Cannabis; extracting the medicine

Arno Hazekamp
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Paper cover: Cannabis Pur 100% (250 grams) from Grafisch Papier, The Nederlands.

Photo cover: Dutch medicinal cannabis, variety “Bedrocan”.

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Proefschrift

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Contents

CHAPTER 1

A general introduction to cannabis as medicine

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1.1 Cannabis as a medicine

It is hard to think of a medical topic that so strongly divides the research community as the medicinal use of cannabis. It can probably be said that cannabis is the most controversial plant in the history of mankind. But surely, if the plant *Cannabis sativa* would be discovered today, growing in some remote spot of the world, it would be hailed as a wonder of nature; a new miracle plant with the potential to treat anything ranging from headaches to neurological disorders to cancer. It is therefore interesting to notice that, even after decades of research, cannabis is probably most well known for causing anxiety, agitation and paranoia among politicians, while its medicinal potential continues to be disputed.
Interestingly, delta-9-tetrahydrocannabinol (THC), the main component of the cannabis plant, and one of the most renowned plant compounds of the world, is in fact already acknowledged as a medicine. It has been available to patients since 1986 under the name Marinol®, which is prescribed to treat nausea, pain and loss of appetite. So even if cannabis was nothing more than an herbal receptacle of THC, it should at least be accepted as some generic form of this registered medicine. However, on multiple levels (*in vivo*, *in vitro*, in clinical trials) it is becoming increasingly clear that THC alone does not equal cannabis [Williamson 2000; Russo 2003], pointing out that other components are necessary to explain the claimed medicinal effects.
Cannabis has the potential to evolve into a useful and much needed medicine, but is seriously obstructed by its classification as a dangerous narcotic. However, as shown in the case of the opium plant (*Papaver somniferum*) and the opiates derived from it (e.g. morphine, codeine), the distinction between a dangerous drug of abuse and a medicine can be made by proper, unbiased and well conducted research. Hopefully this thesis can be a contribution to a more rational approach to cannabis as a medicine.

1.2 The cannabis plant and its constituents

1.2.1 Forms of cannabis

Today, cannabis is the most commonly used psychoactive drug worldwide, together with coffee and tobacco, and it is the single most popular illegal drug. Worldwide over 160 million people are using cannabis regularly and these numbers are still rising [World Drug Report, 2006]. But what exactly is cannabis anyway? With such high popular demand, it is not surprising that cannabis and its products are known under a large variety of names. Some of the most widely used ones are defined here.
The commonly used term ‘marijuana’ or ‘marihuana’ traditionally describes the cannabis plant when used as a recreational drug, and is frequently associated with the negative effects or social impact of the drug (figure 1.1). ‘Weed’ is another name for cannabis when used as a recreational drug. When the term ‘hemp’ is used, it usually refers to the use of cannabis as a source of fiber, making the term ‘fiber-hemp’ therefore somewhat superfluous. Because of the

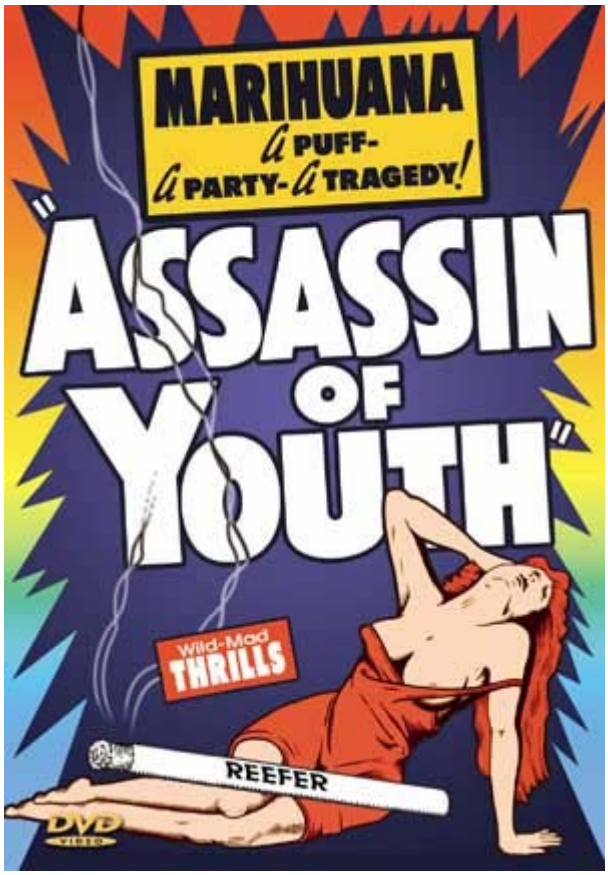


Figure 1.1: Marihuana, the "assassin of youth". Assassin of Youth (1937) is a pre-WWII movie about the negative effects of marijuana, reflecting the hysterical anti-drug propaganda of its time.

inexact and unscientific nature of these terms, they will not be used in this thesis. Instead, the proper scientific name "cannabis" will be consistently used to describe the plant *Cannabis sativa* L. in all its varieties.

When talking about cannabis for either recreational or medicinal use, what is usually referred to are the female flowers ('flos'), being the most potent part of the plant. The dried resin obtained from these flowers is generally known as 'hash', or 'hashish', although a large variety of names exists. This resin is the origin of the most important bioactive components of the cannabis plant, the 'cannabinoids', which will be the main focus throughout this thesis.

Finally, 'dronabinol' is another name for the naturally occurring (-)-*trans*-isomer of THC, often used in a medical context in the scientific and political literature, and adopted by the World Health Organization.

1.2.2 The botany of cannabis

The basic material of all cannabis products is the plant *Cannabis sativa* L (figure 1.2). It is an annual, usually dioecious, more reraly monoecious, wind-pollinated herb, with male and female flowers developing on separate plants. It propagates from seed, grows vigorously in open sunny environments with well drained soils, and has an abundant need for nutrients and water. It can reach up to 5 meters (16 feet) in height in a 4 to 6 month growing season. However, in modern breeding and cultivation of recreational cannabis, the preferred way to propagate the plants is by cloning, using cuttings of a so-called 'mother plant'. As this term indicates, female plants are used for this purpose, as they produce significantly higher amounts of psychoactive compounds than the male plants.

The sexes of Cannabis are anatomically indistinguishable before they start flowering, but after that, the development of male and female plants varies greatly (figure 1.3). Shorter days (or more accurately longer nights) induce the plant to start flowering [Clarke, 1981]. The female plant then produces several crowded clusters of individual flowers (flowertops); a large one at the top of the stem and several smaller ones on each branch, while the male flowers hang in loose clusters along a relatively leafless upright branch. The male plants finish shedding pollen

and die before the seeds in the female plants ripen four to eight weeks after being fertilized. A large female can produce over one kilogram of seed. If the seed survives, it may germinate the next spring.

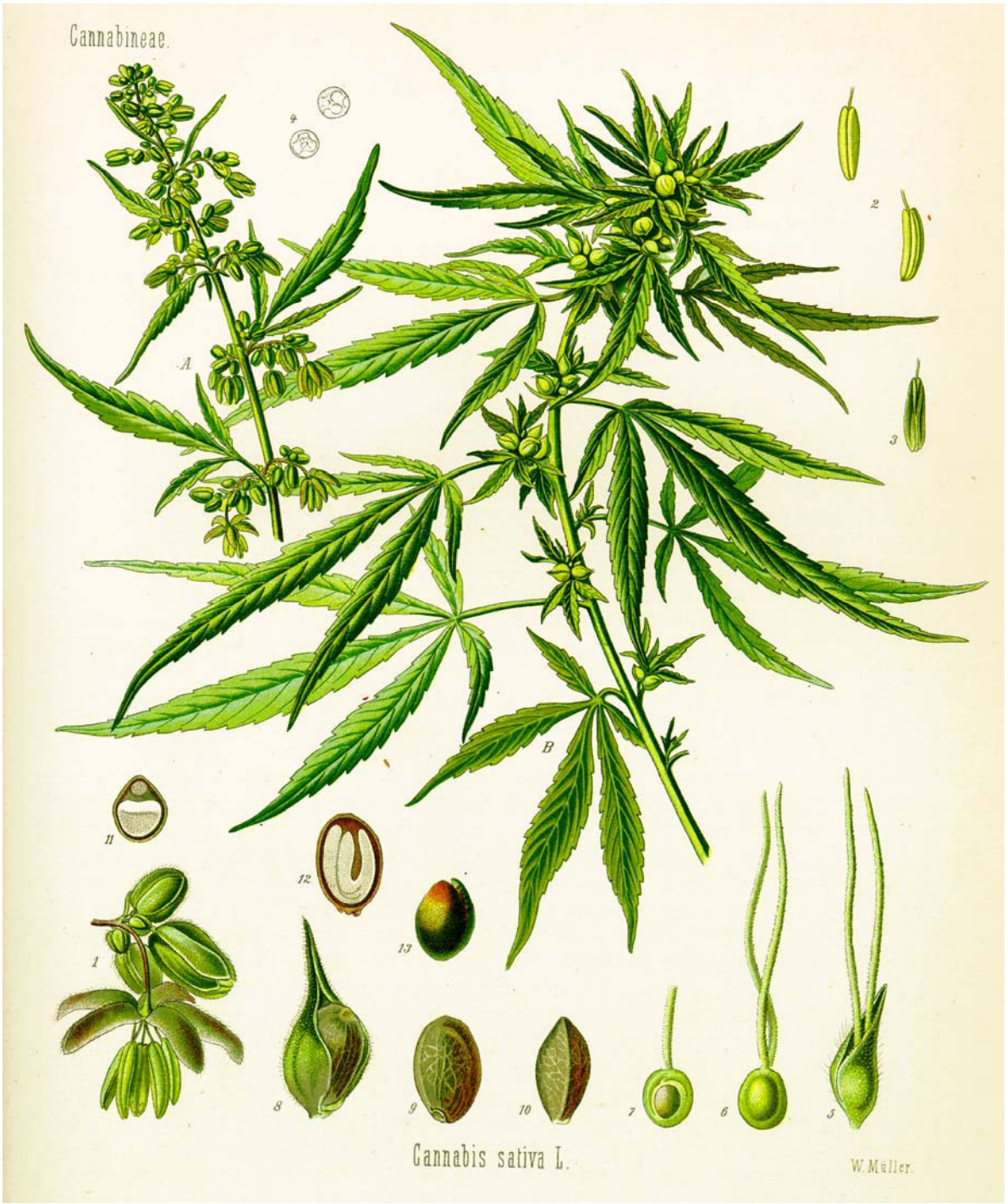


Figure 1.2: *Cannabis sativa* L. Scientific drawing from Franz Eugen Köhler's Medizinal-Pflanzen. Published and copyrighted by Gera-Untermhaus, FE Köhler in 1887 (1883–1914). The drawing is signed W. Müller.

According to current botanical classification, Cannabis belongs with Humulus (hops) to the family of Cannabinaceae (also Cannabaceae and Cannabidaceae [Frohne, 1973; Turner, 1980; Schultes, 1980]. Despite this relationship, cannabinoids can only be found in *Cannabis sativa*. In the genus Humulus and also in crafting experiments between Cannabis and Humulus no cannabinoids have been found [Crombie, 1975; Fenselau, 1976]. The current systematic classification of Cannabis is [Lehmann, 1995]:

Division	Angiosperms
Class	Dicotyledon
Subclass	Archichlamydeae
Order	Urticales
Family	Cannabinaceae
Genus	*Cannabis*
Species	*sativa* L.

Because of centuries of breeding and selection, a large variation of cultivated varieties (or cultivars) has been developed. Recently, more than 700 different cultivars were described [Snoeijer, 2001] and many more are thought to exist. As a result, there has been extensive discussion about further botanical and chemotaxonomic classification. So far, several classifications of cannabis have been proposed: a classification into *Cannabis sativa* L., *C. indica* LAM. and *C. ruderalis* JANISCH [Schultes, 1974; Anderson, 1974; Emboden, 1974] or *Cannabis sativa* L. ssp. *Sativa* and ssp. *Indica* [Small, 1976a,b; Cronquist, 1981]. However, it is becoming commonly accepted that Cannabis is monotypic and consists only of a single species *Cannabis sativa*, as described by Leonard Fuchs in 16th century [Beutler, 1978; Lawi-Berger, 1982a,b; Brenneisen, 1983].

To solve the controversy in a biochemical way, a first chemical classification was done by Grlic [1968], who recognized different ripening stages. Fettermann [1971b] described different phenotypes based on quantitative differences in the content of main cannabinoids and he was the first to distinguish the drug- and fiber- type. Further extension and perfection of this approach was subsequently done by Small and Beckstead [1973], Turner [1979] and Brenneisen [1987]. It was found that a single plant could be classified into different phenotypes, according to age. Although these chemotaxonomic classifications don't strictly define the contents of main cannabinoids for each chemotype, it does provide a practical tool for classification. A final validation of Cannabis classification awaits further chemotaxonomic and genetic research.

For forensic and legislative purposes, the most important classification of Cannabis types is that into the fiber-type and the drug-type. The main difference between these two is found in the content of the psychotropically active component Δ9-tetrahydrocannabinol (THC): a high content of THC classifies as a drug-type cannabis, while a low THC content is found in fiber-type cannabis. All cannabis varieties presently used for medicinal purposes belong to the drug-type, because of their high content of the biologically active THC. But although fiber-type

cannabis is commonly not used for medicinal or recreational purpose, it does contain components that have been found to be biologically active, indicating that the distinction between the two types has limited relevance for medicinal research into cannabis.

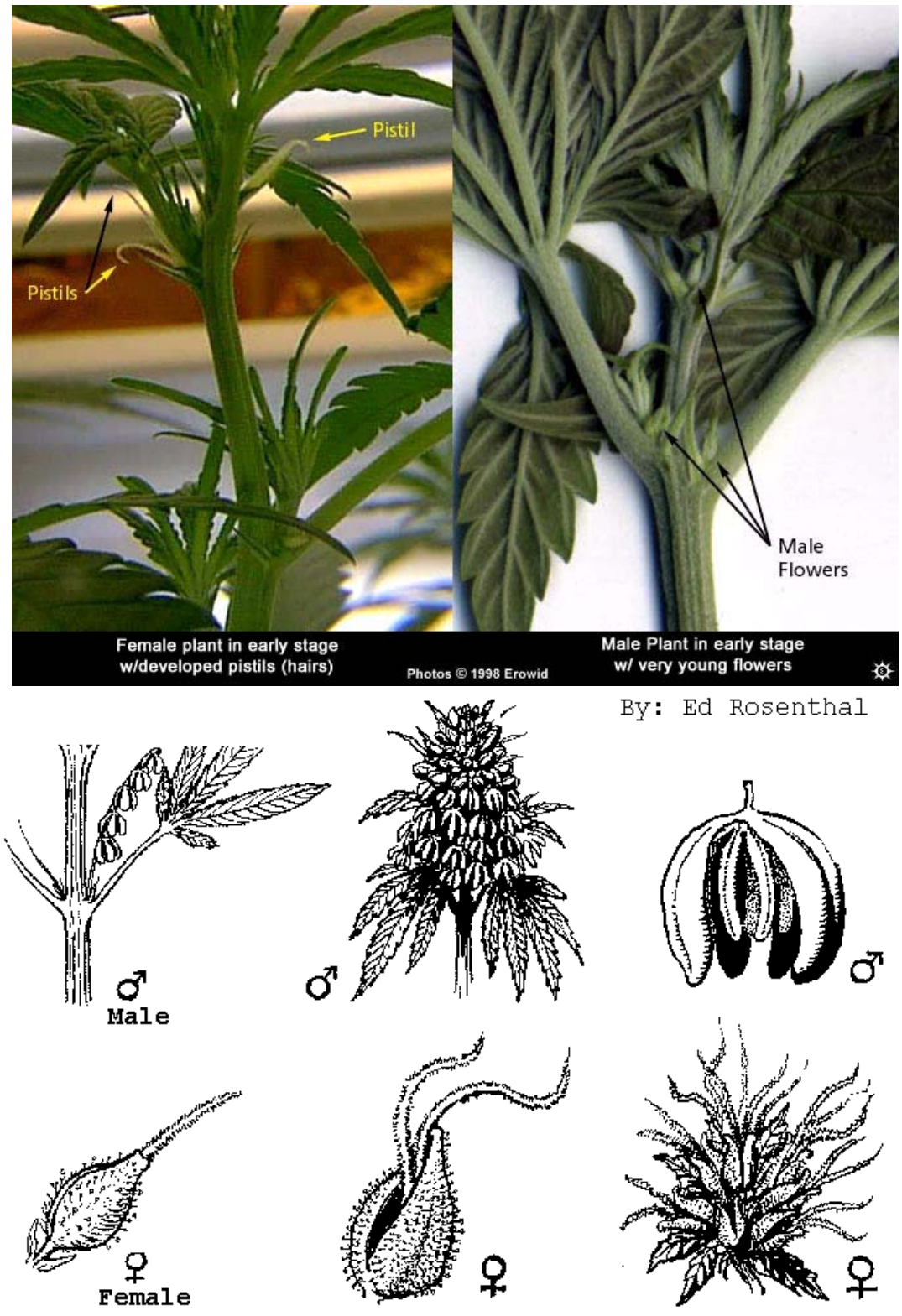
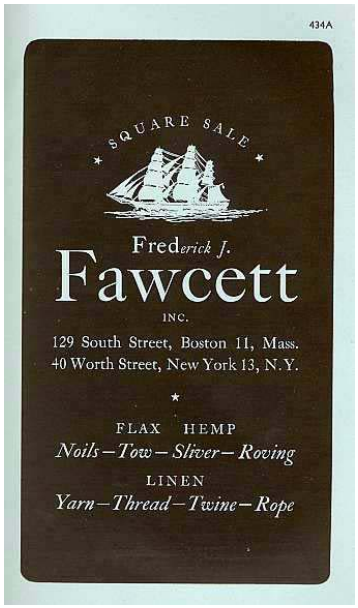


Figure 1.3: Photograph and drawing of male and female flowers of cannabis. Reprinted with permission of Ed Rosenthal.

1.2.3 History of cannabis as a useful plant



Cannabis most likely originates from Central Asia, as archeological evidence indicates it was cultivated in China for food and fiber already 10.000 years ago. Also in ancient Egyptian mummies clues have been found for the use of cannabis as food or medicine [Balabanova, 1992]. In fact, cannabis is one of the oldest known medicinal plants and is described in almost every ancient handbook on plant medicine, most comonly in the form of a tincture or a tea [Zuardi, 2006; Grotenhermen, 2002]. Some religions were closely related with the properties of the cannabis plant. For example, in Hindu legend cannabis is believed to be the favorite food of the god Shiva, because of its energizing properties. As cannabis spread from Asia towards the West, almost every culture came into contact with this miracle plant. Nowadays, cannabis can be found in all temperate and tropical zones, except in humid, tropical rainforests [Conert, 1992].

As a fiber plant cannabis produces some of the best and most durable fibers of natural origin. For a long time in history these fibers were used to produce sails for sea-ships, paper, banknotes and even the first Levi's jeans. The oil of the hempseed has been suggested to be well balanced in regards to the ratio of linoleic and linolenic acids for human nutrition. Furthermore, the oil because of this feature and the presence of gamma-linolenic acid, is ideal as an ingredient for body oils and lipid-enriched creams [Oomah, 2002].

Despite the fact that cannabis was grown on a large scale in most countries, the abuse as a narcotic remained uncommon in Europe or the United States untill relatively recently. People were largely unaware of the psychoactive properties of cannabis and it is unlikely that early cultivars, selected mainly for their fiber qualities, contained significant amounts of the psychoactive compound THC. The medicinal use of cannabis was only introduced in Europe around 1840, by a young Irish doctor, William O'Shaughnessy, who served for the East India Trading Company in India, where the medicinal use of cannabis was widespread. Unlike the European fiber cannabis, these Indian varieties did contain a reasonable amount of bioactive compounds. In the following decades cannabis knew a short period of popularity both in Europe and the United States. At the top of its popularity, more than 28 different medicinal preparations were available with cannabis as active ingredient, which were recommended for indications as various as menstrual cramps, asthma, cough, insomnia, support of birth labor, migraine, throat infection and withdrawal from opium use [Grotenhermen, 2002].

However, difficulties with the supply from overseas and

varying quality of the plant material made it difficult to prepare a reliable formulation of cannabis. Because no tools existed for quality control it was impossible to prepare a standardized medicine, so patients often received a dose that was either too low, having no effect, or too high, resulting in serious side effects. Moreover, cannabis extract was not water-soluble and could not be injected, while oral administration was found to be unreliable because of its slow and erratic absorption. Because of such drawbacks the medicinal use of cannabis increasingly disappeared in the beginning of the twentieth century. When finally a high tax was imposed on all cannabis-based products (seeds and fibers excluded) and increasingly restrictive legislation was introduced for cannabis abuse, the medicinal use of cannabis gradually disappeared from all Western pharmacopoeias in the period from 1937 [Grotenhermen and Russo, 2002]. In contrast to the alkaloid drugs codeine and morphine, which are derived from opium, isolation of the pure active substances from cannabis was not achieved until the 1960s [Gaoni, 1964a].

Only since the flower-power-time of the 1960s, the smoking of cannabis as a recreational drug has become a widely known phenomenon in the Western world. From then on, import of stronger varieties from the tropics, combined with a growing interest in breeding, initially most notably among American Vietnam war veterans, led to a steady increase in psychoactive potency. Contemporary recreational cannabis has increasingly become a high-tech crop, grown indoors under completely artificial conditions.

