The oven temperature was programmed to start at 80º C. New TP was tested several times with different samples and on every occasion the shapes of the peaks were better than these of the old method (Figures 1 A and B).

Figure 1

A: chromatogram received with the old temperature program



B: chromatogram received with the new temperature program



Blank injections after the injections of standard solutions and sample solutions indicated another problem – the THC peaks appeared on those chromatograms, too. Several blank sample chromatograms were reviewed and it was discovered, that the average hight of the THC peak in

a blank injection was 3% of that of the previous injection. The analysis of two different THC concentrations, 17% and 3.4%, showed that chromatograms of the blank samples after the analyses contained THC peaks with the according areas of 2.1% and 1.3% of the original THC peak. According to references, the memory effect of the on-column injection method can be 0.5% to 3% [33]. When THC concentration in samples is high, this memory effect can be evaluated as a minor systematic error. When QCs are continuously analysed together with the normal samples, the deviation of results of the first sample in the sequence and those in between the samples is included to the uncertainty budget of the results.

Reduction of the memory effect of samples with high THC concentration was tested separately. A sample containing 17% of THC was analysed. The THC peak area received was 537 units. The first blank sample following the analysis showed a THC peak with area of 11.3 units. The THC peak area was 4.2 units in the second and 2.6 units in the third blank sample. Hence, the samples with high THC concentration have a bigger memory effect, but with every consecutive blank sample the contamination reduces significantly.

Memory effect must be taken into account when samples with small THC concentrations are measured, e.g. samples where the THC concentration is close to 0.2%. It is necessary to analyse samples with low THC concentrations separately from the samples with high THC concentrations. Also, blank samples must be analysed prior to the analysis of samples with low THC concentrations.

Likewise, samples with complex matrices (e.g. cannabis resin) caused several peaks in the following blank samples. Cannabis resin samples tested during the test-period of the improved method showed that the retention times of all peaks that transferred to the next sample were different from these of the internal standard, CBN, CBD and THC. It was also discovered that these interfering peaks disappeared after one blank sample was analysed. After each analysis of samples with a complex matrix it is useful to analyse a blank sample. Normally blank samples are analysed only after every fifth analysis.

4.2 Sample preparation

With the old method, 20 mg to 40 mg of a sample was weighed and accordingly 1 ml to 5 ml of internal standard solution (0.5 mg/ml tetracosane in heptane) was added.

Due to practical reasons, only minor improvements were made to the sample preparation procedure. As all laboratory staff uses the old procedure, it was more efficient to keep it similar. Also all chemicals used (internal standard, heptane) were already present in laboratory.

The internal standard solution volumes were changed because of the changes in calibration procedure.

4.2.1 Internal standard solution preparation

The internal standard solution is the solution of tetracosane in heptane at concentration of 0.5 mg/ml. 500 mg tetracosane is weighed into the 1000 ml volumetric flask and it will be filled with heptane.

These chemicals were also used as an internal standard in the previous method. As they were present in the laboratory, it was not reasonable and economical for us to replace them.

According to the results of quality tests carried out with the old method, the solution of tetracosane in heptane is stable. In a dark glass bottle with a hermetic stopper it can be preserved at room temperature for 12 months. Taking into account the number of THC analyses in our laboratory, the internal standard solution runs out sooner than 12 month.

4.2.2 Cannabis sample preparation

As cannabis products usually arrive at the laboratory as complete cannabis plants or its parts (flowering tops, resin peaces etc), it is important to comminute them and prepare homogenised samples that represent as actual a chemical composition of the herbal products as possible. To do so, the plant material must be dried and homogenised. In our practice, the use of mortar and sieves is sufficient for separating the pieces of stalk and for homogenisation. Samples taken from the agricultural cannabis fields (50 and more plants) are pre-prepared for us in ARIB and do not need further homogenisation.

The volumes of the sample and internal standard solution for the sample solution preparation are chosen so as to ensure that the results do not exceed the upper limit of the calibration graph (2.0 mg/ml). If the expected THC concentration is between 0.050% and 5.0%, 40 mg of the sample and 1 ml of internal standard solution are sufficient. If the expected THC concentration is bigger than 5.0%, 20 mg of the sample and 3 ml of the internal standard solution suffice. It is also possible to make solutions at concentrations different to those described above, but then the analyst must be certain that the result will be in the working range of the calibration curve.