1.2.4 Cannabis constituents

With over 420 known constituents, Cannabis is one of the chemically best studied plants [Turner, 1980; Ross, 1995]. Most interesting among these constituents are the secretions of the head cells of glandular hairs (trichomes) distributed across the surface of the cannabis plant (figure 1.4). Although trichomes can be found all over the male and female plants, they are particularly concentrated at some parts of the female inflorescence. Solitary resin glands, consisting of one or two dozen cells, most often form at the tips of slender trichome stalks which form as extensions of the plant surface. These glands secrete an aromatic terpenoid-containing resin with a very high content of cannabinoids, which collects under a thin waxy membrane surrounding the secretory head cells. The secreted resin is largely segregated from the secretory cells, which isolates the resin from the atmosphere as well as membrane bound enzymes, protecting it from oxidative degradation and enzymatic change. A layer of abscission cells at the base of each secretory head allows the gland to be easily removed [Kim, 2003].
The resin excreted by the trichomes contains a variety of constituents, any of which might play a role in the biological activities of the cannabis plant. Among these are terpenoids, flavonoids and cannabinoids. Because it would be too complex to study all these components in a single

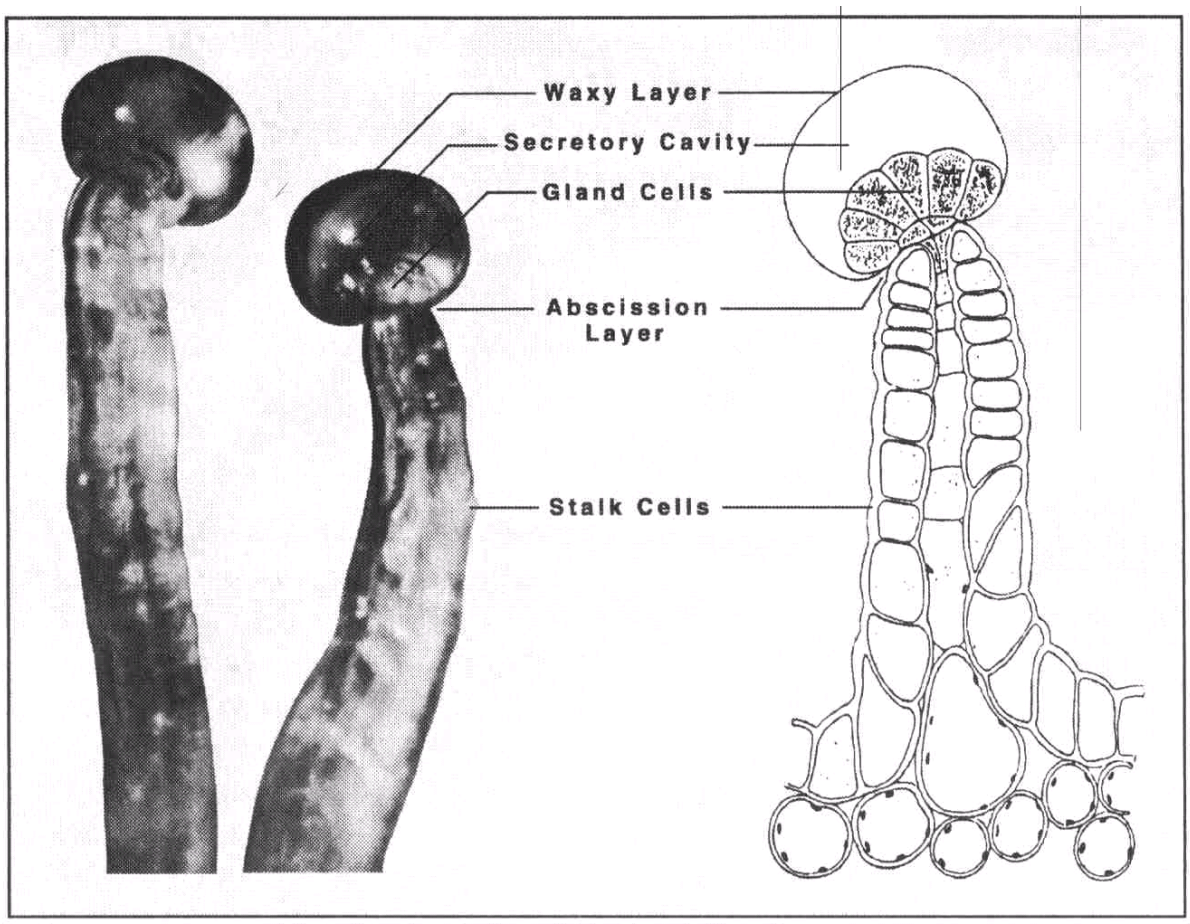


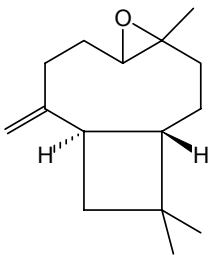
Figure 1.4: Microscope photograph and drawing of a cannabis resin gland, with secretory head cells visible underneath the transparent cannabinoid- and terpenoid-rich resin.
Source: drawing from RC Clarke. Hashish! Los Angeles: Red Eye Press, 1998. Reprinted with permission.

PhD-project, this thesis is particularly focused on the cannabinoids. Hopefully the other classes of compound will (again) receive their share of scientific attention in the near future.
The adaptational significance of the resin glands remains speculative. Although the resin gives a certain defense against insect and fungal attack, cannabis crops are still vulnerable to attack by a wide variety of pests, particularly under greenhouse conditions. Certainly, the intoxicating effects of Cannabis resin have increased cannabis predation by humans, as well as encouraged its domestication, thus dramatically widening its distribution. Recently, it has been shown that the cannabinoids cannabigerolic acid (CBGA) and tetrahydrocannabinolic acid (THCA) induce cell death via apoptosis in plant cells but also in insect cells. Furthermore, formation of THCA is linked to hydrogen peroxide formation which may contribute to self-defense of the Cannabis plant [Sirikantaramas, 2005]. These results strongly suggest that cannabinoids act as plant defense compounds, like many other plant secondary metabolites.
An extensive review of cannabis constituents has been made [Turner, 1980; Ross, 1995]. Besides at least 66 cannabinoids, compounds that have been identified in cannabis products are listed in table 1.1 [Grotenhermen, 2002].

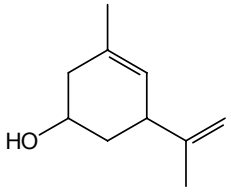
Table 1.1: An overview of compounds identified in cannabis.

120	terpenoids
50	hydrocarbons
34	sugars and related compounds
27	nitrogenous compounds
25	non-cannabinoid phenols
22	fatty acids
21	simple acids
21	flavonoids
18	amino acids
13	simple ketones
13	simple esters and lactones
12	simple aldehydes
11	proteins, glycoproteins and enzymes
11	steroids
9	elements
7	simple alcohols
2	pigments
1	vitamin

So far, more than 100 terpenoids have been found in cannabis, including 58 monoterpenoids, 38 sesquiterpenoids, one diterpenoid, two triterpenoids and four other terpenoids [Turner, 1980]. They can be studied after steam-distillation of cannabis material or by headspace-gas chromatography, although large qualitative differences are seen between these two techniques [Hood, 1973; Strömberg, 1974; Hendriks, 1978]. While cannabinoids are odorless, the volatile mono- and sesquiterpenoids are the compounds that give cannabis its distinct smell. The sesquiterpenoid β-caryophyllene-epoxide (figure 1.5), for example, is the main compound that search-dogs are trained to recognize [Stahl, 1973]. Only one unusual terpenoid can be found in cannabis: the monoterpenoid *m*-mentha-1,8(9)-dien-5-ol (figure 1.5). All others can be found ubiquitously in nature. For this reason the terpenoids of cannabis did not receive much scientific interest, until it was found that the terpenoid spectrum of cannabis products can help in determining the origin of cannabis in custom seizures [Brenneisen, 1988].



β-caryophyllene-epoxide



m-mentha-1,8(9)-dien-5-ol

Figure 1.5: Two special constituents of the cannabis plant

1.3 Cannabinoids

1.3.1 Cannabinoids defined

Cannabinoids are considered to be the main biologically active constituents of the cannabis plant. In spite of the fact that THC is often erroneously referred to as the 'active ingredient' of cannabis preparations, currently at least 66 different cannabinoids have been described. The most important ones are shown in figure 1.6. Mechoulam and Gaoni [1967] defined cannabinoids as: the group of C_{21} compounds typical of and present in *Cannabis sativa*, including their carboxylic acids, analogs, and transformation products. But from this rather restricted pharmacognostic definition, considerable expansion is now required. A modern definition will put more emphasis on synthetic chemistry and on pharmacology, and would also include related structures or any other compound that affects cannabinoid receptors. This, however, creates several chemical subcategories of cannabinoids. In this thesis, the focus will be exclusively on the (phyto)cannabinoids, occurring naturally in the cannabis plant.
Chemically, the (phyto)cannabinoids belong to the terpenophenols, which are very common in nature. Cannabinoids are accumulated in the glandular hairs described above, where they typically make up more than 80% of the subcuticular secretion. In general all plant parts can contain cannabinoids, except for the seeds. The traces of cannabinoids found in seeds are most likely a result of contamination with cannabis resin from the flowers [Lawi-Berger, 1982; Ross, 2000]. Essentially there are no qualitative differences in cannabinoid spectrum between plant parts, only quantitative differences [Fetterman, 1971b; Field, 1980]. The highest cannabinoid concentrations (in % of dry weight plant material) can be found in the bracts of the flowers and fruits. In the foliage leaves the content is lower, and in the stems and, even more so, the roots the content is very low [Hemphill, 1980]. Cannabis grown outdoors generally has lower levels of cannabinoids when compared to indoor grown plants. When grown under artificial, high yielding conditions, cannabis flowering parts can be obtained with a resin content of up to 25-30%, mainly consisting of THC (in the form of its acidic precursor THCA, see below). This high abundance of a single type of secondary metabolite is virtually unparalleled in the plant kingdom.
Interestingly, THC, the psychotropically active principle of cannabis, contains no nitrogen atom and therefore is no alkaloid. This is rare amongst the psychotropically active compounds.

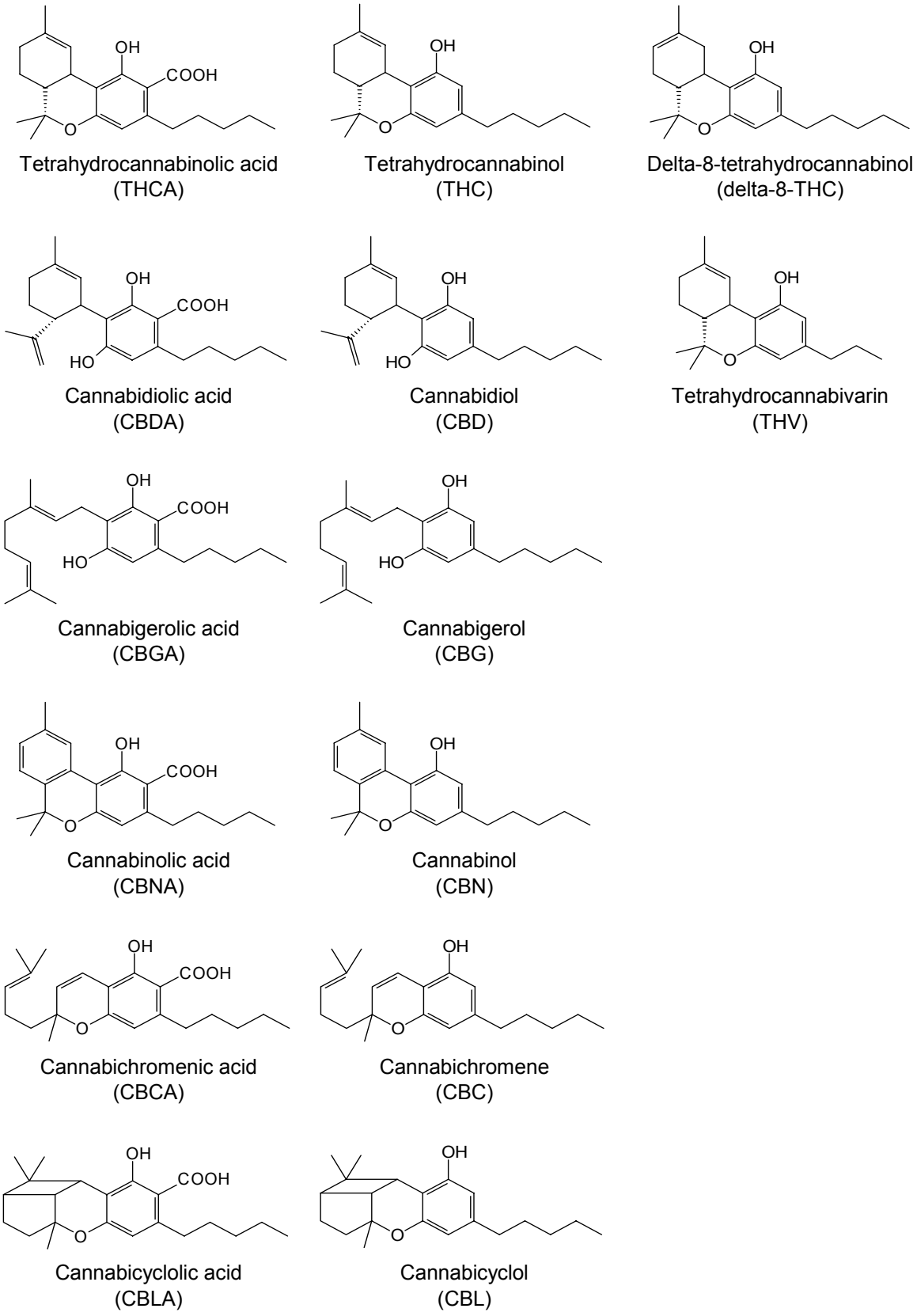


Figure 1.6: Structures of the cannabinoids most commonly found in cannabis plant materials

1.3.2 Biosynthesis

For the chemical numbering of cannabinoids 5 different nomenclature systems have been used so far [Eddy, 1965], but the most commonly used system nowadays is the dibenzopyran numbering, which is also adopted by Chemical Abstracts. In Europe the monoterpenoid system based on p-cymene has also been widely used. As a result, the main psychoactive cannabinoid delta-9-THC is sometimes described as delta-1-THC in older manuscripts. In this thesis, the dibenzopyran numbering is consistently used, therefore THC is fully described as (-)-*trans*-Δ^9-tetrahydrocannabinol (figure 1.7).

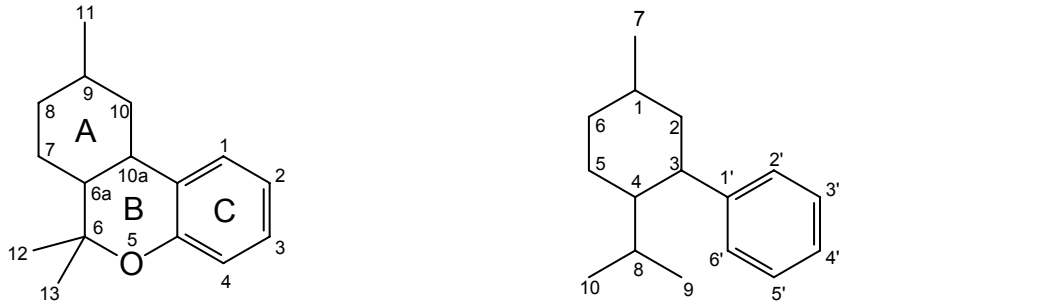


Figure 1.7: Two most commonly used numbering systems for the cannabinoids. The dibenzopyran system is used in this thesis.

In all biosynthetic pathways for cannabinoids that were postulated until 1964 ,CBD or CBDA was regarded as key intermediate, which was built from a monoterpene, and olivetol or olivetolic acid, respectively. Other cannabinoids were then derived from this common precursor. However, Gaoni and Mechoulam [1964b] showed that CBG is the precursor of CBD, which was biosynthesized through the condensation of geranylpyrophosphate (GPP), and olivetol or olivetolic acid. Subsequently, they concluded that CBD, THC and CBN all derive from CBG and differ mainly in the way this precursor is cyclized [Mechoulam, 1965; 1967; 1970; 1973]. Shoyama [1970; 1975] further concluded that neither the free phenolic forms of the cannabinoids nor CBNA were produced by the living plant. Instead, he postulated a biosynthetic pathway based on geraniol and a polyketoacid. The same conclusion was reached by Turner and Hadley [1973] after study of African cannabis types. This biosynthetic pathway could explain the different contents of cannabinoids in cannabis products of different origins and the occurrence of homologues and derivatives.
Currently, the hypothesis that the C_{10}-terpenoid moiety is biosynthesized via the deoxyxylulose phosphate pathway, and the phenolic moiety is generated by a polyketide-type reaction sequence is widely accepted. More specifically, incorporation studies with ^{13}C-labeled

glucose have shown that geranyl diphosphate (GPP) and the polyketide olivetolic acid are specific intermediates in the biosynthesis of cannabinoids, leading to the formation of CBGA (figure 1.8) [Fellermeier, 1998; Fellermeier, 2001]. Further biosynthetic pathways of cannabinoid production have finally become clear by identification and subsequent cloning of the responsible genes [Taura, 1995b; Taura, 1996; Morimoto, 1998]. A major structural variation for the cannabinoids is found in the alkyl sidechain of the olivetolic acid moiety: although the pentyl (C5)-sidechain is usually present, also shorter sidechains can be found, ranging from C4 to C1. It is interesting to note that free olivetolic acid has never been detected in cannabis plant material.

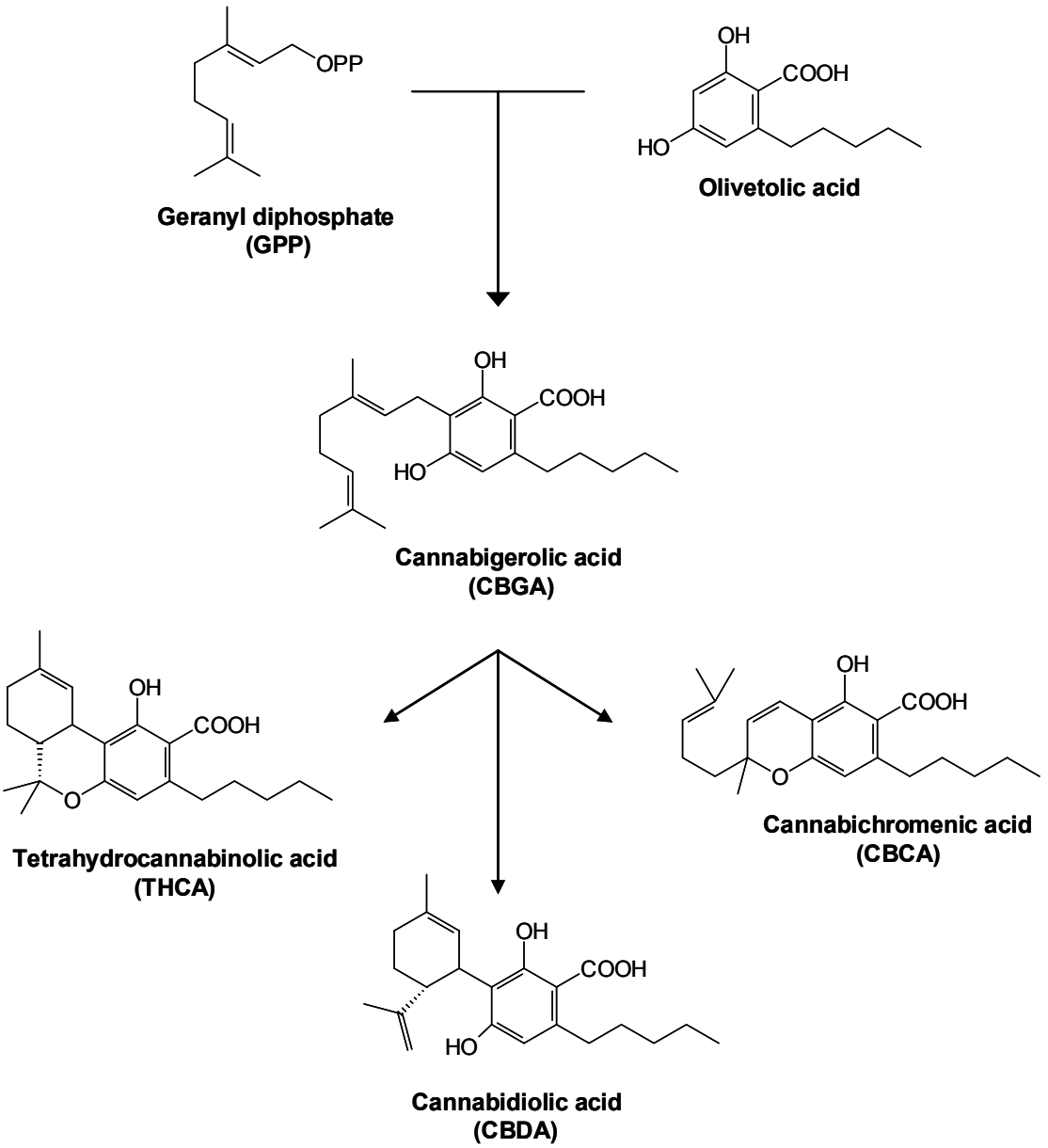


Figure 1.8: Biosynthetic pathway for the production of the cannabinoids

The main biosynthetic steps are shown in figure 1.8. Based on this pathway, cannabinoids are produced by the cannabis plant as carboxylic acids, where the substituent at position 2 is a carboxyl moiety (–COOH). Consequently, in fresh plant material almost no neutral cannabinoids can be found, but theoretically all cannabinoids are present in this acidic form. However, the carboxyl group is not very stable and is easily lost as CO_2 under influence of heat or light, resulting in the corresponding neutral cannabinoid. In this way the acidic precursor THCA can be converted into the psychoactive THC, which is the reason why all forms of (recreational) cannabis consumption include some form of heating of the material (i.e. smoking, vaporizing, making tea or baked products).

1.3.3 Classifications of cannabinoids

Although more than 60 cannabinoids are known, it should not be concluded that all cannabinoids are detectable in all cannabis products. They were identified over several decades of cannabis research, studying many different cannabis products and different and sometimes rare types of cannabis plants from a variety of origins and qualities.
The main cannabinoid types that are usually detected in each breeding strain or cultivar of cannabis are THC, CBD, CBN, CBG and CBC. However, there can be an enormous variation in their quantitative ratios. The different chemical types of cannabinoids have been well described [Turner, 1980, ElSohly 1983] and will therefore not be extensively discussed here. However, understanding how the cannabinoids are (chemically) related to each other is important when studying cannabis samples, as degradation and changes in the cannabinoid profile might occur as a result of storage or breeding conditions, variations in preparation of medicines, mixing with other components (e.g. tobacco when smoking), heating etc. For the phytochemical work in this thesis, the cannabinoids can most conveniently be divided in three groups (see also figure 1.9):

1) cannabinoids produced by metabolism of the plant (acidic cannabinoids);
2) cannabinoids present in the plant resulting from decarboxylation (neutral cannabinoids);
3) cannabinoids occurring as artefacts by degradation (e.g.: oxidation, isomerization, UV-light).