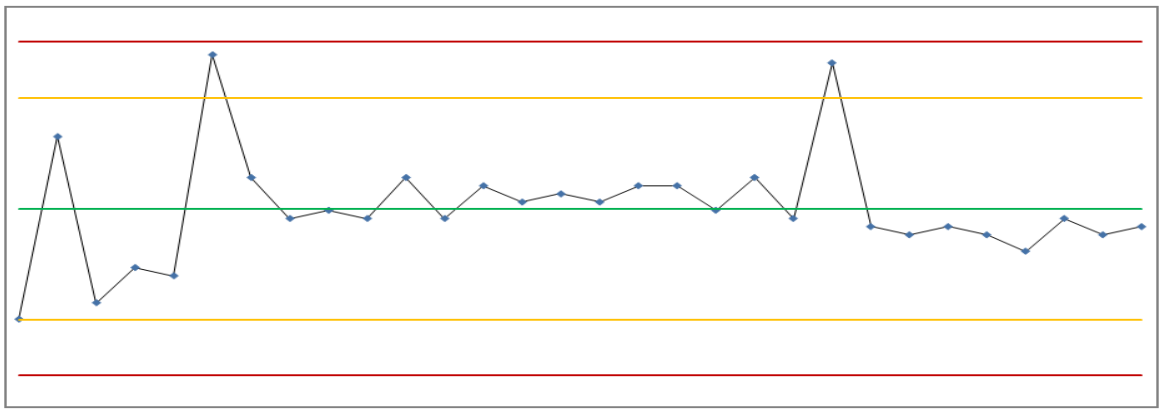
4.2.3 Quality control system and quality control samples

QCs were implemented to the new method for quality monitoring (calibration stability, method accuracy). Two different QCs were prepared for different purposes.

QC No 1 (QC1) is CBD solution in the internal standard solution at concentration of approximately 0.5 mg/ml. Approximately 5 mg of certified reference material (CBD) is weighed into a 10 ml volumetric flask, which is then filled with internal standard solution. The solution is divided into 50 vials, closed with the stoppers and stored in the freezer (temperature -20º C). The actual concentration must be calculated and documented. A new QC1 must be prepared every time when new internal standard solution is prepared.

QC1 can be used to evaluate the method's systematic error and to monitor the condition of the internal standard solution as it is always made using the same internal standard solution used for routine analysis. When the concentration (mg) is entered into the formula for concentration calculations, the result must theoretically be equal to the purity of CRM. Average concentration and limits of the control chart (X-chart) of QC1 is calculated from 30 independent analyses. The X-chart of QC1 is shown on figure 2.

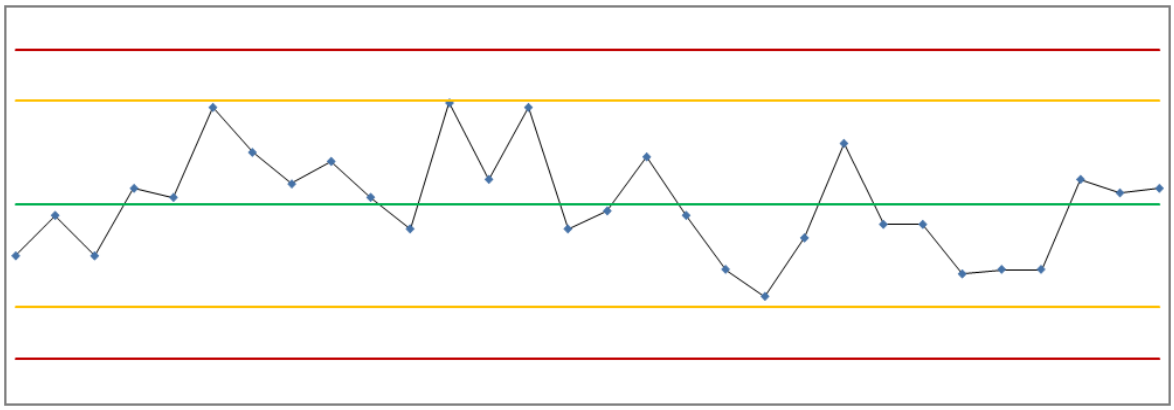
Figure 2: X-chart of 30 analyses of QC1 (blue points). Other graph components: mean (101.02%, green line), warning lines (99.67% and 102.38%, yellow lines), control lines (98.99% and 103.05%, red lines)



QC No 2 (QC2) is homogenised cannabis product prepared in the laboratory and stored in closed dark glass bottles in the freezer (temperature -20º C). QC2 is prepared for GC analyses in the same way as routine cannabis samples.