The group of cannabinoids that occur as a result of degradative conditions deserve some special attention, because their presence is largely the result of variable and unpredictable conditions during all stages of growing, harvest, processing, storage and use. As a result, a well-defined cannabis preparation may change rapidly into a product with significantly different biological effects. Particularly in samples that have been stored for an extended period, CBN can be found in relatively large amounts. Cannabinoids of the CBN type are not formed by biosynthesis, but rather by oxidative degradation of THC- and CBD types. Also the types Δ^8-THC and CBL are not naturally occurring, but artifacts. The isomerization of Δ^9-

THC to Δ^8-THC is well documented [Mechoulam, 1970; Mechoulam, 1973; Razdan, 1973]. Since Δ^8-THC is more thermostable than Δ^9-THC, it will accumulate during heating of Δ^9-THC. The cannabinoid CBL arises by exposure of CBC to UV-radiation, leading to crosslinking of two double bonds in the molecule [Crombie, 1968].

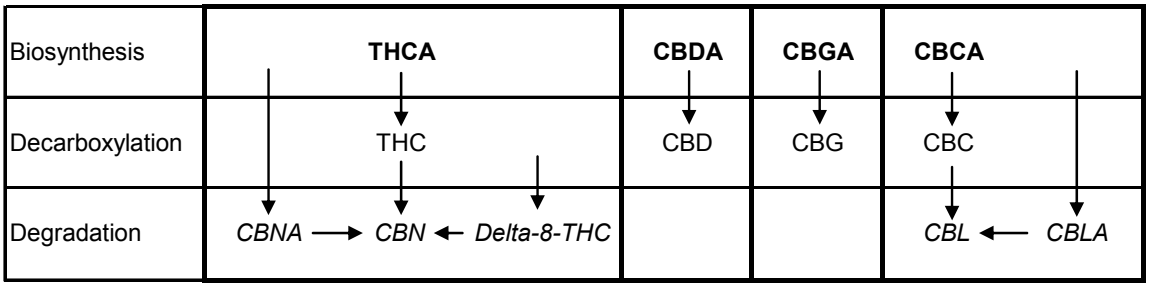


Figure 1.9: Relationships between the major cannabinoids found in cannabis plant materials. Three different groups are distinguished: cannabinoids produced by biosynthesis of the plant; cannabinoids resulting from natural decarboxylation of acidic cannabinoids; degradation products resulting from various influences, such as UV-light, oxydation or isomerization. Arrows indicate the routes of conversion.

1.3.4 Studying cannabinoids

Medicines based on natural products are usually hard to study. Plant materials may contain many (structurally) closely related compounds, and often it is unclear what the active ingredient is, if indeed there is only one. Sometimes the biologically active components of the plant have only been partially characterized (e.g. *Ginkgo biloba*, St. John's Wort, *Hypericum perforatum, Echinacea purpurea*). Because of this complexity of medicinal plants, some important conditions for reliable study of natural products are: the availability of analytical methods that can study the components without sample degradation; reference standards of the compounds of interest; and a clear overview of physicochemical, spectroscopic and chromatographic properties of the sample components.

For the study of cannabinoids, the analytical methods that are available have recently been extensively reviewed by Raharjo [2004]. By far the most commonly used chromatographic methods have been high performance liquid chromatography (HPLC) and gas chromatography (GC). The use of GC, commonly coupled to flame ionization detection (FID) or mass (MS)-detection, permits the analysis of a large variety of cannabinoids with very high resolution. However, a major disadvantage of GC is in the fact that the acidic cannabinoids can not be analyzed without prior derivatization to protect the labile carboxyl function. Because it is hard to perform a quantitative derivatization for all components in a complex mixture, GC analysis has only limited value when studying the authentic composition of cannabis products. When analyzing cannabinoids in their authentic form, HPLC is the preferred method. Making use of a UV- or photodiode-array detector (PDA), cannabinoids can be efficiently analyzed without causing degradation of sample components.

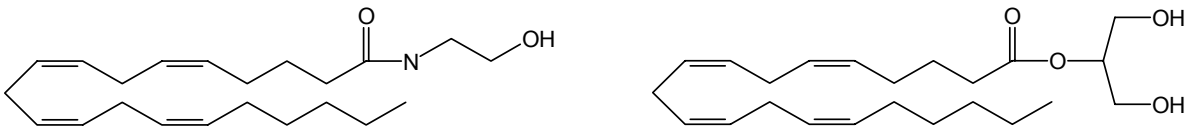
However, it is difficult to separate all major cannabinoids in a single run. To overcome this problem, the use of mass-detection (LC-MS) to distinguish between overlapping chromatographic peaks is becoming increasingly important [Stolker, 2004; Hazekamp, 2005].
Independent of the method used for cannabinoid analysis, reliable standards are needed for the compounds to be studied, in order to allow high quality, quantitative research on the pharmacological and medicinal aspects of cannabis. However, at the time the work for this thesis was started, only a few of the major cannabinoids were commercially available (THC, CBD, CBN and Δ^8-THC). Even the cannabinoid present in the highest concentration in any drug-type cannabis plant, THCA, had not been made commercially available yet. Without a doubt, this lack of reference standards is a great obstacle for a detailed study and understanding of cannabis.
Although spectroscopic and chromatographic data have been published for most known cannabinoids during isolation and identification experiments (see Turner *et al.* [1980] for an overview), they are scattered over a huge amount of scientific papers. Moreover, standardized data obtained under identical analytical conditions have not been reported yet. This is regrettable, because when studying a complex phytomedicine like cannabis, it is important to communicate about the subject in a standardized way. After all, differences in analytical methods, or in the interpretation of results make it hard to discuss the science behind cannabis. Such differences can be prevented by the development of validated methods, which are agreed upon by all scientists involved. For other important drugs (such as cocaine, opioids, LSD) such standardized methods have been developed and cross-validated between laboratories, commonly resulting in official Pharmacopoeia texts. For cannabis, such a text has not been available since several decades.
In conclusion, a lot of data on cannabis and the cannabinoids have been published, but their value is only limited. There is a clear need to put all the pieces of the cannabis puzzle together and come up with reliable, validated results.

1.4 Cannabinoids as active compounds

1.4.1 Mechanisms of cannabinoid action

Until the discovery of specific cannabis receptors, the biochemical mode of action of cannabinoids was much disputed. Because of their lipophilic character, cannabinoids can penetrate cellular membranes by diffusion. Initially, possible explanations for cannabinoid activity included unspecific membrane binding resulting in fluidity- and permeability changes of neural membranes, the inhibition of acetylcholine-synthesis, an increase in the synthesis of catecholamines, and an interaction with the synaptosomal uptake of serotonin [Dewey, 1986; Pertwee, 1988]. However, it was established in the mid 1980s that cannabinoid activity is highly stereoselective [Mechoulam, 1992], indicating the existence of a receptor mediated mechanism.

The first reliable indications that cannabinoids act through receptors came when it was shown that cannabinoids can act as inhibitors of the adenylate cyclase second messenger pathway in brain tissue and neuroblastoma cell lines. This activity was dose-dependent, stereospecific, and could be modulated by pertussistoxin [Howlett, 1985, 1986, 1987; Devane, 1988; Bidaut-Russell, 1990]. Finally, a stereospecific G-protein-coupled cannabinoid receptor (CB-1) was found and cloned [Matsuda, 1990].
The CB-1 receptor is most clearly present in the central nervous system, but it is also found in certain peripheral organs and tissues. Amongst others, it inhibits adenylate cyclase activity and the opening of N-type calcium channels [Mackie, 1992]. Shortly after that, a second, periferous cannabinoid receptor (CB-2) was found with a possible role in immunological processes [Munro, 1993]. It is primarily expressed by immune tissues like leukocytes, spleen and tonsils, and it shows a different selectivity than centrally acting CB-1. So far, the physiological roles of CB-2 receptors are proving difficult to establish, but at least one of these seems the modulation of cytokine release (Molina-Holgado, 2003). Surprisingly, there is only a mere 45% homology between the CB-1 and CB-2 receptors.
Based on the observation that all natural cannabinoids are highly lipid soluble, an attempt was made to isolate endogenous ligands for the cannabinoid receptors from fatty tissues of animals. Finally, a single compound could be isolated from porcine brain tissue, with a high affinity for the CB1 receptor, named anandamide (arachidonic acid ethanolamine) [Devane, 1992]. Later, a related compound was isolated from canine gut with an affinity for cannabinoid receptors; 2-arachidonyl glycerol (2-AG, see figure 1.10)) [Mechoulam, 1995]. In recent years, a large variety of compounds with endocannabinoid activity have been isolated or synthesized [Mechoulam, 1998; Pertwee, 2006b], interestingly all having an eicosanoid structure. Cannabinoid receptors and their endogenous ligands together constitute what is referred to as the endogenous cannabinoid (endocannabinoid) system.



Anandamide 2-arachidonylglycerol

Figure 1.10: Structures of the two major endocannabinoids

Not all of the effects of cannabinoids can be explained by receptor-mediated effects, and it is believed that at least some effects are non-specific and caused through membrane turbation [Makriyannis, 1995], or by binding to yet unknown targets in the cell. It has been found in isolated blood vessel preparations that some endocannabinoids can activate vanilloid receptors on sensory neurons [Zygmunt, 1999], which raises the possibility that endocannabinoids are endogenous agonists for vanilloid receptors [Pertwee, 2005]. These receptors might therefore be putatively regarded as CB-3 receptors. The cannabinoid signaling

system is teleologically millions of years old, as it has been found in mammals, fishes, and invertebrates down to very primitive organisms, such as the hydra [De Petrocellis, 1999]. Indeed, there are indications that CB receptors are evolutionary related to the vanilloid receptors [McPartland, 2002].

1.4.2 Therapeutic potential

Cannabis preparations have been employed in the treatment of numerous diseases, with marked differences in the available supporting data. Clinical studies with single cannabinoids (natural or synthetic) or whole plant preparations (e.g. smoked cannabis, encapsulated extract) have often been inspired by positive anecdotal experiences of patients using crude cannabis products for self-treatment. The antiemetic [Dansak, 1997], appetite enhancing [Plasse, 1991], analgesic [Noye, 1974] and muscle relaxant effects [Clifford, 1983], and the therapeutic use in Tourette's syndrome [Muller-Vahl, 1999] were all discovered or rediscovered in this manner. Incidental observations have also revealed therapeutically useful effects. The discovery of decreased intraocular pressure with THC administration, potentially useful in the treatment of glaucoma, was made serendipitously during a systematic investigation of healthy cannabis users [Hepler, 1971]. However, anecdotes as to the efficacy of Cannabis or THC in indications that have not been confirmed in controlled studies have to be judged with caution.

Although most known cannabinoids have been tested to describe their relative potency in comparison to THC (in receptor binding assays or in THC specific assays), up to very recently virtually nothing was known about their own biological activities. However, testing non-THC cannabinoids as serious candidates for new leads, can sometimes lead to completely counter-intuitive results, as shown in the case of THV. Its potency is about ¾ of that of THC in classical *in vitro* assays, [Turner, 1980; Hollister, 1974], while only very recently *in vivo* testing showed THV to be rather an antagonist of THC activity [Thomas, 2005]. And although CBN was initially considered an inactive degradation product of THC, it was later found to have some interesting activities of its own [Herring, 2001; Jan, 2002]. And even while, in potent plant material, THCA can be present at levels of more than 20% of dry weight, its activities remained unstudied for decades. The therapeutic value of the acidic cannabinoid THCA as an immuno-modulating agent has only been discovered very recently [Verhoeckx, 2006], and its effect has been patented. Examples like these show that the study of medicinal cannabis should include the whole array of cannabinoids present, as far as possible [McPartland, 2001].

The therapeutic potential of cannabinoids can be further clarified by pointing out the central physiological importance of the endocannabinoid system, and its homology to, and interaction with the endorphin system. In addition to the role as modulator of food intake, the cannabinoid system is involved in several physiological functions and might be related to a general stress-recovery system. This variety of effects was concisely summarized by Di Marzo *et al.* [1998], who stated that cannabinoids help you 'feel less pain, control your movement, relax, eat, forget (posttraumatic), sleep, and protect your neurons'. The activation of the

endogenous cannabinoid system could represent a crucial and important component for each of these functions. One yet unproven but intriguing idea is that endocannabinoids may set the "analgesic tone" of the body, with the level of their production acting as a kind of pain thermostat. It is likely that such a system relies on the combined activities of a range of compounds. Strategies to modulate endocannabinoid activity include inhibition of re-uptake into cells and inhibition of their degradation to increase concentration and duration of action. The effect of plant cannabinoids interacting with such an endocannabinoid system could be on multiple levels, other than receptor binding alone. Some of such interactions have already been described [Watts, 2004].

The endocannabinoid system that is responsible for our physiological response to cannabis use is in many respects analogous to the endorphin system. It is widely known that opioids and cannabinoids share several pharmacological effects, including antinociception, hypothermia, inhibition of locomotor activity, hypotension, and sedation [Cichewicz, 2004]. Furthermore, crosstalk between the two systems has been shown [Corchero, 2004]. Cannabinoids and opioids both produce analgesia through a G-protein-coupled mechanism, and the analgesic effect of THC is, at least in part, mediated through opioid receptors, indicating an intimate connection between cannabinoid and opioid signaling pathways in the modulation of pain perception [Cichewicz, 2004]. Although both cannabinoids and opioids are accompanied by undesirable side effects at high doses, it was found that THC can enhance the potency of opioids such as morphine, thereby dramatically reducing the dose needed for pain control [Williams, 2006].

In the past, opium abuse led to the study of the physiological effects of opium constituents, which in turn prompted the discovery of opioid receptors. The result was one of our most significant medicines in use today: morphine. The story of cannabis has been exactly analogous to the opium story, up to the point of discovery of the endocannabinoid system. However, there seems to be a reluctance to make the final step and turn cannabinoids into real medicine. A review by the US Institute of Medicine has commented on how little we know about cannabinoids in comparison with opiates [Joy, 1999]. However, the brain has more CB1- than opioid-receptors. The analogy between the history of research into the two groups suggests good reason for optimism about the future of cannabinoid drug development [Vigano, 2005; Pertwee, 2006].

1.4.3 Cannabis medicines

A major obstacle in the development of cannabinoid-based drugs has been the low water solubility of the cannabinoids [Garrett, 1974], which makes it difficult to develop effective formulations for human use [Hazekamp, 2006]. Nevertheless, an increasing number of pharmaceutical companies start to pick up the idea of cannabinoids or their antagonists as therapeutic drugs. At present a number of medicines based on the biological activities of the cannabinoids are available, such as Marinol, Nabilone, and Sativex. Marinol (dronabinol, synthetic Δ^9-THC) and Cesamet (nabilone, a THC-derivative) are registered for the indication

of nausea and vomiting associated with cancer chemotherapy. Marinol is also approved for anorexia and cachexia in HIV/AIDS. Although there are some clear indications that some effects may vary according to the fact if a cannabinoid is taken alone, or in combination with other cannabinoids, virtually no work has been done on the activities of combined cannabinoids. One important exception is the clinical testing of combinations of THC and CBD in the medicinal product Sativex [Russo, 2006], which is currently registered only in Canada.

Several new cannabinoid-based products are expected to be introduced in the near future. Among them are Rimonabant (Acomplia, by Sanofi-Aventis) [van Gaal, 2005], and the potent analgesic ajulemic acid [Burstein, 2004]. Rimonabant was developed based on the observation that cannabis consumption commonly leads to an insatiable feeling of hunger, also known as 'the munchies'. Rimonabant is an antagonist of the CB1 receptor, and causes the opposite to occur. To be launched in the near future, it is expected to become a major drug in the fight against obesity. Ajulemic acid (AJA) is a synthetic analog of the human THC metabolite, THC-11-oic acid. Although the mechanism of AJA action remains largely unknown, it has potent analgesic and anti-inflammatory activity, without the psychotropic action of THC. Unlike the nonsteroidal anti-inflammatory drugs, AJA is not ulcerogenic at therapeutic doses, making it a promising anti-inflammatory drug.

Although it seems clear that the Cannabis plant still has a highly relevant potential for medicine, it is also clear that the medicinal use of cannabis is not a panacea. Cannabis, as any other medicine, can have its side effects, especially when consumed in high amounts. But a widely expressed opinion on the unwanted actions of cannabis and THC has been formulated in a 1999 report of the US Institute of Medicine on the medical use of cannabis: "Marijuana is not a completely benign substance. It is a powerful drug with a variety of effects. However, except for the harms associated with smoking, the adverse effects of marijuana use are within the range of effects tolerated for other medication" [Joy, 1999]. The toxic properties of cannabis are mostly dependent on the content of cannabinoids. The toxicity of cannabis drugs and cannabinoids is considered to be generally low, and comparable to socially accepted psychoactive products like coffee, alcohol and tobacco [Hollister, 1986]. So even though the role of cannabinoids in modern therapeutics remains uncertain, there are enough clues to realize it would be irrational not to explore it further.

In general, there are 5 major concerns about cannabis use: 1) the unabated increase in use, 2) the constant decrease of the age of first use, 3) the increased risk of psychosis in vulnerable people, 4) the constant increase of cannabis heavy users searching help for quitting cannabis use, and 5) the increased risk of driving accidents. However, these worries should not prevent any scientific research on cannabis use in medicine. Instead, a clear distinction must be made between therapeutic and recreational use.

1.5 Cannabis and the law

1.5.1 Political cannabis

Figure 1.11: Medicinal cannabis: requested by a large group of patients, but feared by the authorities.

Starting from 1954, the World Health Organization (WHO) has claimed that cannabis and its preparations no longer serve any useful medical purpose and are therefore essentially obsolete. Up to that moment, cannabis legislation had been based on a large number of conventions, causing considerable confusion in the execution of treaties. Under pressure of increasing reports that cannabis was a drug dangerous to society, it was proposed to combine all in single convention, the draft of which was finally accepted by the United Nations in 1961. In following years several complementary treaties were made to strengthen it. Under the "Single Convention on Narcotic Drugs" cannabis and its products were defined as dangerous narcotics with a high potential for abuse and no accepted medicinal value. It reflected the belief that cannabis was a dangerous narcotic with a threat that was equal to the most dangerous opiates, as it was strongly believed that cannabis use could serve as stepping stone to the use of such drugs.

Since the Single Convention, the potential danger of cannabis abuse by recreational users has been much higher on the political agenda then any of its benefits as a source for fiber, food or medicines (figure 1.11). Nowadays it may be hard to believe, but according to the American president Nixon, cannabis was a secret weapon of the communists, being spread by the Jews to destabilize the Western world. This sense of cannabis-related fear has been the base for the legislation that is currently seriously obstructing the rediscovery of cannabis as a medicine. Even today, under US law, possession of only several grams of cannabis can lead to imprisonment for life. The distinction between medicinal and recreational use is thereby made only in a handful of US States.

It can be observed that new scientific insights on cannabis are only slowly and reluctantly incorporated into new legislation. However, in the coming years, a large variety of scientific and clinical data is expected to become available, further showing the physiological effects of cannabinoids and the endocannabinoid system. And in several Western countries important obstacles for a real acceptance of medicinal cannabis have already been addressed, as serious steps are taken towards decriminalization of cannabis use or even providing medicinal cannabis products to patients [GW pharmaceuticals, 2003; Duran, 2005; Sibald, 2005; Irvine, 2006]. These shifts constitute the first steps away from the dominant drug policy paradigm advocated by the United States, which is punishment-based prohibition, and it signals that the Single Convention may start to reach its expiry date. The legislation that follows it will depend

for a large part on the quality of the research available. However, good arguments will finally not be enough; what is most needed is a change in mentality [Reinarman, 2004]; in politics, but also in the way research is conducted.

1.5.2 The Dutch situation

The Netherlands have known a liberal drug policy already for several decades, so it is not surprising that the Dutch have been among the first to approach the discussion on medicinal cannabis in a practical way. In the 1990s, it was increasingly acknowledged that a considerable group of people was using cannabis for medicinal purposes, obtained through the illicit market. Simultaneously, a growing number of Dutch health officials judged that, although scientific proof on the effectiveness of cannabis might still be insufficient, the perceived dangers of cannabis use no longer outweighed its potential beneficial effects to certain groups of chronically ill patients. However, its unofficial status made it impossible to make any guarantees on the quality, consistency, or origin of the cannabis found in the illicit market. Therefore, in order to supply these patients with a safe and reliable source of high quality cannabis, the Office of Medicinal Cannabis (OMC) was established in March 2000. It started acting as a national agency on 1 January 2001. The OMC is the organization of the Dutch Government which is responsible for the production of cannabis for medical and scientific purposes, and is in full agreement with international law. After an initial preparation period, medical grade cannabis (in the form of dried female flowertops) finally became available in Dutch pharmacies in September 2003, on prescription only. Based on the availability and quality of clinical data and scientific literature, a selection of indications was made by the OMC for treatment with its medicinal grade cannabis [OMC, 2006].
Right from the start, a reliable source of high quality cannabis materials was considered crucial for the success of the Dutch medicinal cannabis program. Therefore, skilled breeders were contracted for the cultivation of plants under highly standardized conditions, resulting in a product with a very consistent composition. The whole process of growing, processing and packaging of the plant material are performed according to pharmaceutical standards, and supervised by the OMC. The quality is guaranteed through regular testing by certified laboratories. Besides supplying high quality cannabis to medicinal users, the OMC also provides the same material for research and development of medicinal preparations based on cannabis constituents.
The availability of reliable cannabis of consistent quality has proven to be crucial to perform good research, as it opened up the way for long term quantitative studies on cannabis and its constituents on a national level. Currently, a variety of laboratories and research groups cooperate for quality control, fundamental research and clinical development. Cannabis research in The Netherlands is blooming, with a clear focus on scientific outcome, rather than on repression of cannabis use. It is exactly these conditions that have made the work for this thesis possible.