Average concentration and limits of the control chart (X-chart) of QC2 is calculated from 30 independent analyses. The X-chart of QC2 is shown on figure 3.

Figure 3: X-chart of 30 analyses of QC2 (blue points). Other graph components: mean (3.66%, green line), warning lines (3.43% and 3.88%, yellow lines), control lines (3.31% and 4.00%, red lines)



4.3 Validation

During the validation process, CBD's suitability for reference material when measuring THC and CBN concentration was tested together with the properties of calibration graph, LoD, LoQ, repeatability, reproducibility, selectivity and trueness.

4.3.1 Calibration

4.3.1.1 Indirect reference materials

CBD was used as reference material and preparation of calibration solutions instead of THC or CBN.

For testing the CBD/THC ratio, the solution of CBD in ethanol at concentration 1.0 mg/ml was prepared and analysed intermittently with the solution of THC in ethanol at concentration 1.0 mg/ml (Lipomed certification). Each solution was analysed ten times. The CBD/THC ratio was calculated using the peak areas. As a result of calculations the coefficient 0.937 was established and used for correction of the peak areas in the THC calibration graph.

For testing the CBD/CBN ratio, the solution of CBD in the internal standard solution at concentration of 0.5 mg/ml was prepared and analysed intermittently with the solution of CBN in the internal standard solution at concentration of 0.5 mg/ml. Each solution was analysed ten times. The CBD/CBN ratio was calculated using the peak areas. Calculations gave a coefficient 0.994. As the result is very close to the 1.00 and CBN does not have critical importance, it was decided that the peak areas of the CBN calibratioh graph required no corrections.

4.3.1.2 Linear range and working range

Calibration solutions were made at concentrations: 0.020, 0.040, 0.060, 0.081, 0.101, 0.121, 0.201, 0.403, 1.007, 1.511 and 2.014 mg/ml. The calibration graph is linear with the correlation coefficient 0.99997. The calibration graph of CBD and CBN is shown in figure 4 and the calibration graph for THC is shown in the figure 5.

It may be concluded that the improved method is linear in the range from 0.020 mg/ml to 2.012 mg/ml. The corresponding cannabinoid concentration range is between 0.050% to 5.0% with 40 mg of the sample and 1 ml of the internal standard solution; and between 0.30% and 30% with 20 mg of the sample and 3 ml of the internal standard solution. When analysing samples at concentrations above 30%, the result will be out of the linear range and it must be reported that the cannabinoid concentration exceeds 30%.

As the THC concentration level of 0.2% is most critical, there are more calibration points in this area.

Figure 4 Calibration graph of CBD and CBN

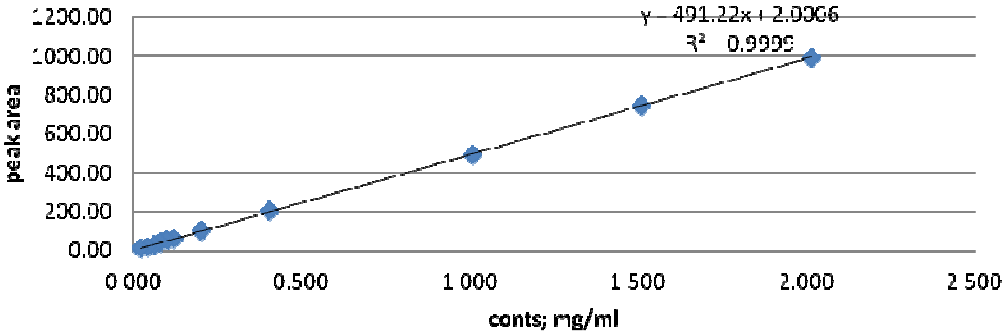
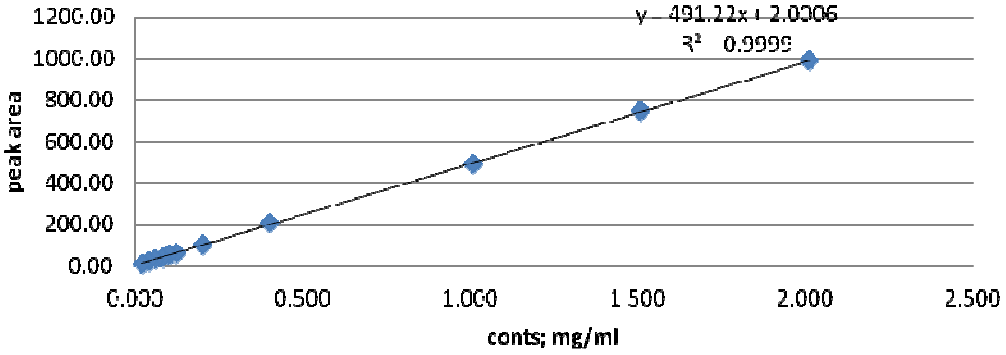