1.6 Outline of this thesis

This thesis is written from an analytical, phytochemical point of view, and deals primarily with biochemical aspects of medicinal cannabis. Because, after all, the cannabinoids are widely considered to be the most important (but not the only!) active components of the cannabis plant, the work has been focused on them. And since of all the cannabinoids, THC is the best studied, this cannabinoid became the focus of several chapters in this thesis. However, the main purpose of this thesis is to bring cannabis, as a whole, back into focus.
The work for this thesis was performed in The Netherlands, which has a well known tradition of accepting cannabis as a recreational drug. Although this makes studying the medicinal aspects of cannabis much easier, it is also confusing because the distinction between the two can not always be clearly made. In **chapter 2** it is shown how to make a difference between medicinal and recreational cannabis, and why a regulated source of high grade cannabis is needed for any pharmaceutical research to succeed.
Once the necessity of medicinal cannabis is established, quantitative research can begin. In **chapter 3** a method is developed for purification of the major cannabinoids from plant material, which is the starting point for the production of standards. In **chapter 4** a method is then described to prepare solutions of cannabinoids reference standards. Unfortunately, one potentially important cannabinoid, CBNA, could not be isolated, so a separate method was developed to produce it by partial chemical synthesis. The procedure is described in **chapter 5**.
All cannabinoid standards were then characterized by their chromatographic and spectroscopic properties. Consequently, **chapter 6** provides cannabis researchers with a synoptic overview of the analytical characteristics of the main cannabinoids. But it is clear that even good quality cannabinoid standards can not be used if no method is available for their reliable analysis. For this purpose, an HPLC-DAD method was developed and validated according to the most recent pharmaceutical requirements, as described in **chapter 7**.
Cannabis as a medicine is consumed in a variety of forms and by different routes. A large proportion of medicinal cannabis users prefers to consume it as a tea, but almost nothing has been published on the characteristics of such tea. Therefore the parameters involved in tea-making were systematically studied in **chapter 8**. Although generally, the easiest way of administering a medicine is orally, the low water solubility of the cannabinoids makes this route of administration rather unconvenient. In **chapter 9**, we studied the use of cyclodextrins for improving the aqueous solubility as well as the stability of THC and other cannabinoids.
The most efficient administration route of cannabis is inhalation (smoking). To decrease the exposure to toxic compounds of cannabis smoke, we evaluated the use of a vaporizer device, that can evaporate the active components of the cannabis plant for inhalation, in **chapter 10**.
As a result of these studies, we now have a much better understanding of the cannabis plant, its main active components the cannabinoids, and its galenic formulations and routes of administration.

CHAPTER 2

An evaluation of the quality of medicinal grade cannabis in the Netherlands

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Abstract

Since 2003, medicinal grade cannabis is provided in the Netherlands on prescription through pharmacies. Growing, processing and packaging of the plant material are performed according to pharmaceutical standards and are supervised by the official Office of Medicinal Cannabis (OMC). The quality is guaranteed through regular testing by certified laboratories. However, in the Netherlands a tolerated illicit cannabis market exists in the form of so-called 'coffeeshops', which offers a wide variety of cannabis to the general public as well as to medicinal users of cannabis. Since cannabis has been available in the pharmacies, many patients have started to compare the price and quality of OMC and coffeeshop cannabis. As a result, the public debate on the success and necessity of the OMC program has been based more on personal experiences, rather than scientific data. The general opinion of consumers is that OMC cannabis is more expensive, without any clear difference in the quality.
This study was performed in order to show any differences in quality that might exist between the official and illicit sources of cannabis for medicinal use. Cannabis samples obtained from 11 randomly selected coffeeshops were compared to medicinal grade cannabis obtained from the OMC in a range of validated tests. Many coffeeshop samples were found to contain less weight than expected, and all were contaminated with bacteria and fungi. No obvious differences were found in either cannabinoid- or water-content of the samples. The obtained results show that medicinal cannabis offered through the pharmacies is more reliable and safer for the health of medical users of cannabis.

2.1 Introduction

The use of cannabis as a medicine is increasingly becoming a topic of public discussion in a growing number of countries around the world. As a result of the United Nations Single Convention on Narcotic Drugs (1961), which was followed by a range of complementary treaties, international legislation has been a major obstacle for developments in this field for the last several decades. However, in recent years there have been some serious efforts to bring cannabis back into scientific and clinical research and to permit its use by medical patients. Initiatives that have been taken range from the decriminalization of medicinal cannabis use in the United Kingdom and Switzerland, to serious efforts to give patients direct access to high quality cannabis, or derivatives such as standardized extracts, like in Spain and Canada.
The Netherlands have become the world's first country to make herbal cannabis available as a prescription drug in pharmacies to treat a variety of patients. Since September 2003, pharmacies dispense medicinal cannabis to patients on prescription. Doctors practicing in the Netherlands are allowed to prescribe cannabis to treat a variety of indications (see below). As a general guideline, cannabis should be prescribed only after conventional treatments have been tried and found to be ineffective. As such, cannabis is effectively treated as a last-resort medication.
Because of the unique, liberal situation in the Netherlands with respect to drug laws, an illicit cannabis market can essentially openly compete with pharmacies, and experienced users of medicinal cannabis naturally compare both sources in terms of quality, medicinal effect, and price. It is therefore not surprising that opinions about the quality and efficacy of the state-grown cannabis emerged in the public media. Because of the popularity of cannabis as a theme in the media, opinions about the pharmacy product quickly found their way to the general public and it became clear that a certain fraction of medical cannabis users were not satisfied with the offered type of cannabis. A group of coffeeshop (see below) owners even started a campaign to promote the quality of their own material at the expense of the pharmacy cannabis. However, such opinions and initiatives were generally based on subjective measures and judgements by a group of authoritative and experienced users. Obviously, the opinion-based nature of this debate complicates the evaluation of the introduction of medicinal grade cannabis in the Netherlands and it clearly shows the need to address this matter in a scientific way.
The research presented here challenges the messages in the media about the dissatisfaction of some users with the medicinal grade cannabis offered by the Office for Medicinal Cannabis. This cannabis has been variously claimed to be too weak, too potent or too dry. According to some patients the ‘official’ cannabis doesn’t work, or it does so in a very different manner from what they are used to. Other users are wary of the treatment of medicinal grade cannabis by means of gamma-irradiation, which is routinely done in order to sterilize the material. The most common complaint, however, concerns the higher price. To address these complaints, we tested samples obtained from randomly selected coffeeshops according to the validated quantitative and microbiological analyses that are routinely used for quality control of

medicinal grade cannabis in the Netherlands. The obtained data was compared with that of the simultaneously obtained pharmacy product. The tests for analysis of medicinal grade cannabis used in this study have been described in the official Dutch monograph for medicinal cannabis.
The results presented in this study are intended as a contribution to the discussion about the necessity or advantage of having a policy of centrally regulated production and distribution of medicinal grade cannabis. We hope it can also assist the users of medicinal cannabis in making a well-informed choice in the selection of their medicine.

2.1.1 The Dutch drug policy

In the current situation in the Netherlands, medicinal users of cannabis can obtain their cannabis material from two distinct sources: informally through the street market and formally through the pharmacy. To understand the choices that medicinal users in the Netherlands have to make in order to decide between these two sources, it is important to have some understanding about the Dutch drug policy concerning cannabis [Netherlands Ministry of Foreign Affairs, 2002]
The basic principles of the Dutch drug policy were largely formulated in the mid-seventies. This policy does not moralise, but is based on the assumption that drug use is an undeniable fact and must be dealt with as practically as possible. The most important objective of this drug policy is therefore to prevent or to limit the risks and the harm associated with drug use, both to the user himself and to society. As a results of this, the Ministry of Health is responsible for co-ordinating drug policy.
The cornerstone of this policy is the law known as the Opium Act, which is based on two key principles. Firstly, it distinguishes between different types of drugs on the basis of their harmfulness (cannabis products on the one hand, and drugs that represent an "unacceptable" risk on the other). The terms ‘soft-drugs’ and ‘hard-drugs’ refer to this distinction. Secondly, the law differentiates on the basis of the nature of the offence, such as the distinction between possession of small quantities of drugs intended for personal use, and possession intended for dealing purposes. Possession of up to 30 grams of cannabis is a minor offence, while possession of more than 30 grams is a criminal offence. Drug use itself is not an offence. This approach offers the scope to pursue a balanced policy through the selective application of criminal law.
Dealing in small quantities of cannabis, through the outlets known as “coffeeshops”, is tolerated (condoned) under strict conditions. There are currently about 700 such coffeeshops in the Netherlands, with the majority located in the bigger cities. Tolerance is a typically Dutch policy instrument which is based on the power of the Public Prosecutor to refrain from prosecuting offences. This principle is formulated in the law and is called the “expediency principle”. The small-scale dealing carried out in the coffee shops is thus an offence from a legal viewpoint, but under certain conditions it is not prosecuted. These conditions are: no advertising, no sales of hard-drugs, no nuisance must be caused in the neighbourhood, no admittance of and sales to

minors (under the age of 18), and no sales exceeding 5 grams of cannabis per transaction. The stock of the coffeeshop should not exceed 500 grams of cannabis. If these rules are violated, the coffeeshop can be closed down by the municipal authorities.
The idea behind the Netherlands' policy towards the coffee shops is that of harm reduction. This is based on the argument that if small-scale cannabis dealing and use is not prosecuted under certain conditions, the users – who are mainly young people experimenting with the drug – are not criminalised (they do not get a criminal record) and they are not forced to move in criminal circles, where the risk that they will be pressed to try more dangerous drugs such as heroin is much greater.
It is widely believed that drugs are legally available in the Netherlands, and that no effort is made to combat the supply side of the drug market. Nothing could be further from the truth. There is constant, intensive co-operation between the drug dependence care system, the judicial authorities and the public administrators. With the exception of small-scale cannabis dealing in coffeeshops, tackling all other forms of drug dealing and production has high priority. The police and customs officials regularly seize large hauls of drugs and collaborate closely with other countries in the fight against organized crime. In 2000 alone, about 40,000 kg of cannabis and about 660,000 marihuana plants were seized and 1372 nursery gardens dismantled.
Tolerance does not mean that cannabis smokers can just light up a smoke anywhere they like outside a coffeeshop. Although no formal rules prohibit cannabis smoking in public places, such as bars, restaurants or train stations, very few people do so. If they do, no sanctions are applied; but the person is likely to be asked by the personnel to put out the cigarette. The absence of formal regulations for the use of cannabis has opened the way for these informal norms, and their existence and effectiveness is an aspect of Dutch drug policy that is often underestimated and difficult to grasp by foreigners. For example, tourists who visit Amsterdam commonly make the mistake of thinking they can smoke cannabis 'everywhere'. It must be noted that the majority of the Dutch population, especially senior citizens, have never consumed cannabis and do not know much about cannabis regulations or habits. It's in this complex situation of written and unwritten rules that consumers of medicinal cannabis in the Netherlands have to make choices about obtaining their medicine.

2.1.2 Medicinal cannabis in the Netherlands

Health Minister Els Borst (1994-2002) acknowledged the fact that a considerable group of people was using cannabis obtained through coffeeshops for medicinal purposes. However, its unofficial status makes it impossible to make any guarantees on the quality, consistency, or origin of the cannabis found in coffeeshops. In order to supply these patients with a safe and reliable source of high quality cannabis, the Office of Medicinal Cannabis (OMC) was established in March 2000 and started acting as a national agency on 1 January 2001. The OMC is the organisation of the Dutch Government which is responsible for the production of cannabis for medical and scientifical purposes. It holds the monopoly in the Netherlands for

the import, export, and wholesale of this cannabis and its preparations on behalf of the Minister of Health, Welfare and Sport, and is notified to the International Narcotics Control Board (INCB) in Vienna. The previously mentioned United Nations Single Convention on Narcotic Drugs obliges the Netherlands to organize its Office in this way.
After an initial preparation period, medical grade cannabis became available in Dutch pharmacies in September 2003 on prescription only. Potential users must visit a medical professional (usually their own General Practitioner), who can grant approval for using cannabis for treatment in the form of a prescription.
Based on the availability and quality of clinical data and scientific literature, a selection of indications was made by the OMC for treatment with its medicinal grade cannabis. These are: nausea and loss of appetite resulting from chemotherapy, radiotherapy or HIV-combination therapy; palliative treatment for cancer and HIV patients; spasticity and pain associated with multiple sclerosis or spinal cord injury; chronic neurogenic pain; and physical or verbal tics caused by Tourette's syndrome. However, if they find it necessary in selected cases, medical professionals are allowed to prescribe cannabis for other indications as well.
The medicinal grade cannabis comes in the form of dried and manicured flowertops of female plants and is produced by an authorized grower (Bedrocan BV, Veendam, the Netherlands). Plants are cultivated indoors according to guidelines that have been derived from the general rules for Good Agricultural Practise of the Working Group on Herbal Medicinal Products of the European Medicines Evaluation Agency (EMEA) [OMC, 2003]. The detailed specifications for medicinal grade cannabis can be found on the website of the OMC [OMC, 2006].

Figure 2.1: The 5 gram package of medicinal grade cannabis as currently available in Dutch pharmacies. The variety shown is 'Bedrocan' with a mean THC content of 18%. (Not shown is the variety 'Bedrobinol', with a mean THC content of 13%).

2.2 Materials and methods

2.2.1 Medicinal cannabis of the OMC

Currently, two different cannabis varieties are available in Dutch pharmacies: Bedrocan, mean THC content 18% (specifications: 15.5-21.0%) and Bedrobinol, mean THC content 13% (specifications: 11.0-14.8%). The product is finally packaged in sealed plastic containers in quantities of 5 grams for distribution (figure 2.1). For this study, two original pharmacy packages (total 10 grams) of each variety were obtained through the OMC.

2.2.2 Cannabis sampling

In order to conduct a statistically acceptable experiment on the quality of cannabis obtained from coffeeshops, 10 different coffeeshops were visited. These were randomly and independently selected by Intraval (Groningen/Rotterdam, The Netherlands). Furthermore, an unofficial Dutch foundation specialized in providing cannabis to medical patients was included in the study, resulting in a total of 11 locations where samples were collected. In order to guarantee that these locations remain anonymous, locations are identified by letters only (A-K). In order to limit traveling time, only coffeeshops in the West and middle of the Netherlands (the provinces of Zuid-Holland, Noord-Holland and Utrecht) were visited. About 70% of al Dutch coffeeshops are located in this most densely populated region of the Netherlands [Snippe, 2004].
The person that visited the coffeeshops for collection of the samples pretended to be a family member of a patient suffering from multiple sclerosis, and asked what type of cannabis was recommended for this indication. The recommended cannabis was then purchased (10 grams) for performing the study.

2.2.3 Determination of cannabinoid composition and water content

In order to compare the potency of the samples, contents of delta-9-tetrahydrocannabinol (THC) and its acidic precursor tetrahydrocannabinolic acid (THCA) were determined by HPLC analysis. For the analysis, we used the validated HPLC-method as described in the official Dutch monograph for medicinal cannabis [OMC, 2006]. In order to confirm the results obtained by HPLC, quantification of THC and THCA was repeated by using a recently developed quantitative ^{1}H-NMR method [Hazekamp, 2004b].
Although THC is known to be the major active compound in the cannabis plant, it is widely believed by researchers, as well as patients, that other components (predominantly the cannabinoids) also could play a role in the medicinal properties of cannabis [Williamson, 2000]. The bioactivity of such compounds has been shown in a large variety of scientific studies. Examples are the cannabinoid cannabidiol (CBD) that was shown to be active in the reduction of neuropathic pain [Notcutt, 2004] and cannabinol (CBN) that acts on the immune system [Jan, 2002]. To include non-THC type cannabinoids in our evaluation, the total profile of cannabinoids present in each sample was measured by HPLC, as described above, and by gas chromatography (GC) [Hazekamp, 2005].
Water content of the samples was determined according to the method of Karl-Fischer and was expressed as % of sample weight. Obtained values were confirmed by determining loss on drying after 24 hours heating at 40^{o}C under vacuum.

2.2.4 Microbiology

Policy of the OMC prescribes that microbiological analysis of the medicinal cannabis must be performed after the plants are harvested and again after the final product is packaged. Packaged material must conform with the European Pharmacopeia (EP), chapter 5.1.4, category 2: "microbiological quality of pharmaceutical preparations", which deals with the requirements for medicinal preparations for inhalation. To prevent the formation of microbial toxins, the product is sterilized shortly after harvest by gamma-irradiation (dose <10 kGy) and subsequently packaged under aseptic conditions. If the packaged product does not conform to the microbiological specifications of the EP, the entire batch is rejected for further medical use.
In order to determine the level of microbiological contamination of the obtained samples, microbiological analysis for the presence of potentially harmful bacteria and fungi was performed by Bactimm BV (Nijmegen, The Netherlands), the company that also performs the routine analyses of medicinal cannabis for the OMC.

2.2.5 Price

The most relevant way to compare prices of medicinal preparations is by expressing the price relative to the amount of active ingredient present (price per dosage). In the case of medicinal use of cannabis, it is widely assumed that the major active constituent is THC, although other cannabinoids are believed to play a role as well. Therefore, prices were corrected for the obtained weight of the samples as well as their content of THC. Corrected prices were expressed per 100 mg of THC.

2.3 Results and discussion

For completion of all the analytical tests, 10 grams of cannabis was needed, but the Dutch policy concerning the toleration of coffeeshops prohibits selling more than 5 grams per client per day. Therefore in most cases the sample collector had to return at a later time to obtain another 5 grams of the same cannabis. However, in 4 out of 11 visits the collector was allowed by the coffeeshop to obtain 10 grams at once. The workers in most coffeeshops were found to have experience answering questions concerning the medicinal use of cannabis and were willing to offer advice on matters such as method and frequency of use, as well as on expected results. Although the cannabis was explicitly purchased for medical use, none of the visited locations asked to see a doctor's prescription before selling the cannabis.
Obtained samples were weighed in order to divide them up in portions for performing the different tests. It was found that less than 9.50 grams were present in the obtained package(s) in 5 out of 11 cases, meaning a deficit of more than 5%. A variation of 5% in content is the tolerance that is usually accepted in trade in the EU. In one case (coffeeshop A) only 7.49 grams (-25%) were delivered. Although it was not an objective of our study, these results

indicate that falsification of weight (whether intentionally or not) is not merely an incidental problem. In contrast, both samples obtained from the OMC contained almost exactly the expected amount of 10 grams (± 0.1 gram). The prices and obtained weights of the samples are listed in table 2.1.

Table 2.1: Prices paid for each sample when '10 grams' was demanded, and amount of sample (in grams) actually obtained in the purchase. For Bedrocan and Bedrobinol, '10 grams' was obtained by combining 2 standard pharmacy packages of 5 grams each.

Cannabis sample	Price (euro)	Obtained weight (gram)
Bedrocan	€ 93.92	9.97
Bedrobinol	€ 81.94	9.90
A	€ 48.00	7.49
B	€ 50.00	9.83
C	€ 60.00	8.37
D	€ 60.00	10.79
E	€ 48.00	9.30
F	€ 60.00	9.63
G	€ 60.00	9.77
H	€ 70.00	9.61
I	€ 50.00	8.81
J	€ 60.00	9.49
K	€ 60.00	9.61

In fresh cannabis plant material, THC is predominantly present in the form of its acidic precursor THC-acid (THCA). Under the influence of heat or storage, THCA can be converted into free THC. For the recreational as well as the medicinal user, THC is the most important bio-active component, and therefore it is common practise in analytical laboratories to determine the total THC content of cannabis (THCA + THC) after heating of the plant material. However, this method is not completely reliable because a full conversion of THCA to THC is difficult to achieve. Furthermore, during the heating process degradation products of THC (such as cannabinol or delta-8-THC) can form or evaporation of THC can occur [Veress, 1990]. During this study these problems were prevented by determining the amount of THCA and THC individually. From these results the total THC content was then calculated. This method has only recently become available, through the development of a reliable THCA reference standard for quantification [Hazekamp, 2004b].
THC-content of the samples is shown in figure 2.2. For all coffeeshop samples, the THC content was found to be in the range of 11.7-19.1% (as percentage of dry weight plant material), which is consistent with values reported earlier [Pijlman, 2005]. The THC content of the pharmacy varieties fell also within this range: variety 'Bedrocan' (16.5% THC) was found in the middle of the range, while variety 'Bedrobinol' (12.2% THC) was at the lower end of the range.

Besides THC and THCA, other cannabinoids were taken into account as well during analysis of the cannabinoid composition of the samples. However, no major differences were observed among the coffeeshop samples when comparing the obtained GC- or HPLC-chromatograms.

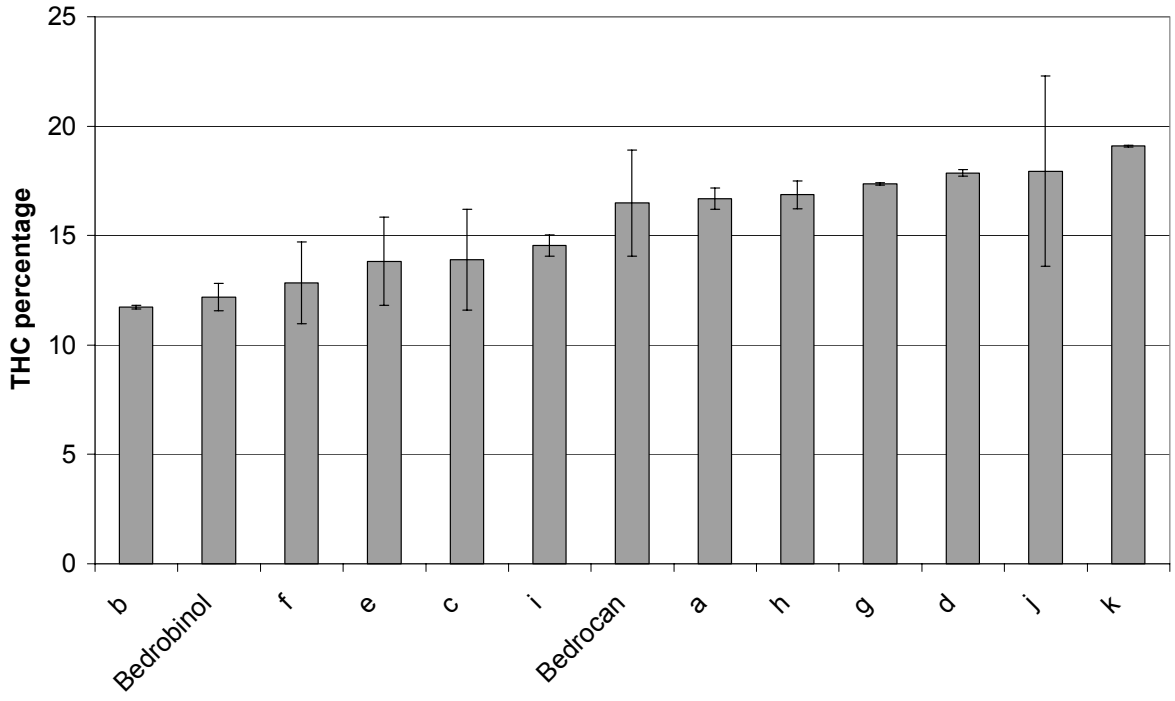

Figure 2.2: Content of total THC for each sample in % of sample weight. Results are shown in increasing order. Values are the mean of 2 determinations. Errorbars indicate standard error.