Figure 5 Calibration graph for THC



4.3.2 Limit of detection and limit of quantification

To determine the LoD, four CBD solutions in internal standard were made at concentrations: 0.0101, 0.0050, 0.0025 and 0.0013 mg/ml. The signals to noise ratios were measured manually from chromatograms. As it was decided that the limit of detection should be with signal to noise ratio equal to 10, the LoD was calculated from the results of two least concentrated solutions with signal to noise ratios of 13.6 and 6.75. As a result the LoD was detected at 0.0018 mg/ml. LoD was validated by analysing the CBD solution at concentration 0.0018 mg/ml six times. Relative standard deviation of the peak area was calculated to be 2%, therefore the repeatability of the 0.0018 mg/ml was found to be adequate for LoD.

LoQ was decided to be the lowest point in the calibration graph at concentration 0.020 mg/ml. When the detected result is smaller than LoQ it must be reported that the cannabinoid concentration is below 0.020 mg/ml. The main purpose of this method is to determine whether the concentration is higher or lower than 0.2% (0.081 mg/ml) and it is not necessary to determine the exact THC content when it is lower than the limit of quantification.

4.3.3 Repeatability and Reproducibility

For repeatability evaluation two different solutions were made from two different cannabis samples. The first sample was prepared and the THC and CBD concentration was measured. Six consecutive analyses were executed. The average THC concentration was 3.71% with the relative standard deviation of 0.5% and the concentration of CBD was 1.18% with the relative standard deviation of 1.0%. The second sample was prepared and analysed one month later. Seven consecutive analyses were executed. The average THC concentration was measured at 10.4% with the relative standard deviation of 0.5%.

For reproducibility calculations QC2 was analysed on different days by different chemists. The average THC concentration of 30 results was 3.66% with the relative standard deviation of 3.13% and average CND concentration was 1.12% with the relative standard deviation of 3.46%.

The relative standard deviations of repeatability and reproducibility were considered to be acceptable.

4.3.4 Selectivity

The matrix of cannabis samples, especially cannabis resins, can be very different and unknown because of the variety of cannabis strains and the cultivation conditions. Therefore it is crucial to be acknowledge that this can influence the analysis results.

As the majority of analyses are done with the so-called “green samples” (cannabis plants, marijuana), there are no THC free hashish samples available and as THC is the most critical substance to be analysed, selectivity tests were carried out with the samples of agricultural cannabis. The samples had been stored three years in transparent plastic bags at room temperature and the THC content was presumed to be minimal or null. These samples were soluted in heptanes and analysed to detect the possible interfering peaks with the same retention times as that of the internal standard (tetracosane) and THC. As a result, no interfering peaks were discovered.

It is also possible to monitor the peak area of the internal standard, which should remain around the same level all the time. It can be monitored every time when the QCs are analysed. When the area of the internal standard is significantly (2 times standard deviation) bigger in the sample, it must be checked if there is a matrix peak with the same retention time as the internal standard.

4.3.5 Trueness

For the trueness evaluation, QC1 was analysed 30 times and as the result the average concentration of CBD was measured 101.02% with the standard deviation of 0.678%. In theory, the CBD concentration should be 99.337%.