Likely, this is the result of decades of cross-breeding and selection of high-THC producing strains of cannabis. Possibly, this process has minimized the variability between the cannabis strains, with some exception for their content of THC. Some representative HPLC chromatograms are shown in figure 2.3.

When coffeeshop samples were compared to the OMC samples, only one noticeable difference was observed: the latter contains a larger proportion of free THC, and a correspondingly lower proportion of its carboxylic acid precursor THCA. We expect this to be the result of handling and packaging, which is likely to convert some THCA into free THC. A higher content of free THC can be beneficial when a patient consumes the cannabis in a form that has not been heated strongly or long enough, like in the case of an infusion (for cannabis tea). Under such conditions THCA will not be completely transformed into THC so a smaller amount of the active component THC will be consumed. However, when the cannabis is consumed by smoking or in the form of strongly heated products (e.g. baked products such as cookies), the transformation of THCA into THC will be virtually complete and the observed differences in initial free THC content will become irrelevant.

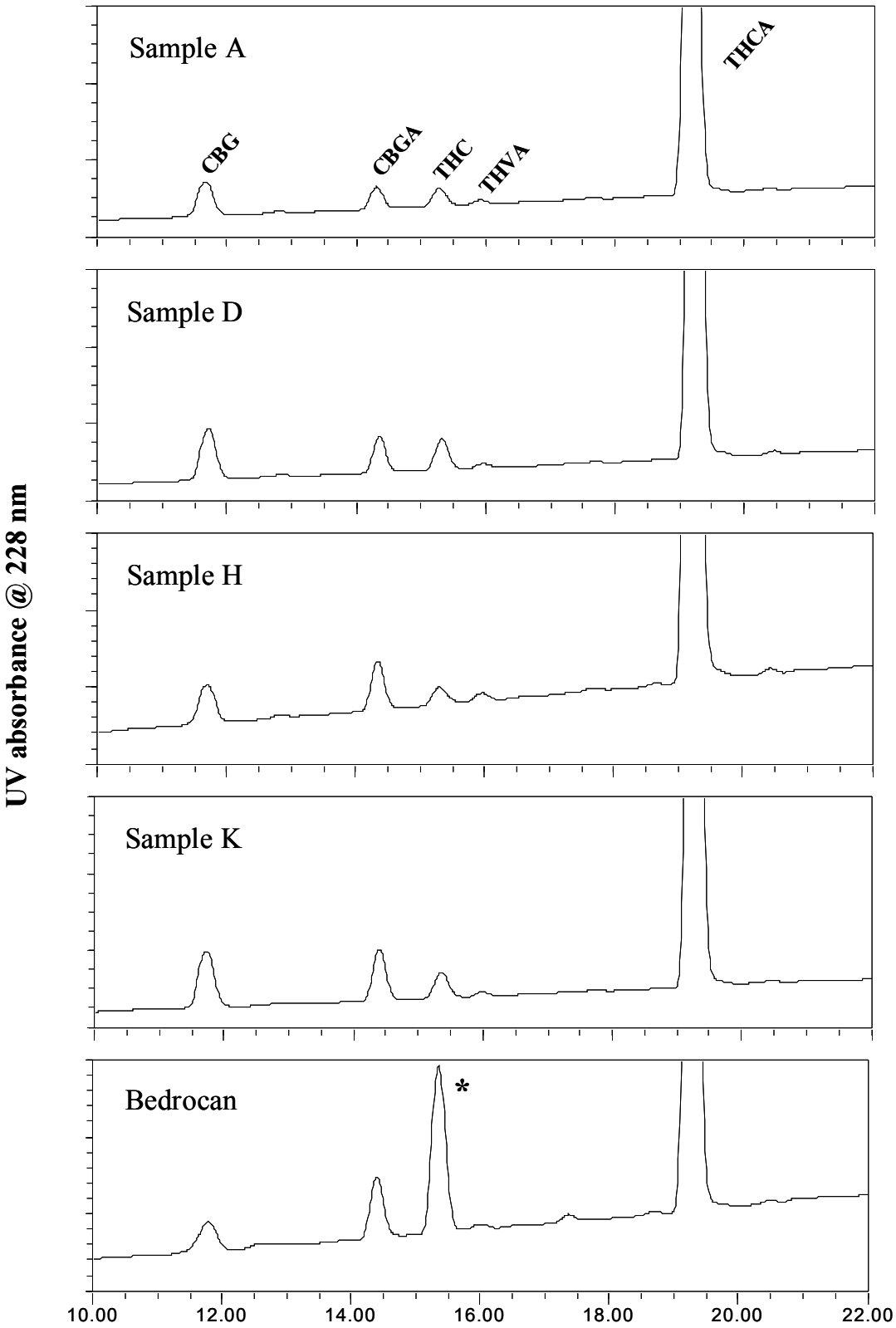

Figure 2.3: HPLC chromatograms (228 nm) of selected samples. No cannabinoids were observed outside the shown region of the chromatograms. Pharmacy cannabis contains a larger proportion of free THC (*). CBG: cannabigerol; CBGA: cannabigerolic acid; THVA: tetrahydrocannabivarinic acid.

When water content of the samples was compared, it was found that the OMC-variety 'Bedrocan' (water content 4.7%) was not significantly different compared to the coffeeshop samples, where water contents ranged from 3.9-5.5%. For the variety 'Bedrobinol' however, a significantly higher water content of 8.0% was found. According to the OMC, this value was intentionally higher, after comments from users, in order to make the inhalation of this variety more pleasurable. According to OMC specifications the water content of the cannabis at the time of quality control (directly after packaging) must be between 5-10%.
The EP requirements with regard to microbiological purity for inhalation preparations set the following limits for sample contamination: total molds and aerobic bacteria: ≤10 colony forming units (CFU) per gram; total enterobacteria and gram-negative bacteria: ≤100 CFU per gram. The infectious bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* must be completely absent. As shown in table 2.2, all samples obtained from coffeeshops carried contamination levels of bacteria and/or fungi above these limits. In contrast, both cannabis varieties from the OMC were found to be clear of such contaminations. According to the OMC, rejection of its medicinal cannabis based on microbiological contamination has never occurred to date.

Table 2.2: Presence of bacteria and fungi (in cfu per gram) in the studied samples.
[1)] CFU per gram = colony forming units present in one gram of the sample.
[2)] The contaminants on sample K were further identified to be the bacterium *E. coli*, and fungi of the types *Penicillium*, *Cladosporum* and *Aspergillus*.

Cannabis sample	Enterobacteria and Gram-negative bacteria (cfu/gram) [1)]	Molds and aerobic bacteria (cfu/gram) [1)]
Bedrocan	<10	< 100
Bedrobinol	<10	< 100
A	<10	480000
B	4500	900
C	<10	1000
D	70	120
E	13000	6500
F	80000	4800
G	180	350
H	27000	1300
I	350	4200
J	23000	91000
K [2)]	5900	3600

The mycological laboratory of Centraal Bureau voor Schimmelcultures (CBS, Utrecht, the Netherlands) further analyzed the contaminants present in one of the samples (sample K), and identified several known pathogens, including the intestinal bacterium *Escherichia coli*, and fungi of the *Penicillium*, *Cladosporum* and *Aspergillus* types. Some of these microbes are

capable of producing hazardous mycotoxins, such as aflatoxin B, ochratoxin A and B, and sterigmatocystine.

Aflatoxins, in particular, are known to be extremely potent carcinogens [Ricordy, 2002]. They are not completely destroyed by heat during smoking, and thus may be inhaled [Kagen, 1983; Georggiett, 2000]. The presence of potentially hazardous fungi on recreationally-used cannabis has been repeatedly described and increasingly these fungi are being acknowledged as an underestimated source of neurological toxicity [Carod Artal, 2003] or infections such as aspergillosis [Llewellyn, 1977; Hamadeh, 1988; Wallace, 1998]. There are some indications that the use of anti-inflammatory steroids can increase the susceptibility to fungal infections [Marks, 1996] and it should be noted that a significant fraction of the population of patients that uses medicinal cannabis also uses such drugs. Moreover, medicinal cannabis is relatively commonly used by HIV/Aids patients and other types of patients who, because of their compromised immune systems, are specifically vulnerable to infections. Opportunistic lung infections with *Aspergillus* have already been suggested as a serious contribution to morbidity in this subgroup of patients [Wallace, 1988; Johnson, 1999].

Even for consumers who are not immuno-compromised, neurological toxicity of contaminated cannabis samples is pointed out as a health risk [Carod Artal, 2003]. Therefore, these combined data indicate that medicinal use of cannabis that has been purchased from uncontrolled sources could be considered as a potential health risk for the population of medicinal users, particularly for those who consume larger amounts of cannabis on a daily basis.

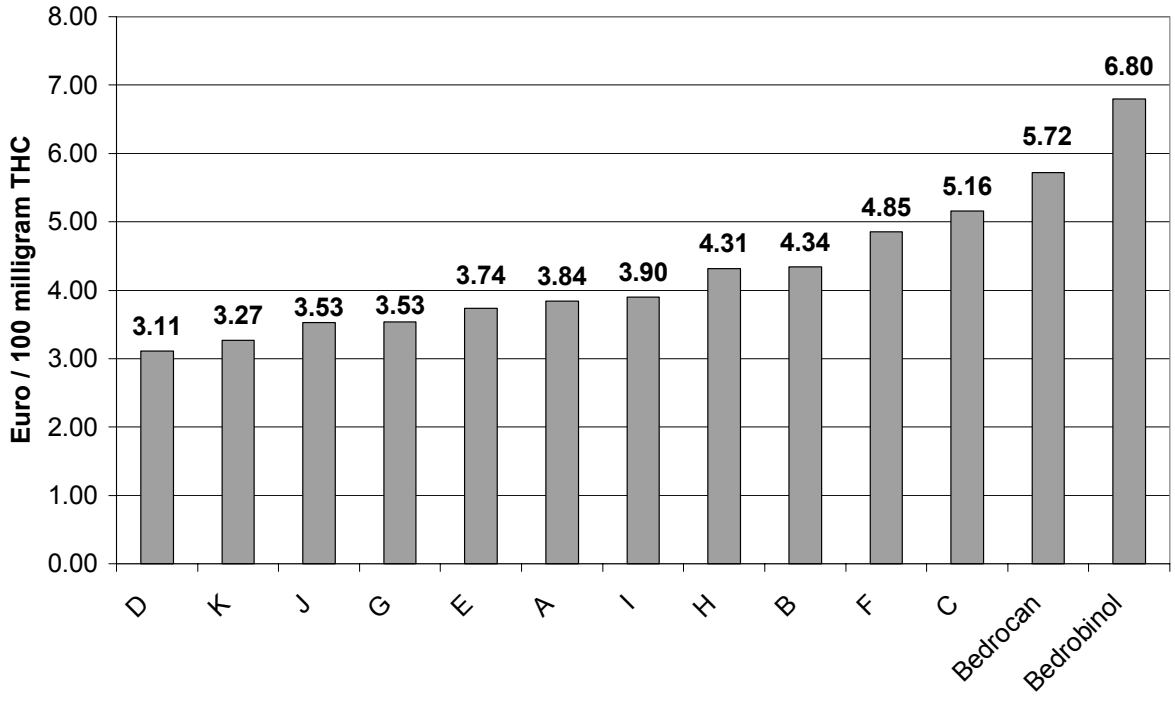

Figure 2.4: Price of each sample, expressed as price (in euros) paid per equivalent of 100 mg THC. Results are shown in increasing order.

The higher price of medicinal cannabis has proven to be a major drawback for medical patients in the Netherlands to obtain their cannabis from pharmacies. By expressing the price of the samples relative to the level of THC present, a fair comparison between the obtained samples is possible. Results are shown in figure 2.4. It is shown that the price of the pharmacy variety 'Bedrocan' (€ 5.72 per 100 mg THC) is somewhat above the range of prices that were paid for coffeeshop samples (€ 3.11–5.16). The relative price of the 'Bedrobinol' variety, however, is significantly higher (€ 6.80). According to OMC, the higher costs of medicinal grade cannabis are the result of maintaining a high quality standard for the product. Included are: production according to pharmaceutical standards, aseptic packaging, distribution and costs made by pharmacies. Moreover, costs accrue as a result of constant quality controls and microbiological analyses. Finally, pharmacy cannabis includes a 6% VAT charge, while the EU VAT system does not allow that VAT is charged on the illicit (although tolerated) cannabis from coffeeshops.

2.4 Conclusion

The simple rules of supply and demand usually result in the consumer buying the product with the best quality-to-price ratio. As a result, the unique situation in the Netherlands has led to a confusing situation for medicinal users of cannabis. Price comparisons and superficial inspection easily lead to favouring the cheaper material from the coffeeshops over the more expensive, but seemingly equal, pharmacy grade. The fact that only the quality of the latter is guaranteed through regular controls does not seem to impress most consumers. However, it is obvious that the standards for any medicinal preparation are high and that these can be enforced only by appropriate analytical testing. According to the OMC, another reason why the price of Cannabis available in pharmacies is currently somewhat higher than expected, is because sales are relatively low. If the number of patients would increase, this could reduce the price because the fixed costs per sold unit would drop.

Because the number of coffeeshop samples that were used for this study was limited, conclusions must be drawn with some precaution and results presented here should be reported as incidental findings. Still, based on the obtained results we concluded that the price paid for medicinal cannabis distributed through the Dutch pharmacies must be considered reasonable. The cannabinoid strength and composition of the pharmacy products and the water content are not significantly different from other types of cannabis. In contrast, the pharmacy product is guaranteed to have a consistent potency, and potentially harmful microbial contaminations are absent. These results indicate that routine analysis of the cannabis results in a significantly safer product of high and reproducible quality. Delivery of medicinal cannabis to patients through the OMC and pharmacies results in a reliable product without the health risks commonly associated with coffeeshop cannabis.

Some patients have claimed that the official cannabis simply is not as good as their personal choice of 'medi-weed'. Certainly, the possibility remains that cannabis varieties with a similar cannabinoid profile can have different strengths or effectiveness, based on the presence of

other components such as terpenoids or flavonoids. Nevertheless, the current scientific consensus is that mainly the cannabinoids are responsible for the bioactivity of cannabis, and testing of the samples by two different methods did not show obvious differences in cannabinoid composition. In conclusion, it seems that there remains some room for discussion on this point.

When patients choose to obtain cannabis from an uncontrolled source, they must realize that they do so with a certain risk to their health. In this test, we did not check for the presence of pesticides, fungicides or heavy metals, but there are multiple indications that these are frequently present in cannabis samples from uncontrolled sources [McPartland, 1997; Ware, 2005]. The same lack of quality control makes it impossible to determine whether products that are claimed to be grown organically, like in some coffeeshops, are really that much more trustworthy. Ultimately, it is the consumer that makes the choice. We hope that the research presented in this article may help the consumer to make an informed and safe choice.

Tests for the presence of heavy metals and pesticides are routinely performed for the OMC cannabis. Therefore the medicinal grade cannabis in Dutch pharmacies is guaranteed to be free (below official standard limits) of such contaminants. Unfortunately, because such tests are very costly, they could not be carried out as part of this study. Future studies should therefore include a larger number of sampled locations, and could include analysis for the presence of heavy metals, pesticides or fungicides.

CHAPTER 3

Preparative isolation of cannabinoids from *Cannabis sativa* by centrifugal partition chromatography

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Abstract

A simple method is presented for the preparative isolation of seven major cannabinoids from *Cannabis sativa* plant material. Separation was performed by centrifugal partition chromatography, a technique that permits large scale preparative isolations. Using only two solvent systems, it was possible to obtain purified samples of the cannabinoids; (-)-Δ^9-(*trans*)-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), cannabinol (CBN), cannabigerol (CBG), (-)-Δ^9-(*trans*)-tetrahydrocannabinolic acid-A (THCA), cannabigerolic acid (CBGA) and cannabidiolic acid (CBDA). A drug-type and a fiber-type cannabis cultivar were used for the isolation. All isolates were shown to be 90-95% pure by gas chromatography. This method makes new cannabinoids available on a large scale for biological testing. The method described in this report can also be used to isolate additional cannabinoids from cannabis plant material.

3.1 Introduction

In recent years, a lot of research on the medical applications of *Cannabis sativa* L. has been initiated, as several, mostly European, countries move towards a more liberal view on the use of cannabis as a medicine. Research on the cannabis plant and on the patients using cannabis products demands reference compounds in the form of purified cannabis constituents. Although more than 400 compounds have been identified in cannabis [Turner, 1980], most studies focus on the effects of the cannabinoids, in particular (-)-Δ^9-(*trans*)-tetrahydrocannabinol (Δ^9-THC). Most of the effects of cannabis have been attributed to Δ^9-THC, and synthetic Δ^9-THC (dronabinol, Marinol©) has been approved for some medical applications. However, in several medical studies the effect of Δ^9-THC or dronabinol alone could not match the effect of a total cannabis preparation [Williamson, 2000], indicating there might be other active compounds present. More than 60 cannabinoids have been found in cannabis [Turner, 1980], and occasionally new cannabinoids are still being discovered [Taura, 1995a]. Only a few of the known cannabinoids have been studied in some detail, although many of these have been shown to possess some biological activity (reviewed by Grotenhermen, 2002).

Although it seems justified to investigate cannabinoids other than Δ^9-THC alone, the biggest obstacle is the availability of sufficient amounts of highly pure reference standards for calibration of analytical tools and for medical studies. Only a few of the naturally occurring cannabinoids are commercially available today: Δ^9-THC, Δ^8-THC, CBD and CBN. In fresh plant material of cannabis, most cannabinoids are present as their carboxylic acid form, known as acidic cannabinoids [Shoyama, 1975]. The free phenolic forms of the cannabinoids are also known as neutral cannabinoids. Of the acidic cannabinoids, only (-)-Δ^9-(*trans*)-tetrahydrocannabinolic acid (THCA) has been studied biologically to some extent [Tampier, 1973], as far as we know. Although it is the most abundant cannabinoid found in drug-type cannabis, it is not yet commercially available. For THCA and other acidic cannabinoids several isolation methods or synthetic routes have been described, but most of these methods were inefficient, time-consuming or not suitable for preparative isolations [Mechoulam, 1965; Yamauchi, 1967; Mechoulam, 1969a; Gaoni, 1971; Lehmann, 1992].

In this study centrifugal partition chromatography (CPC) was tested for the large scale isolation of cannabinoids. It is a countercurrent liquid-liquid partitioning chromatography technique in which the stationary phase is immobilized by centrifugal force, while the mobile phase is pumped through at high flow rates. During a separation, sample components are partitioned between the mobile and the stationary phases, and are separated on the basis of differences in their partition coefficients. CPC offers particular advantages in the isolation of compounds; there is no irreversible retention, it can cover a broad scale of polarities and it has a very high capacity because of the large volume of stationary phase involved in the separation process. CPC can be used on a preparative scale with an injection size up to several grams. The method was first described by Murayama *et al.* [1982] and the theoretical aspects were discussed by Foucault [1994]. Another countercurrent chromatography technique, droplet

counter-current chromatography (DCCC) was used for the first isolation of THCA as a complex with dimethylformamide [Korte, 1965].
The isolated seven different acidic and neutral cannabinoids were analyzed for purity by GC and additional analysis was done by HPLC and thin layer chromatography (TLC). Purity of all isolates was 90% to 95%. The isolated cannabinoids are suitable as standards for quantification experiments, or as reference compounds in biological assays. The use of different cannabis cultivars for the isolation of additional cannabinoids is discussed.

3.2 Materials and methods

3.2.1 Standards and solvents

A standard of Δ^8-tetrahydrocannabinol (Δ^8-THC) was obtained from Sigma (St. Louis, MO, USA). Standards of CBD and CBN were a kind gift of the Dutch Forensic Institute (NFI, Rijswijk, The Netherlands). Reference compounds of Δ^9-THC, cannabigerol (CBG), cannabigerolic acid (CBGA), cannabidiolic acid (CBDA) and THCA were isolated previously in our laboratory by using preparative HPLC and identified as described below. **T**he structures of these cannabinoids can be found in chapter 1 of this thesis, figure 1.6.
All organic solvents (analytical or HPLC reagent grade) were purchased from J.T. Baker (Deventer, The Netherlands).

3.2.2 Plant material

Cannabis sativa L. plant material of the drug type (cultivar SIMM02) was obtained from Stichting Institute for Medical Marijuana (SIMM) in Rotterdam, The Netherlands. After harvest, the plant material was air-dried in the dark under constant temperature and humidity for 4 weeks. Fiber-type cannabis (cultivar Kompolti) was grown outdoors in the garden of our institute. Plant material was harvested in October 2002 and air-dried as described above. No pesticides or other chemicals were applied to the plants.
For isolation of cannabinoids only female flowertops were used. These were manicured to remove other plant parts such as leaves and stems. Plant material was stored at –20°C until used.

3.2.3 Thin Layer Chromatography (TLC)

Samples were manually spotted on 10x20 cm reversed phase (C_{18}) silica gel plates F254 No. 105559 (Merck, Darmstadt, Germany) and developed in saturated normal chambers (saturation time 15 minutes). Eluent was methanol : 5% acetic acid, 19:1 (v/v). After development, visual inspection was done under UV 254nm. General visualization of compounds was done by spraying with modified anisaldehyde-sulphuric acid spray reagent [Stahl, 1967]. For selective visualization of cannabinoids the TLC plate was sprayed with 0.5%

fast blue B salt (Sigma) in water, followed by 0.1M NaOH [Corrigan, 1980]. Reference standards were used for identification of chromatographic spots.

3.2.4 High-Performance Liquid Chromatography (HPLC)

The HPLC profiles were acquired on a Waters (Milford, MA) HPLC system consisting of a 626 pump, a 717plus autosampler and a 2996 diodearray detector (DAD), controlled by Waters Millennium 3.2 software. The profiles were recorded at 285nm to keep a stable baseline during the gradient. Full spectra were recorded in the range of 200-400nm. The analytical column was a Vydac (Hesperia, CA) C_{18}, type 218MS54 (4.6x250 mm, 5 µm), with a Waters Bondapak C_{18} (2x20 mm, 50 µm) guard column. The mobile phase consisted of a mixture of methanol-water containing 25 mM of formic acid in gradient mode; methanol:water in ratios from 65:35 to 100:0 over 25 minutes, then isocratic to 28 minutes. The column was re-equilibrated under initial conditions for 4 minutes. Flowrate was 1.5 ml/min and total runtime was 32 min. All determinations were carried out at ambient temperature.

3.2.5 Gas Chromatography (GC-FID) and Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-FID profiles were generated with a Chrompack (Middelburg, The Netherlands) CP9000 gas chromatograph, fitted with a Durabond fused silica capillary column (30 m x 0.25 mm inner diameter) coated with DB-1 (J&W scientific Inc., Rancho Cordova, CA) at a film thickness of 0.1 µm. The (FID) signal was recorded on a Shimadzu (Kyoto, Japan) CR3A integrator. The oven temperature was programmed from 100°C to 280°C at a rate of 10°C/min. The oven was then kept at 280°C until the end of the runtime of 30 minutes. The injector and the detector temperatures were maintained at 280°C and 290°C, respectively. Nitrogen was used as the carriergas at a pressure of 70 kPa. Air and hydrogen were used as detector gases. The injection split ratio was 1/50.
To obtain mass-spectral data of isolated compounds, GC-MS analyses were performed on a Varian (Bergen op Zoom, The Netherlands) 3800 gas chromatograph, coupled to a Varian Saturn 2000 mass spectrometer operating in the electron impact (EI) mode. The GC was fitted with a Varian VA5MS capillary column (30 m x 0.25 mm inner diameter) coated with DB1 at a film thickness of 0.25 µm. The oven temperature was programmed as described above. Helium was used as the carriergas at a pressure of 65 kPa. The injection split ratio was 1/50. The system was controlled by Varian Saturn GC/MS workstation version 5.2 software. All GC-MS samples were analyzed without prior derivatization.

3.2.6 Extraction

Dried flowertops of SIMM02 (50 gr) and Kompolti (100 gr) were extracted three times by maceration with 1.25 L of *n*-hexane for several hours. Each extraction was started by 5

minutes of sonication. Finally, the three sequential extracts were combined and filtered over a glass-filter. HPLC analysis showed that SIMM02 extract contained mainly THCA and CBGA, while the Kompolti extract contained mainly CBDA.

3.2.7 Separation of acidic and neutral Cannabinoids

A glass-filter (mesh size 2) of about 5 cm in diameter and 7 cm in height was filled for 2/3 with acid-washed see-sand (Sigma) and topped with glass pearls (±1 mm diameter). Before use the sand was sequentially washed with 200 ml of hexane, ethanol and water. Cannabis hexane extract was concentrated to about 5 ml of hexane, placed drop-wise on top of the sandfilter and evaporated by using a warm air blower. The sandfilter was then placed onto a suction Erlenmeyer and acidic cannabinoids were eluted by washing the sandfilter under vacuum with a 0.1 M NaOH solution. The elution was continued until the eluate turned from deep-orange to colorless. Neutral cannabinoids and other compounds were then eluted with ethanol (200 ml), followed by hexane (200 ml). Acidic cannabinoids were precipitated in the aqueous eluate by adding HCl until the pH reached 2 and then filtered through the (dried) sandfilter. The precipitate that remained on top of the sandfilter was finally eluted with ethanol (200 ml). Neutral and acidic cannabinoid fractions were both concentrated into a small volume by evaporation under reduced pressure and analyzed by GC and HPLC.

3.2.8 Centrifugal Partition Chromatography (CPC) apparatus

A Sanki (Kyoto, Japan) centrifugal partition chromatograph (type LLB-M), equipped with a 100 ml cartridge was used. It was connected to a Shimadzu LC-10ADvp pump, a Rheodyne (Cotati, CA, USA) manual injector with a 5 ml loop and a Pharmacia (Roosendaal, The Netherlands) FRAC-100 fraction collector. Pressure was limited to 100 bar.

3.2.9 Isolation of acidic cannabinoids

For the isolation of THCA and CBGA by CPC the two-phase system hexane/methanol/water, 5:3:2 (v/v/v, solvent system 1) was used. The aqueous phase of the solvent system was acidified with 25mM of formic acid. During the run the methanol/water ratio of the mobile phase was linearly increased from 3:2 to about 4.5:0.5 (by addition of methanol) to speed up the elution of retained compounds. The CPC was operated in descending mode, by using the upper (hexane-rich) layer as stationary phase, and the lower (aqueous) layer as mobile phase. The flowrate was set at 4 ml/min and rotation speed was 500 rpm. The volume of stationary phase was 70 ml under these conditions. The sample (2.5 gr of the acidic cannabinoids fraction of SIMM02) was dissolved in upper layer until a final volume of 5 ml for injection. Fraction size was 10 ml and 50 fractions were collected. Each fraction was analyzed by TLC and selected fractions were further analyzed by HPLC. Fractions containing a high proportion (>90%) of a

single cannabinoid (THCA or CBGA) were combined and evaporated to dryness. Isolates were redissolved in 5 ml of ethanol and kept at –20°C for qualitative analysis.
CBDA was isolated from the acidic cannabinoid fraction of Kompolti extract as described above, using the same CPC two-phase system (solvent system 1).

3.2.10 Isolation of neutral cannabinoids

Slightly different methods were used to isolate the neutral cannabinoids Δ^9-THC, CBN, CBD and CBG. For the isolation of CBN, 600 mg of THCA (isolated as described above) was decarboxylated by heating; the sample was placed in a heat-resistant open glass vial and ethanol was evaporated by flushing with nitrogen-gas. The open vial was then placed into a preheated oven at 135°C overnight. The color of the sample darkened considerably during heating. Total decarboxylation of THCA was confirmed by HPLC. The resulting mixture of CBN, Δ^9-THC, and some Δ^8-THC was fractionated by CPC.
For the isolation of CBD, the acidic cannabinoids fraction of Kompolti extract was used. After evaporation of the solvent, 600 mg was heated at 180°C for 10 minutes in an open glass container. Total decarboxylation of CBDA was confirmed by HPLC. The resulting mixture of CBD and lower amounts of other neutral cannabinoids was fractionated by CPC. Isolation of CBG was performed according to the same protocol using 1.0 gr of the acidic cannabinoids fraction of SIMM02 extract.
Isolation of Δ^9-THC was done from the neutral cannabinoids fraction of SIMM02 extract. After evaporation of the solvent, 510 mg of the neutral cannabinoids fraction of SIMM02 extract was directly fractionated by CPC.
Fractionation of neutral cannabinoids was performed by CPC using the two-phase system hexane/acetone/acetonitrile, 5:2:3 (v:v:v, solvent system 2). The CPC was operated in ascending mode, with the lower (acetonitrile-rich) phase used as stationary phase and the upper (hexane-rich) upper phase as mobile phase. The flow-rate was set at 5 ml/min and rotation speed was 600 rpm. The volume of stationary phase was 65 ml under these conditions. The sample was dissolved to a final volume of 5 ml of upper phase for injection. Fraction size was 10 ml and 50 fractions were collected. Each fraction was analyzed by TLC and selected fractions were further analyzed by HPLC. Fractions containing a high proportion (>90%) of the desired compound were combined and subsequently evaporated under reduced pressure. The final sample was redissolved in 5 ml of ethanol and kept at –20°C for qualitative analysis.

3.2.11 Confirmation of identity and purity of isolated cannabinoids

The identity of isolated cannabinoids was confirmed by comparing retention times (HPLC and GC) and spectroscopical data (UV, MS) with reference compounds and literature data [Budzikiewicz, 1965; Mechoulam, 1969b; Gaoni, 1971; Brenneisen, 1988; Lehmann, 1992]. Purity of isolated cannabinoids was determined by GC-FID at a concentration of 1 mg/ml (by

weight). To visualize compounds that cannot be detected by GC, samples were also qualitatively analyzed by HPLC and TLC.

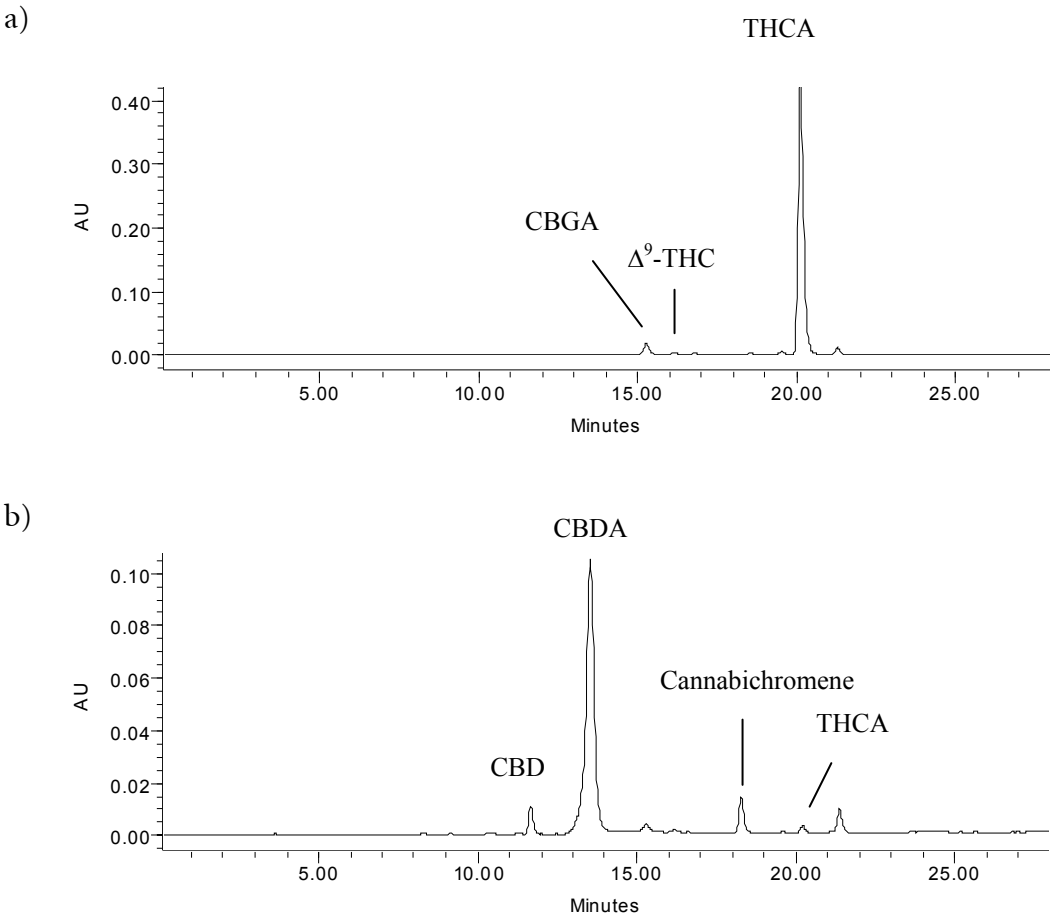
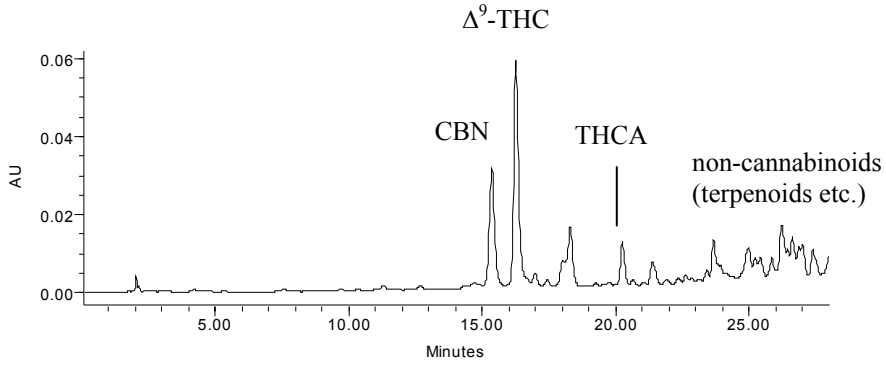


Figure 3.2: HPLC-chromatograms of the hexane extract of cannabis cultivars SIMM02 (a) and Kompolti (b). Main cannabinoid peaks are indicated

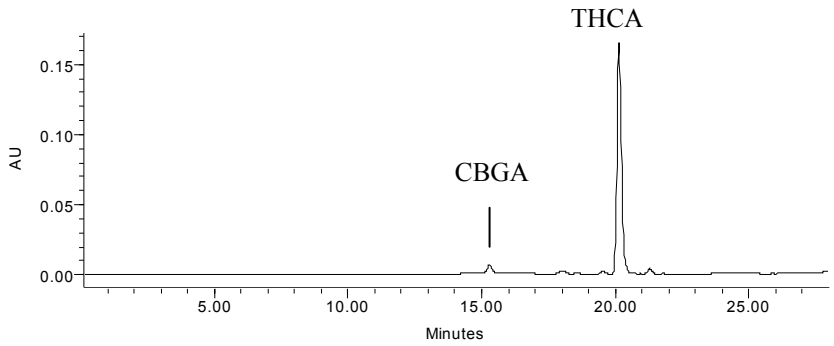
3.3 Results and discussion

For the isolation of seven different cannabinoids, two different types of *Cannabis sativa* L. were used. The structures of the isolated cannabinoids can be found in chapter 1, figure 1.6. Analysis of the hexane extracts by HPLC showed that the main compounds of SIMM02 were THCA and CBGA, while CBDA was the main compound for the Kompolti cultivar (figure 3.2). *n*-Hexane was chosen as the extraction solvent because it is easy to evaporate, it is relatively non-toxic, and it didn't extract chlorophyll, which interferes with most of the chromatography techniques. The extraction yields for the drug-type cannabis SIMM02 and the fiber-type cannabis Kompolti were 17% and 3%, respectively. By making use of the solubility of acidic cannabinoids in water under basic conditions, the acidic cannabinoids could efficiently be separated from neutral cannabinoids and other plant compounds in the hexane extract as shown by HPLC analysis (figure 3.3).

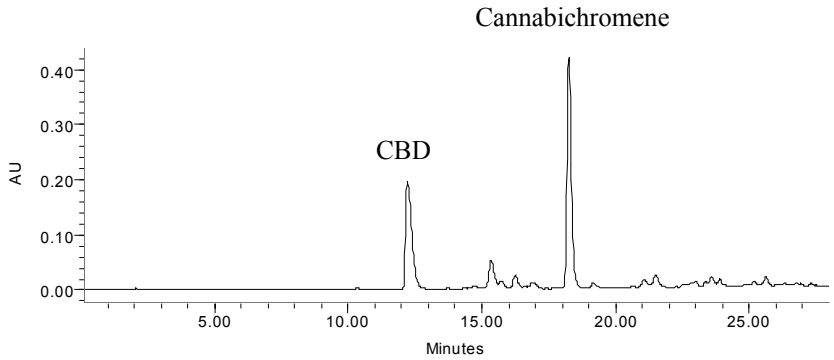
1a)



1b)



2a)



2b)

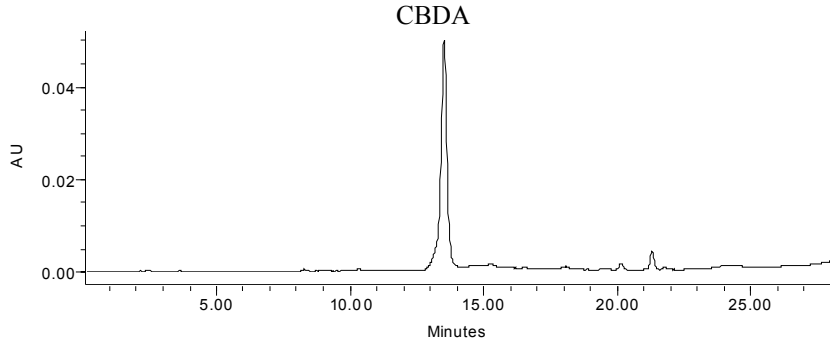


Figure 3.3: HPLC-chromatograms of neutral (a) and acidic (b) cannabinoid fraction after sandfilter fractionation of hexane extracts. 1) SIMM02; 2) kompolti

The acidic cannabinoids fraction, resulting from the sandfilter separation, was the preferred starting material for the isolation of cannabinoids, because it is free of interfering compounds such as lipids or terpenoids, and it contains the highest yield of extracted cannabinoids. About 2/3 of the weight of the total hexane extract was recovered in the acidic cannabinoids fraction. A schematic overview of the isolation of the different cannabinoids can be seen in figure 3.4.

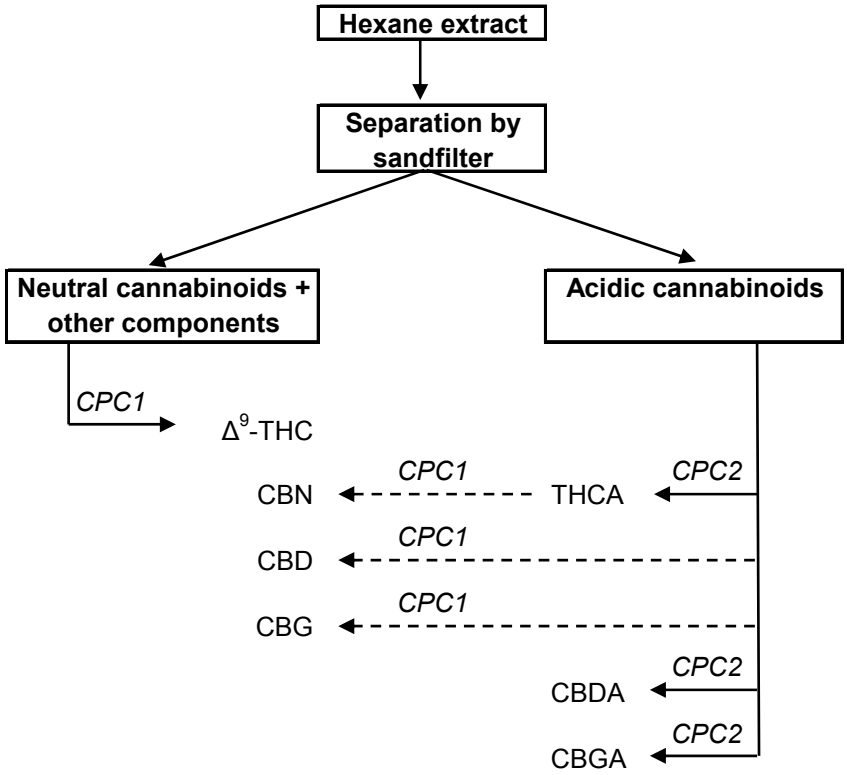


Figure 3.4: Scheme of the preparative scale isolation of cannabinoids from Cannabis sativa hexane extract. CPC; separation by centrifugal partition chromatography using the indicated solvent system. A dashed line indicates a heating step as described in this chapter.
CPC 1: hexane/methanol/water/formic acid
CPC 2: hexane/acetone/acetonitrile

The CPC two-phase systems used in this study were selected based on their polarity, stability and absence of (very) toxic solvents. The performance of selected CPC systems was evaluated according to Ingkaninan *et al.* [2000]. It should be noted that the retention volume in CPC is strongly dependent on the size of the injection sample, i.e. a higher amount results in a larger retention volume. Therefore, not the absolute retention (in ml), but the relative elution order of the cannabinoids in the used solvent systems is shown in figure 3.5. The amount of each cannabinoid isolated per gram of dry-weight plant material and the total amount isolated in this study are shown in table 3.1.

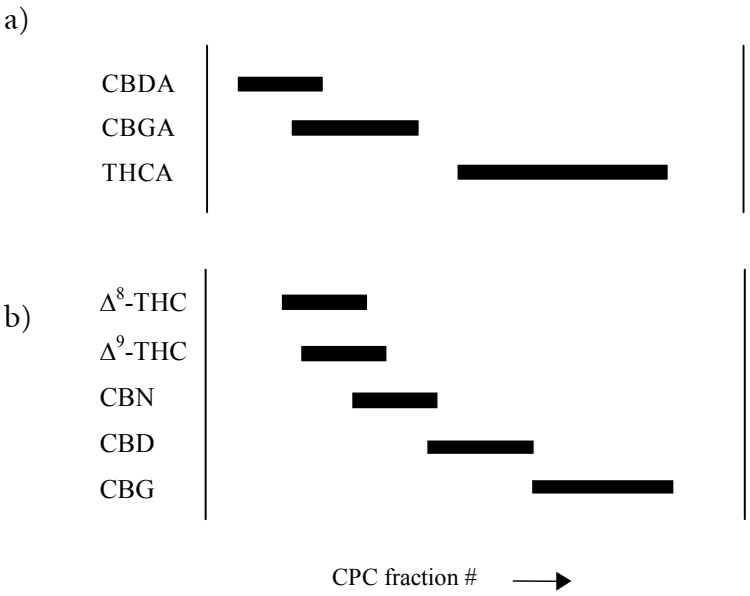


Figure 3.5: Schematic overview of the elution order of cannabinoids in CPC. a): acidic cannabinoids in CPC solvent system 1; b): neutral cannabinoids in CPC solvent system 2

Using CPC solvent system 1, the acidic cannabinoids THCA and CBGA could be well separated in a single experiment. This solvent system has the advantage that the concentration of methanol in the mobile phase can be increased during the run, without causing instability of the two-phase system. In this way the retention volume of the strongly retained THCA could be reduced from more than 800 ml (isocratic CPC, data not shown) to about 500 ml (gradient CPC). Because CBDA was the single major compound in the Kompolti extract, it was fairly simple to isolate it. Increasing the methanol concentration of the mobile phase could also reduce the elution volume of CBDA considerably.

Table 3.1: Identification, yields and purity of the isolated cannabinoids.
a): mg yield per 100 mg of dry weight plant material; b): Purity determined at a concentration of 1mg/ml.