4.4 Uncertainty estimation

For uncertainty estimation the Nordtest method [35] was used with data from the validation procedure (trueness and reproducibility estimation) and from the certificate of the CRM (cannabidiol).

Combined uncertainty (u_c) was calculated using the following formula:

$$u_c = \sqrt{(uR_w)^2 + u(bias)^2}$$ where:

uR_w is the relative standard deviation of THC results from the reproducibility estimation and u(bias) is calculated with the trueness estimation data using the following formula:

$$u(bias) = \sqrt{(bias)^2 + \left(\frac{s_{bias}}{\sqrt{n}}\right)^2 + u(Cref)^2}$$

where:

$\text{bias} = \sqrt{\frac{\sum(\text{bias}_i)^2}{n}}$ (square mean of the relative bias);

s_{bias} is the relative standard deviation of the CRM analysis results; n is the number of CRM analysis and u(Cref) is purity of the CRM with 95% confidence level. Expanded uncertainty (U) was calculated by multiplying combined uncertainty with the coverage factor k=2.

The relative combined uncertainty of the method was estimated to be u_c = 3.6% and relative expanded uncertainty U = 7%; (k=2).

5. Summary

The purpose of this master's theses is to validate a method to quantificate mainly THC and also CBD and CBN in cannabis plants and its products with GC-FID. For validation the following parameters were evaluated for the final method: suitability of CBD as the reference material for measuring THC and CBN concentration, properties of the calibration graph, limit of detection, limit of quantification, repeatability, reproducibility, selectivity and trueness. Finally the uncertainty was evaluated by using the Nordtest method.

According to the results, CBD as a crystalline and rather stable compound acts in the FID in the same way as THC and CBN. The THC/CBD ratio was found to be 0.937 and CBN/CBD ratio was found to be 0.994. According to these results CBD is suitable for the calibration of the other named cannabinoids. With the calibration of THC, the coefficient 0.937 was used.

The calibration graphs produced were linear within the range from 0.020 to 2.014 mg/ml and with the correlation coefficient 0.99997. The working range of the method is from 0.050% to 30% of THC. The working range is wide enough to determine the THC level in the real cannabis samples.

LoD was determined on a rather low level, 0.0018 mg/ml, to which corresponds the THC concentration of 0.0045%. LoQ was decided to be the lowest point in the calibration graph. As the critical THC concentration to be detected is 0.2%, the very low and very high concentrations are not of great importance.

Repeatability and reproducibility were detected to be sufficient and acceptable. The relative standard deviation of repeatability was between 0.5% and 1.0% and the relative standard deviation of reproducibility was 3.13% (THC) and 3.46% (CBD).

As the matrix of the cannabis samples can be very different and unknown because of the variety of the cannabis strains and the cultivation conditions, it is very important to acknowledge that this can influence the results of the analyses. This was the most difficult validation parameter to evaluate due to the absence of blank cannabis samples and its products samples. Selectivity was evaluated using the agricultural cannabis samples where the THC content was presumed to be minimal or null. There were no interfering peaks with the same retention times as that of the internal standard or THC.

Trueness was tested by analysing QC containing known amount of CBD (CRM). The results were acceptable. The average CBD concentration was measured at 101.02% (theoretical concentration 99.337%) with the relative standard deviation of 0.678%.

Relative combined uncertainty was estimated to be u_c = 3.6% and expanded uncertainty U = 7%; (k=2).

In conclusion, it may be stated that the validated method is appropriate for quantification of THC, CBN and CBD in cannabis and its products with GC-FID. There are some interesting aspects in this work that can be used for further research and evaluation – different matrices and selectivity evaluation; different dissolving agents that can be used for extraction; optimising the GC parameters.

THC, CBD ja CBN kvantitatiivse gaasikromatograafilise määramise metoodika valideerimine

Gert Suurkuusk

Kokkuvõte

Käesoleva magistritöö eesmärgiks oli Eesti Kohtuekspertiisi Instituudis kasutatava analüüsimetoodika valideerimine. Metoodika on mõeldud kanepis ja selle produktides kolme kannabinoidi, peamiselt THC, aga ka CBN ja CBD kvantitatiivseks määramiseks, kasutades leekionisatsioonidetektoriga varustatud gaasikromatograafi.