Isolated cannabinoid	Isolated in this study (mg)	Relative yield [a)]	purity GC [b)]
Δ^9-THC	90,0	0,83	93.1%
THCA	1590	8,34	94.0%
CBD	232	0,46	92,7%
CBDA	326	0.65	90,2%
CBG	40,3	0,54	92,2%
CBGA	37,9	0,46	92,9%
CBN	99,4	1,38	95,0%

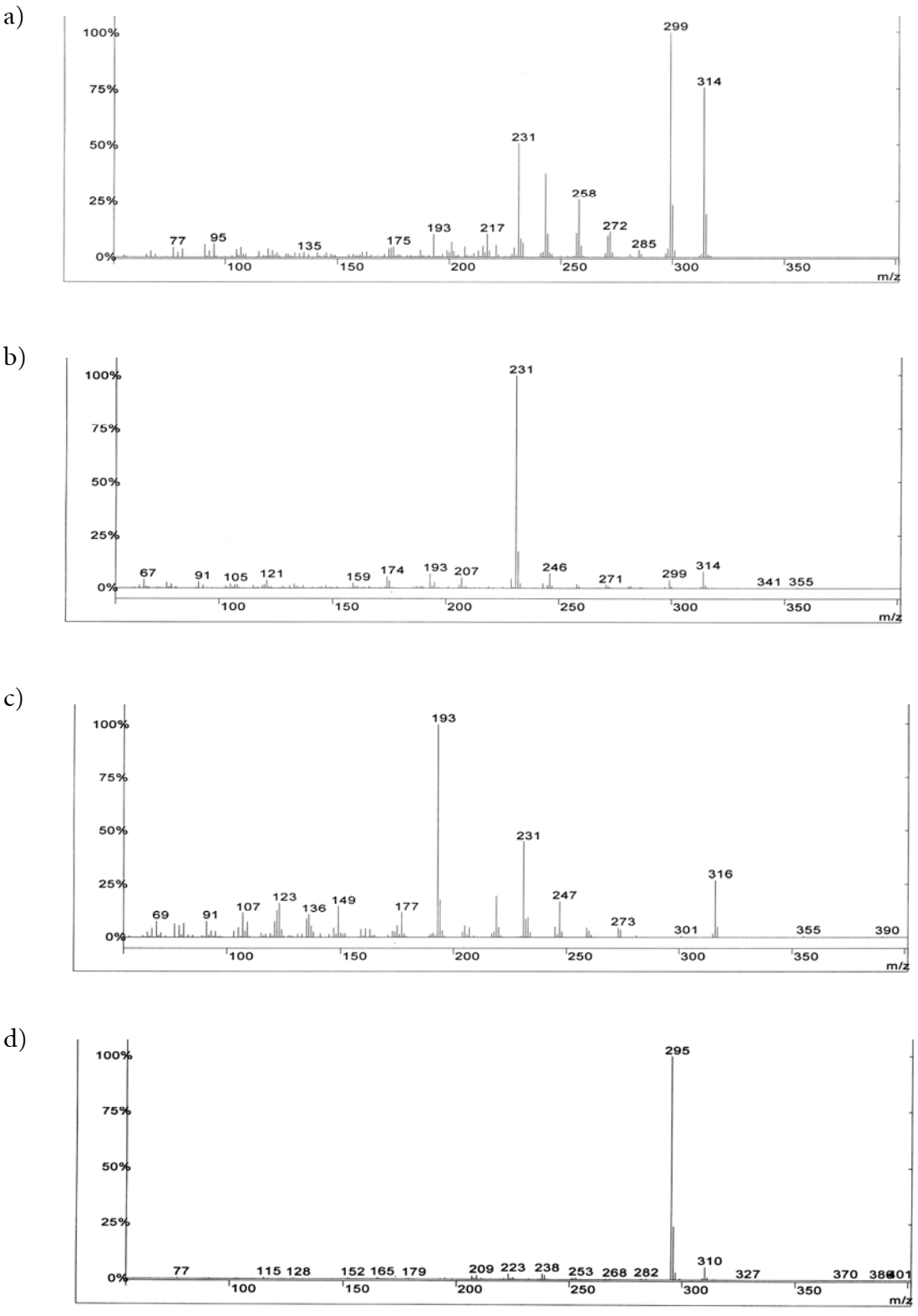


Figure 3.6: GC-MS spectra of the isolated cannabinoids. Only spectra of the neutral cannabinoids are shown. Acidic cannabinoids are decarboxylated in the GC-injector and MS-spectra similar to the corresponding neutral cannabinoids are obtained.
a): Δ9-THC and THCA; b): CBD and CBDA; c): CBG and CBGA; d): CBN

For the isolation of the neutral cannabinoids, slightly different methods had to be used. Neutral cannabinoids can be obtained by heating acidic cannabinoids to produce their corresponding neutral analogs by decarboxylation. This method is commonly used for the analysis of the total cannabinoids content in cannabis samples by HPLC [Kanter, 1979]. The heating temperature is about 180°C and samples are heated for several minutes. To obtain the neutral cannabinoid Δ^9-THC, initially a small amount of THCA was decarboxylated at 180°C for 5 minutes in an oven. However, after analysis by GC it was found that a considerable amount of Δ^8-THC had formed during the heating process. The structural isomers Δ^8- and Δ^9-THC could not be well separated by the CPC system used (data not shown). It was also noted that an increasing amount of CBN was formed during the heating period because of oxidation. Subsequently Δ^9-THC was isolated directly from the neutral cannabinoids fraction. But given the low abundance of neutral cannabinoids in the extracts, only a small amount of Δ^9-THC could be isolated. The observed degradation of THCA into CBN was subsequently exploited for the isolation of CBN, since the plant material used was naturally very low in CBN content.

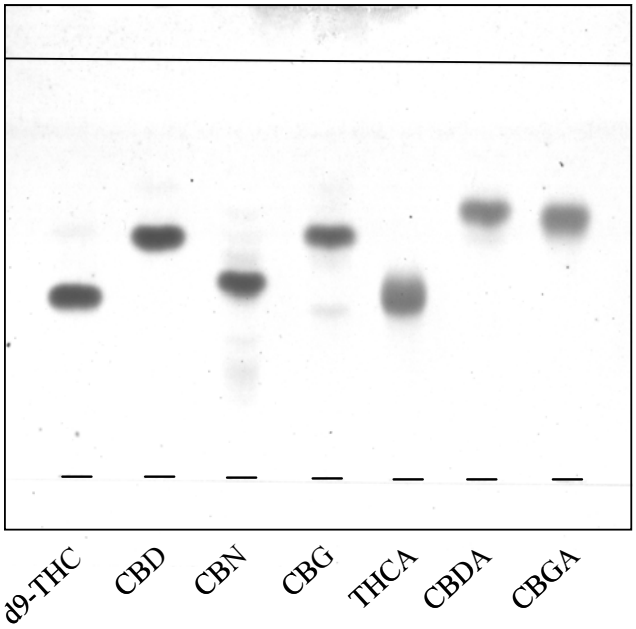


Figure 3.7: TLC of the isolated cannabinoids. Compounds were visualized by spraying the plates with modifiedanisaldehyde-sulphuric acid spray reagent to visualise cannabinoids as well as non-cannabinoids.

For the isolation of CBG, the acidic cannabinoids fraction of SIMM02 was heated, resulting in a mixture of several neutral cannabinoids. Because CBG is very well separated from the other neutral cannabinoids by CPC system 2 (see figure 3.5), CBG could be isolated directly from the mixture. Therefore an amount of the acidic cannabinoids fraction was heated directly (so without prior removal of THCA and other cannabinoids) and separated by solvent system 2. Because of its high abundance in Kompolti extract, CBD could be isolated in the same way.

All isolates could be positively identified by comparison with reference compounds and literature data. The GC-MS spectra of the isolated cannabinoids are shown in figure 3.6. The MS-spectra of acidic cannabinoids and their corresponding neutral cannabinoids are similar because of decarboxylation of acidic cannabinoids in the injector-part of the GC. The purity of isolated cannabinoids was determined by GC-FID and expressed as percentage of peak area compared to the total peak area in the chromatogram (table 3.1). All isolated cannabinoids could be well separated by the GC system used. No additional impurities could be detected in the samples after qualitative analysis by HPLC (data not shown) or TLC (figure 3.7).
It was reported that THCA can be stored at least for one year at –20°C [Gaoni, 1971], so the isolated cannabinoids were kept in ethanol at –20°C. Our preliminary data (HPLC) shows all isolated cannabinoids to be stable for at least 6 months under these conditions (data not shown).

3.4 Conclusion

Preparative isolation of seven different major cannabinoids could be achieved by using CPC as the single technique, with two different solvent systems. The quality of the isolated cannabinoids (>90% pure by GC-FID) is sufficient for many purposes. Additional HPLC and TLC data support the purity of the isolated compounds. This method can make the isolated cannabinoids available for biological testing on a large scale. Also other cannabinoids can probably be isolated in this way by choosing a cannabis variety with a high content of the desired cannabinoid and simultaneously a low content of cannabinoids that are known to overlap with the desired cannabinoid in the CPC separation. The vast diversity in cannabis varieties should make it possible to find a suitable variety for most cannabinoid isolations. It should be possible to isolate several cannabinoids in just one chromatographic run, but the efficiency depends on peak overlap and contamination of the sample with non-cannabinoids. To ensure a high yield the acidic cannabinoid fraction of a cannabis extract should be used.

3.5 Acknowledgements

Stichting Institute for Medical Marijuana (SIMM) is gratefully acknowledged for their kind gift of plant material. The financial support by the Van Leersum Fonds for purchasing the CPC solvent pump is highly appreciated. We would like to thank the Dutch Forensic Institute (NFI), Rijswijk, The Netherlands for their kind gift of CBD and CBN standards.

CHAPTER 4

Quantitative analysis of cannabinoids from *Cannabis sativa* using ^{1}H-NMR

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Abstract

A ^{1}H-NMR method has been developed for the quantitative analysis of pure cannabinoids and for cannabinoids present in Cannabis sativa plant material without the need for chromatographic purification. The experiment was performed by the analysis of singlets in the range of 4.0-7.0 ppm in the ^{1}H-NMR spectrum, in which distinguishable signals of each cannabinoid are found. Quantitation was performed by calculating the ratio between the peak area of selected proton signals of the target compounds and the internal standard anthracene. For this method no cannabinoid reference standards are needed. It allows rapid and simple quantitation of cannabinoids with a final analysis time of only 5 minutes without the need for a pre-purification step.

4.1 Introduction

The cannabis plant has been of medicinal interest for centuries. In recent years a lot of research on the medical applications of *Cannabis sativa* L. has been initiated, as several, mostly European, countries move towards a more liberal view on the use of Cannabis as a medicine. Many different pharmacological properties have been associated with cannabis use, including increased heart rate, drop of body temperature, ataxia and a loss of time-space perception [Grotenhermen, 2002]. Amongst the constituents of *Cannabis sativa*, the cannabinoids have been widely recognized as the active constituents for most clinical activities. The cannabinoids make up a large family of closely related C_{21} compounds and their carboxylic acids and are unique to the cannabis plant [Turner, 1980]. Clinically interesting properties of the cannabinoids are very diverse, ranging from analgetic and antiemetic to the treatment of glaucoma and multiple sclerosis [Williamson, 2000; Grotenhermen, 2002]. However, only four of the 66 known natural cannabinoids [Ross, 1995] are currently commercially available as certified reference standards, i.e.: delta-9-tetrahydrocannabinol (Δ^9-THC or THC), delta-8-tetrahydrocannabinol (Δ^8-THC), cannabidiol (CBD) and cannabinol (CBN). There are indications that also these reference compounds have to be re-quantified regularly because of degradation and differences between batches during production [Poortman-van der Meer, 1998].

Recently, our laboratory developed a method for the large scale isolation of highly pure cannabinoids from *Cannabis sativa* flower tops [Hazekamp, 2004a]. For the quantitative analysis of these compounds, gas chromatography with FID or other detection has been widely used, but this method can not distinguish between cannabinoids and their carboxylic counterparts without prior derivatization [Fetterman, 1971a; Turner, 1974]. HPLC with UV detection is more suitable for simultaneous analysis of these compounds, but it has proven to be very difficult to separate all components in a single chromatographic run [Lehmann, 1995; Ferioli, 2000] and some contaminations may not be detected because they lack UV absorbance. Furthermore, both methods are sensitive to impurities in the sample such as chlorophyll or lipids, and therefore they usually require a sample clean-up step prior to analysis. Most importantly, the reference compounds needed for the preparation of a calibration curve are not available for many cannabinoids. A review of methods for cannabinoids analysis in biological materials is given by Raharjo et al [2005].

To solve the problems associated with these analytical techniques, the development of a reliable and easy method is required as alternative to the conventional analyses. In this study, we developed an analytical method using ^{1}H-NMR for cannabinoids without the need for any chromatographic purification. Quantitative NMR has been shown to be very accurate and highly reproducible, within a very short analysis time. The usefulness of quantitative NMR for the validation of natural product reference compounds as well as its theoretical aspects have been shown by Maniara *et al.* [1998] and by Pauli *et al.* [2001].

The developed method was applied on the quantitative analysis of five different isolated cannabinoids. A similar method has been recently described by our laboratory for the

quantitative analysis of bilobalide and ginkgolides in *Ginkgo biloba* leaves and products [Choi, 2003]. The usefulness of this method was further shown by quantitation of major cannabinoids present in four different types of *Cannabis sativa* plant material.

4.2 Materials and methods

4.2.1 Plant material

Plant material of *Cannabis sativa* was obtained from Stichting Institute for Medical Marijuana (SIMM) in Rotterdam, The Netherlands, and from Bedrocan BV, The Netherlands. Four different cannabis cultivars were used. After harvest the plant material was air-dried in the dark under constant temperature and humidity for 4 weeks. Only flowertops of female plants were used. These were manicured to remove leaves and stems, and stored at –20°C.

4.2.2 Solvents and chemicals

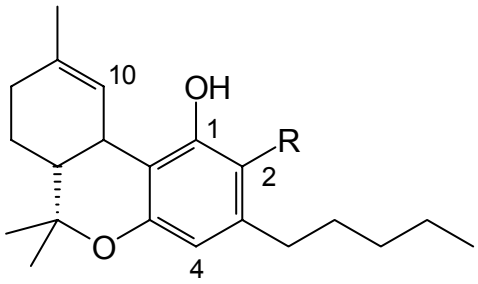
Anthracene was purchased from Sigma (St. Louis, MO). Deuterated chloroform ($CDCl_3$, 99.8%) was obtained from Eurisotop (Gif-sur-Yvette, France). All organic solvents were analytical grade and obtained from Merck Biosolve Ltd. Valkenswaard, The Netherlands.
Pure cannabinoids were previously isolated by us [Hazekamp, 2004a]. They were stored as ethanolic solutions at –20°C. The following isolated cannabinoids were used for quantitation: delta-9-tetrahydrocannabinol (THC), delta-9-tetrahydrocannabinolic acid A (THCA), cannabidiol (CBD), cannabidiolic acid (CBDA) and cannabinol (CBN), (figure 4.1). Commercially obtained certified standards were used for recovery studies; THC was from Cerilliant, (Round Rock, TX), while CBD and CBN were obtained from Sigma.

4.2.3 ^{1}H-NMR parameters

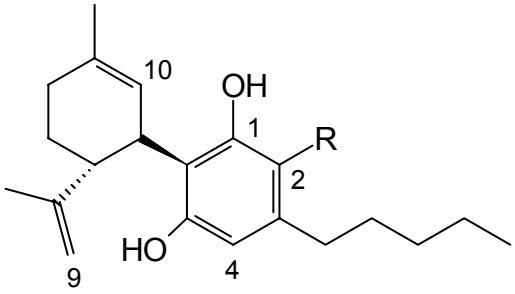
^{1}H-NMR spectra were recorded in $CDCl_3$ using a Bruker DPX 300MHz spectrometer, equiped with an Indy Silicon Graphics computer. For each sample, 64 scans were recorded with the following parameters: 32K data points, pulse width of 4.0μs and relaxation delay of 1s. FID's were Fourier transformed with LB of 0.5Hz. For quantitative analysis, peak area was used after baseline correction.

4.2.4 Determination of accuracy

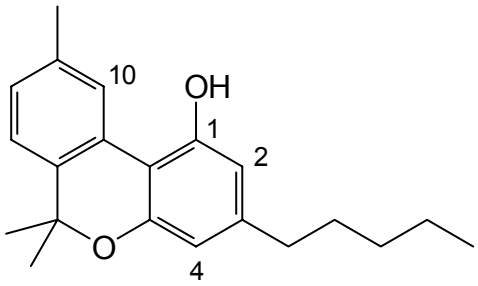
Certified cannabinoid standards were used to evaluate the accuracy of the developed method. From newly opened vials containing THC (1.0 mg/ml), CBD (0.99 mg/ml) and CBN (0.98 mg/ml) 100 μl was mixed with 1.0 mg of anthracene as internal standard (all in triplicate). These samples were evaporated using a vacuum centrifuge and redissolved in 1.0 ml of $CDCl_3$ for NMR analysis.



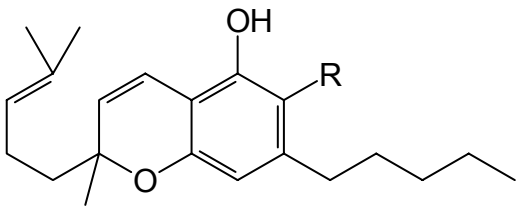
Δ^9-tetrahydrocannabinol (THC) : R = H
Δ^9-tetrahydrocannabinolic acid A (THCA) : R = COOH



Cannabidiol (CBD) : R = H
Cannabidiolic acid (CBDA) : R = COOH



Cannabinol (CBN)



Cannabichromene (CBC): R = H
Cannabichromenic acid (CBCA): R = COOH

Figure 4.1: Structures of the studied cannabinoids. Numbering of the carbon-positions is indicated

An aliquod of the ethanolic solutions of cannabinoid standards, isolated by our own lab (isolates), were diluted in ethanol to a concentration of about 0.5 mg/ml (based on weight after extensive evaporation of solvent). After this they were quantified as described above.

4.2.5 Evaluation of recovery of cannabinoids

Cellulose filter paper spiked with pure cannabinoids was used to mimic the plant material for evaluation of extraction recovery. Fivehundred milligram of cellulose filter paper (Schleicher & schuell GmbH, Cassel, Germany) was cut into pieces of ca. 0.5 cm diameter and placed in the extraction vessel. Each isolated cannabinoid (1.0 mg in ethanol) was spiked into the filter paper disks and the spiked samples were dried at room temperature for 24 h before extraction.

4.2.6 Extraction and quantification of cannabinoids from plant materials

For each analysis plant material (350 mg dry weight) or recovery control was extracted two times for 10 minutes with 15 ml methanol/chloroform (9:1, v/v) under constant agitation. Extractions were started by 2 min of ultrasonication and were performed at 4°C. Both extracts were combined and the volume was brought to 50 ml with extraction solvent. Then 0.5 ml of extract was mixed with 1.00 mg of anthracene as internal standard. These samples were evaporated using a vacuum centrifuge and redissolved in 1.0 ml of $CDCl_3$ for ^{1}H-NMR analysis. All experiments were based on triplicates. For the plant materials, the amount of the major cannabinoids THCA and CBDA was determined.
To evaluate the linearity between sample size of the plant material and the quantification result, different amounts of plantmaterial (100, 300, 500 mg, all in triplicate) were extracted and quantified.

4.2.7 Gas Chromatography (GC) for comparison

Quantification of THCA or CBDA, using a certified standard of THC or CBD was performed with a Chrompack (Middelburg, The Netherlands) CP9000 gas chromatograph, fitted with a Durabond fused silica capillary column (30 m x 0.25 mm inner diameter) coated with DB-1 (J&W scientific Inc., Rancho Cordova, CA) at a film thickness of 0.1 µm. The (FID) signal was recorded on a Shimadzu (Kyoto, Japan) CR3A integrator. The oven temperature was programmed from 100°C to 280°C at a rate of 10°C/min. The oven was then kept at 280°C until the end of the runtime of 30 minutes. The injector and the detector temperature were maintained at 280°C and 290°C, respectively. Nitrogen was used as the carriergas at a pressure of 70 kPa. Air and hydrogen were used as detector gasses. The injection split ratio was 1/50.

4.3 Results and discussion

In this study we developed a ^{1}H-NMR method for the quantitative analysis of pure cannabinoids and cannabinoids present in *Cannabis sativa* plant material, in order to perform quantitative analysis of cannabinoids without the need of chromatographic separation or the use of certified reference standards. The ^{1}H-NMR spectra of the studied cannabinoids have been published [Fellermeier, 2001; Choi, 2004]. Five cannabinoids commonly found in Cannabis plant materials were used for this study. However it must be noted that one major cannabinoid, cannabichromenic acid (CBCA, figure 4.2) was not studied because there was no reference standard available for this compound. CBCA is commonly found in fiber-type, as well as drug-type Cannabis.

The proton signals selected for this study were in the range of δ 4.0 – 7.0, as this is the range where the ^{1}H-NMR spectra are most distinguishable. As internal standard anthracene was selected because it is a very stable compound with a simple ^{1}H-NMR spectrum consisting of a singlet (δ 8.43) and two quartets (δ 8.01, δ 7.48). These signals do not overlap with signals of the cannabinoids that were used in this study. For the quantification experiments, the singlet of anthracene was always used.

Based on the chemical structure of the molecule the most suitable proton signals for quantification were selected for each cannabinoid. Using known amounts of certified standards for THC, CBD and CBN, the developed method was shown to be highly accurate, as can be seen in table 4.1. Following this, the studied cannabinoids were quantified by preparing a solution of 0.5 mg/ml in $CDCl_3$ (based on weight after extensive drying to remove solvent) and performing a preliminary quantification of these solutions using the described ^{1}H-NMR method. The NMR spectra obtained in these experiments are shown in figure 4.2.

The quantified cannabinoid solutions were subsequently used for preparing calibration curves in the concentration ranges as shown in table 4.2, in order to evaluate the accuracy of this method depending on the different concentrations. The highest concentration used was at least two times higher than the 0.5 mg/ml used for the preliminary quantification. The calibration curves were made using the ratio of the peak integral of the compound and the internal standard. The linearity of the calibration curves was determined by plotting the least squares regression lines (table 4.2). All calibration curves were highly linear with a r^2-value of more than 0.99. Because all preliminary quantifications were well within the linear range of this method, we can conclude that these values were accurate.

For testing the recovery of cannabinoids from a plant matrix (consisting mainly of cellulose) during the extraction step, 1.0 mg of each compound was extracted from cellulose papers onto which the compounds were adsorbed [Smith, 1992; Choi, 2003]. The extraction procedure was kept as simple as possible and needed no sample clean-up steps before ^{1}H-NMR analysis. The recovery was more than 92% for each cannabinoid, as shown in table 4.3.

Finally, extracts of four different *Cannabis sativa* cultivars were analyzed for their THCA content using the ^{1}H-NMR method developed in this study (figure 4.3). THCA was a major component of three of these extracts, as shown by HPLC analysis (data not shown).