Kuna THC ei sobi oma omadustelt kuigi hästi referentsaineks, millega GC-FID süsteemi kalibreerida, ja selleks kasutati CBD, tuli valideerimisel eelkõige kontrollida CBD sobivust selleks. Teiste parameetritena hinnati eksperimentaalse osa raames kalibreerimisgraafiku omadusi, avastamispiiri, määramispiiri, korduvustäpsust, korratavust, selektiivsust, tõesust ning lõpuks mõõtemääramatust.

Läbiviidud analüüside tulemusena selgus, et CBD kui kristalne ning küllaltki stabiilne ühend käitub leekionisatsioonidetektoris sarnaselt THC ja CBN-ga. Mõõdetud THC/CBD suhe oli 0,937 ja CBN/CBD suhe 0,994. Saadud tulemuste põhjal järeldati, et CBD sobib kalibreerimisgraafiku koostamiseks. THC kalibreerimisgraafiku koostamiseks tuleb piigipindalasid korrigeerida koefitsiendiga 0,937.

Koostatud kalibreerimisgraafik on vahemikus 0,020 kuni 2,014 mg/ml lineaarne ja selle korrelatsioonikoefitsient on 0,99997. Metoodika tööala on vahemikus 0,050% kuni 30% THC. Tööala on piisavalt lai ja sobib kasutamiseks, kuna EKEI kanepi sõeluuringute tulemuste põhjal jäävad reaalsetes proovides THC sisaldused vahemikku 0,030% kuni 28%.

Metoodika avastamispiir on küllaltki madal, jäädes tasemele 0,0018 mg/ml, millele vastaks proovides THC kontsentratsioon 0,0045%. Määramispiiriks otsustati jätta madalaim punkt kalibreerimisgraafikul, kuna äärmiselt madalate ning kõrgete THC sisalduste täpne määramine ei oma nii suurt tähtsust kui 0,2% THC sisalduse ümbruses olevad väärtused.

Kordustäpsust ning korratavust hinnati vastavate katsete tulemuste suhteliste standardhälvetega, mis saadi vastavalt kordustäpsuse puhul 0,5% kuni 1,0% ning korratavuse puhul 3,13% (THC) ja 3,46% (CBD), millest järeldub, et metoodikaga saadud tulemused on piisavalt kordustäpsed.

Kanepiproovide maatriks võib olla küllaltki keeruline, mille põhjuseks on kanepis sisalduvate kemikaalide arvukus (üle 400 keemilise ühendi), erinevate kanepisortide ning lõpuks ka kasvutingimuste rohkus. Kindlasti tuleb seda silmas pidada ning arvestada ohuga, et mõni komponent võib mõjutada analüüsitulemusi. Selektiivsuse hindamine oli antud töö raames ka kõige keerulisem ülesanne. Põhjuseks eelkõige CBN-, CBD- ja THC-vabade kanepiproduktide (eelkõige hašiš) puudumine. Selektiivsust hinnati THC suhtes, kasutades analüüside tegemiseks põllumajanduslikku kanepit, mille THC sisaldus eeldati olevat väga väike. Katsete tulemusena saadud kromatogrammidel puudusid segavad piigid sisestandardi ja THC kohal.

Metoodika tõesuse hindamiseks analüüsiti kindla CBD sisaldusega kontrollproovi, mille analüüsimisel saadud tulemused olid vastuvõetavad. Keskmine CBD sisaldus saadi 101,02% (teoreetiline sisaldus 99,337%) standardhälbega 0,678%. Tulemustest järeldub, et metoodikaga saadud tulemused on tõesed.

Määramatus arvutati, kasutades korratavuse ja tõesuse hindamisel saadud andmeid ja Nordtest meetodit. Metoodika suhteliseks liitmääramatuseks hinnati u_c = 3,6% ja laiendatud määramatuseks U = 7%; (k=2).

Kokkuvõttes võib öelda, et valideeritud metoodika on kohane THC, CBN ja CBD kvantitatiivseks määramiseks kanepis ja selle produktides. Tulevikus on võimalik kõnealust metoodikat parendada, eelkõige täiendades selektiivsuse hindamist erinevate maatriksite puhul, samuti uurides erinevate lahustite kasutamist ning optimeerides GC parameetreid.

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