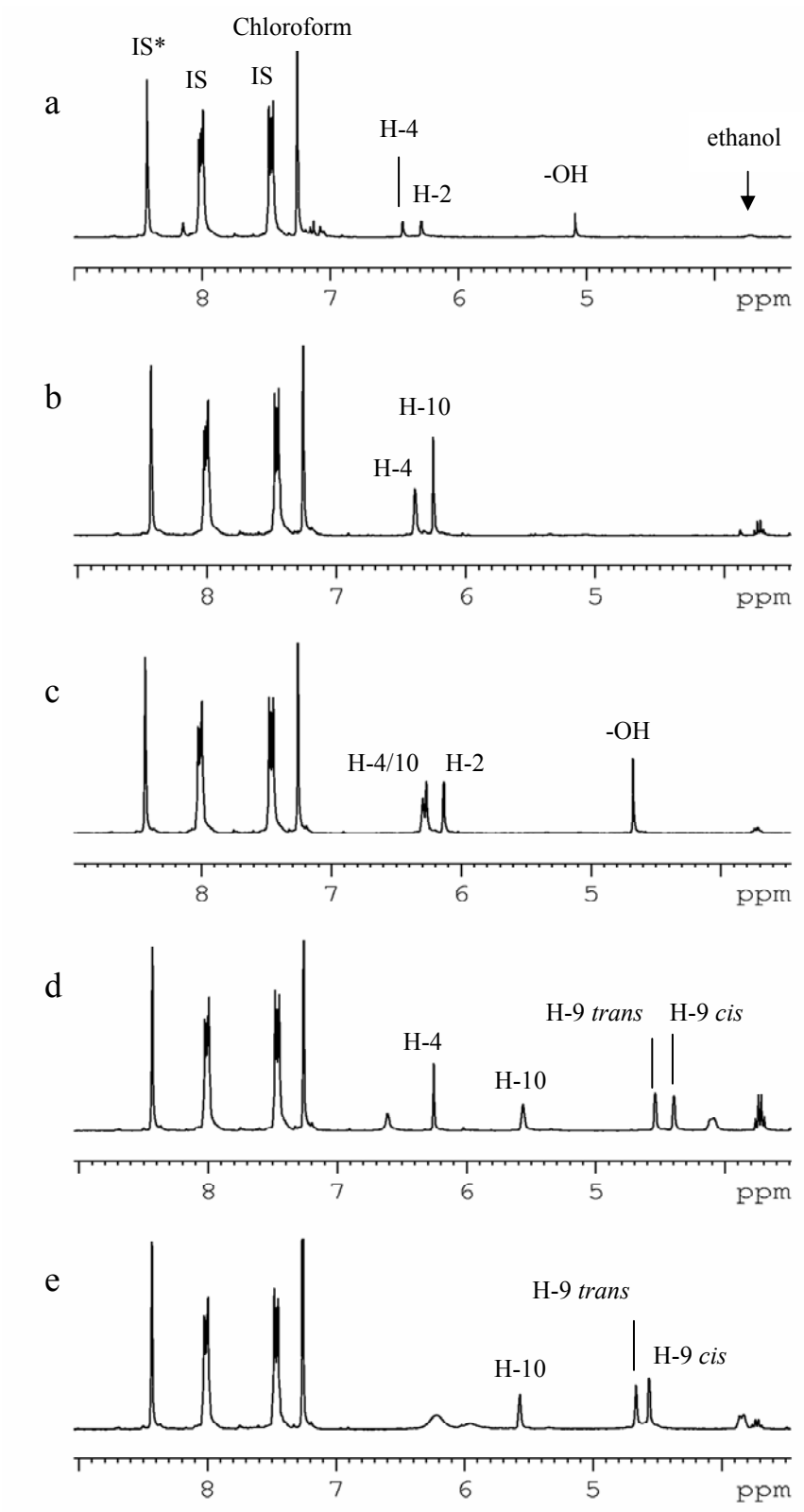


Figure 4.2: [1]H-NMR spectra of 0.5 mg (by weight) of each cannabinoid mixed with 1 mg of anthracene as internal standard. Quantitation was performed by calculating the ratio of the peak area of selected proton signals of the targetcompounds to the singlet of anthracene (*). In some spectra a residue of ethanol is visible.
a: CBN, b: THCA, c: THC, d: CBDA, e: CBD, IS = signals of internal standard.

The fiber-type cannabis contained almost no THCA, but a high level of CBDA. For this type CBDA was quantified. The results were found to be very reproducible with a standard deviation of less than 6%. The results could be confirmed by gas chromatography (see table 4.4). Figure 4.4 shows the high linearity between the amount of cannabis plant material used for extraction (up to 500 mg) and the THCA quantification results (not done for CBDA).

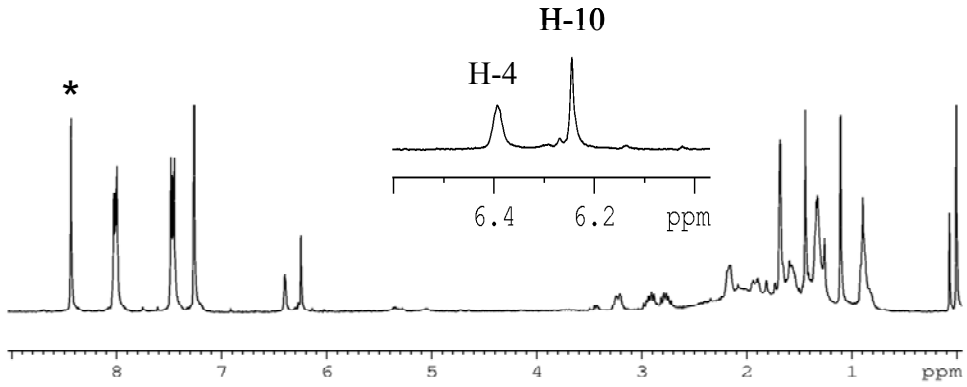


Figure 4.3: ^{1}H-NMR spectrum of a drug type Cannabis extract together with 1 mg of anthracene as internal standard. Part of the spectrum is enlarged to show the overlap of proton signal H-10 of THCA with signals of minor compounds. For quantitation the singlet of anthracene (*) and H-4 of THCA were used.

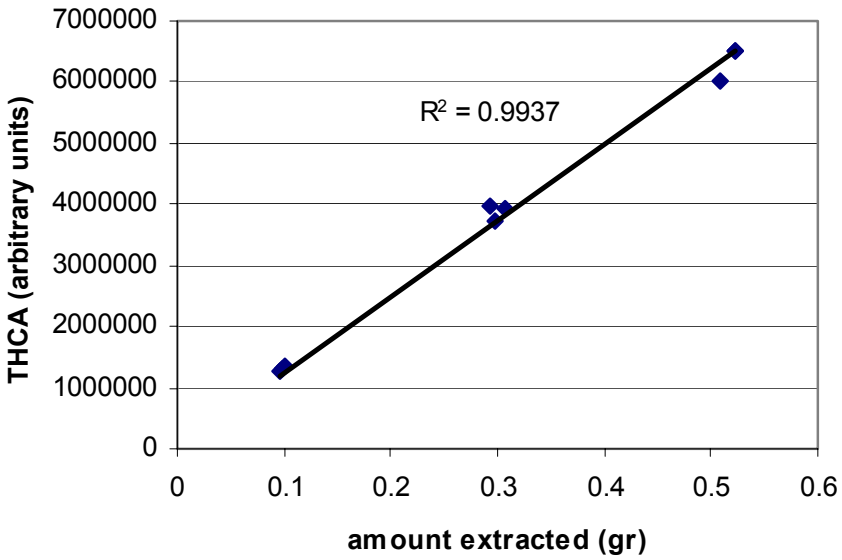


Figure 4.4: Linearity between the amount of extracted cannabis plant material and the amount of THCA (in arbitrary units) quantified by the developed NMR method.

Table 4.1: Quantitation of known amounts of commercially obtained cannabinoid standards

cannabinoid	Added (µg)	Calculated (µg)
THC	100	99 (± 2.9%)
CBD	99	99 (± 2.0%)
CBN	98	99 (± 1.2%)

Table 4.2: Linearity of the calibration curves of the cannabinoids. Listed are the concentration range of the calibration curves and the proton signals that were tested. The linearity of each calibration curve was determined by plotting the least squares regression line. Each sample was measured in duplicate.

Cannabinoid	Investigated range (mg)	Proton signal	δ in ppm	Linearity
CBN	0.1 - 1.0	H-4	6.44	0.9985
		H-10	8.16	overlap with internal standard
THCA	0.2 - 4.0	H-4	6.39	0.9996
		H-10	6.24	0.9998
THC	0.1 - 1.0	H-2	6.14	0.9993
		H-4/H-10	6.27/6.29	0.9999; partially overlapping
CBDA	0.2 - 4.0	H-4	6.26	0.9999
		H-10	5.55	0.9999
		H-9 *trans*	4.54	0.9999
		H-9 *cis*	4.40	0.9999
CBD	0.2 - 4.0	H-10	5.57	0.9992
		H-9 *trans*	4.66	interaction with -OH
		H-9 *cis*	4.56	interaction with -OH

Table 4.3: Recovery of the cannabinoids (%) after extraction from filterpaper with methanol/chloroform, 9:1 (v:v). Each experiment was performed in triplicate.

THC	CBD	CBN	THCA	CBDA
99.2 (± 6.7)	98.0 (± 6.7)	92.1 (± 4.2)	99.6 (±5.1)	100.4 (± 6.2)

Table 4.4: Quantitation of the amount of THCA in four different cannabis types, by NMR and GC. For the fiber type also CBDA was quantified. Values are expressed as mg of cannabinoid per gram dry weight plant material. Each experiment was performed in triplicate.

	Cultivar type	**THCA by NMR**	**used proton signal**	**THCA by GC**
Extract 1	Drug	179 (± 10)	H-4	198 (± 3)
Extract 2	Drug	229 (± 1)	H-4	234 (± 14)
Extract 3	Intermediate	118 (± 3)	H-4	103 (± 6)
Extract 4	Fiber	Too low	H-4	0.88 (0.09)
		CBDA by NMR		**CBDA by GC**
Extract 4	Fiber	22.0 (± 1.4)	H-4	21.4 (± 1.9)

4.4 Conclusion

The content of the major cannabinoid of *Cannabis sativa* plant material and the concentration of purified cannabinoid solutions could be analyzed with a simple method. Analysis time was only 5 minutes, which is much shorter than conventional chromatographic methods. Moreover, cannabinoids could be quantified which are not available as reference compounds and can therefore not be quantified by other methods (i.e.: CBDA and THCA). Preliminary results show that this method is also suitable for the quantitation of cannabigerol (CBG) and cannabigerolic acid (CBGA) and probably additional cannabinoids as well. The ^{1}H-NMR method for the quantitative analysis of cannabinoids has the additional advantage that an overall profile is obtained of the extract so that the purity of an isolated cannabinoid can be determined simultaneously with the identity of impurities.
It seems clear that the quantitation of cannabinoids in isolated samples or simple mixtures can easily and quickly be performed by quantitative ^{1}H-NMR. However, for the quantification in complex plant extracts, the preferred proton signal for quantification should be a singlet which shows a high linearity in the measured concentration range. Furthermore it should not overlap with a proton signal of another component of the extract. Because the composition of extracts can be variable, the most suitable proton signal should be selected after inspection of the total ^{1}H-NMR spectrum.

4.5 Acknowledgements

We gratefully acknowledge Stichting Institute for Medical Marijuana and Bedrocan BV for supplying the *Cannabis sativa* plant material. RIVM (Bilthoven, The Netherlands) is acknowledged for their kind gift of the certified cannabinoid standards.

CHAPTER 5

Synthesis and spectroscopic characterization of cannabinolic acid

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Abstract

Cannabinoids, the main constituents of the cannabis plant, are increasingly studied for their medicinal properties. Cannabinolic acid (CBNA) was synthesized from tetrahydrocannabinolic acid (THCA), a major constituent of the cannabis plant, by aromatization using selenium dioxide mixed with trimethylsilyl polyphosphate as catalyst in chloroform. Purification was achieved by centrifugal partition chromatography and the final product had a purity of over 96% by GC analysis. Spectroscopic data on CBNA such as ^{1}H-NMR- and IR-spectrum, and UV spectral analysis, as well as chromatographic data are presented as useful reference for further research on CBNA. The developed method allows production of CBNA on a preparative scale, making it available for further studies on its biological activities and as reference standard for analytical procedures.

5.1 Introduction

The cannabis plant (*Cannabis sativa* L., Cannabaceae) is under intense study for its medicinal properties in a variety of illnesses such as multiple sclerosis, Tourette's syndrome, chronic pain, wasting syndrome associated with AIDS/HIV and anorexia [Grotenhermen, 2002]. Although so far at least 489 compounds have been identified in cannabis [Elsohly, 2005], most studies focus on the effects of the cannabinoids. As a contribution to the study of the lesser known cannabinoids, we recently published the standardized spectroscopic and chromatographic data of a variety of natural cannabinoids [Hazekamp, 2005]. In that study, data on cannabinolic acid (CBNA) was incomplete, due to unavailability of a calibrated standard. CBNA is formed during storage and aging of plant samples by degradation of tetrahydrocannabinolic acid (THCA), a major component of cannabis resin [Shoyama, 1970; Hanus, 1985]. The biological activities of CBNA have not been studied in detail, and analytical study is complicated by the fact that published spectroscopic data is incomplete. Although a full synthesis of the closely related cannabinol (CBN) has been described [Adams, 1940], the synthesis or preparative isolation of CBNA has not been reported.
In this chapter, we describe the production of CBNA by dehydrogenation of THCA using selenium dioxide mixed with trimethylsilyl polyphosphate (PPSE) as the catalyst in carbon tetrachloride (see figure 5.1) [Lee, 1992]. As a minor modification, we found that carbon tetrachloride could be replaced by the less toxic chloroform without effects on the final transformation yield. Finally, the significant amount of 26 mg purified CBNA was obtained in a single experiment. Final product was highly pure (96% by GC analysis), therefore rendering a quantified CBNA solution suitable for use as reference standard for analytical or biological studies.
Full spectroscopic data for CBNA (UV, fluorescence, IR, ^{1}H-NMR, MS) is presented, facilitating study on the role of CBNA as a component of cannabis products. The spectroscopic and chromatographic data were published in a systematic manner, complementing the data that was earlier obtained on 16 natural cannabinoids [Hazekamp, 2005].

5.2 Materials and methods

5.2.1 Chemicals and solvents

Selenium dioxide (SeO_2, purity >98%, reagent grade), hexamethyldisiloxane (HMDSO, purity >98%) and phosphorus pentoxide (P_2O_5, purity >97%) were purchased from Sigma-Aldrich (St. Louis, MO). Organic solvents (analytical or HPLC reagent grade) were purchased from J.T. Baker (Deventer, The Netherlands). Cannabinoid standards for THCA and CBN (purity ≥98%) were produced and quantified as previously reported [Hazekamp, 2004a,b]. Structures of the cannabinoids are shown in figure 5.1.

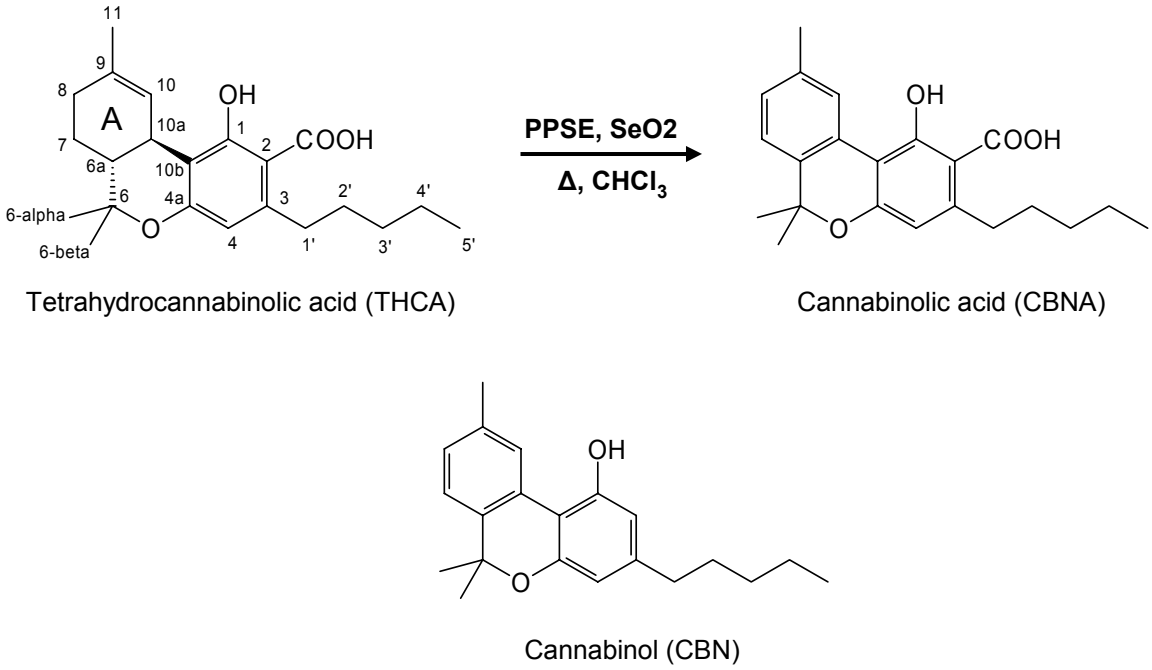

Figure 5.1: Chemical structures of the studied cannabinoids. The formation of CBNA by dehydrogenation of ring A of THCA is indicated. Carbon-numbering for THCA is indicated for interpretation of the ^{1}H-NMR results.

5.2.2 Synthesis

PPSE was prepared from P_2O_5 and HMDSO [Imamoto, 1981]. Thus, HMDSO in chloroform (12% v/v) was refluxed for 30 minutes under nitrogen gas, followed by addition of P_2O_5 (50 mg/ml) and additional refluxing for 2 hours. The clear chloroform phase, containing PPSE, was separated from residual solid P_2O_5 and transferred to a reaction vessel. SeO_2 (30 mg/ml final concentration) and THCA (dissolved in chloroform, 50mg/ml final concentration) were added, giving a molar ratio between SeO_2 and substrate of circa 2:1 [Lee, 1992]. The resultant mixture was mildly refluxed for 6-8 hours to allow dehydrogenation of THCA. Subsequently, the liquid phase containing the cannabinoids was separated from the solid SeO_2. Liquid phase was evaporated under vacuum and reconstituted in hexane, resulting in precipitation of PPSE. Hexane fraction contained crude CBNA.

5.2.3 Isolation and characterization

Purified CBNA (26 mg) was obtained by fractionation of the crude synthesis sample by centrifugal partition chromatography, using hexane/methanol/water, 5:3:2 (v/v/v) with 0.1% formic acid [Hazekamp, 2004a]. The eluent was monitored at the maximal UV-absorption wavelength for CBNA of 261nm. Fractions containing CBNA were detected by LC-DAD-MS. The purified compound was positively identified by comparing retention times in HPLC and GC [Hazekamp, 2005], and spectroscopic data (HPLC-DAD-MS) to literature data

[Hazekamp, 2005; Smith, 1975; Brenneisen, 1988]. A quantitative ^{1}H-NMR method was used to prepare a quantified ethanolic solution of CBNA [Hazekamp, 2004b]. The purity of isolated CBNA was determined by GC analysis at a concentration of 1 mg/ml (5 µl injected). The quantified solution was used to measure the molar extinction coefficients of CBNA in the range of 200-400 nm, and infrared (IR)-spectrum in FT-IR [Hazekamp, 2005].

5.2.4 LC-DAD-MS analysis

LC-DAD-MS data were obtained with an Agilent 1100 series HPLC system consisting of an auto sampler, low-pressure mixing pump, column oven and DAD detector, connected to an Agilent single-quadrupole mass-spectrometer equipped with an Agilent APCI ion probe.
HPLC conditions: Vydac (Hesperia, CA, USA) RP18 column (type 218MS54, 4.6x250 mm, 5 µm); Waters Bondapak RP18 (2x20 mm, 50 µm) guard column. Solvent system: A = H_2O, 0.1% formic acid, B = MeOH, 0.1% formic acid. Gradient: 65% to 100% B in 25 min, then 100% B for 3 min; flow-rate: 1.5 ml/min; injection volume: 10 µL. DAD conditions: 228, 261 nm, and full spectra 210-400 nm.
APCI-MS conditions: Positive ion mode; scan range: 200-400 amu; fragmentor voltage: 100 and 240 V; gas temperature: 350 °C; vaporizer temperature: 400 °C; drying gas (N_2) flow rate: 4 liters min^{-1}; nebulizer pressure: 45 psig (lb/in^2); capillary voltage: 4000 V; corona current: 4.0 µA.

5.2.5 Nuclear Magnetic Resonance spectroscopy (^{1}H-NMR)

Spectra were recorded in $CDCl_3$ using a Bruker DPX 300 MHz spectrometer. 64 scans were recorded with the following parameters: 32K datapoints for zero filling, pulse width of 4.0 µs and relaxation delay of 1 second. FID's were Fourier transformed with LB of 0.5 Hz. Peak assignment was done by comparison to the NMR-spectrum of CBN [Choi, 2004] (table 5.1).
Quantification of isolated CBNA in ethanol solution was performed by the quantitative ^{1}H-NMR method described in chapter 4 of this thesis [Hazekamp, 2004b].

Cannabinolic acid: greenish oil; R_f 0.25, silica gel 60 F_{254}, MeOH/H_2O/acetic acid (19:1: 0.05); R_f 0.54, RP-18 F_{254}, $CHCl_3$/MeOH (19:1); UV (EtOH) λ_{max} (log ε) 261 (4.70), 298 (4.30), 324 (4.11); IR (KBr) νmax 2925, 1620, 1260 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 8.40 (1H, s, H-10), 7.11 (2H, dd, J = 12.31, 8.58 Hz, H-7, H-8), 6.40 (1H, s, H-4), 2.96 (2H, t, J = 7.78 Hz, H-1'), 2.38 (3H, s, H-11), 2.15 (2H, m, H-2'), 1.60 (6H, s, H-6α, H-6β), 1.32 (4H, m, H-3', H-4'), 0.83 (3H, t, J = 6.91 Hz, H-5'); APCI-MS: *m/z* = 355.2 [M+H^+], 337.2 [M-H_20], 311.2 [M-CO_2].

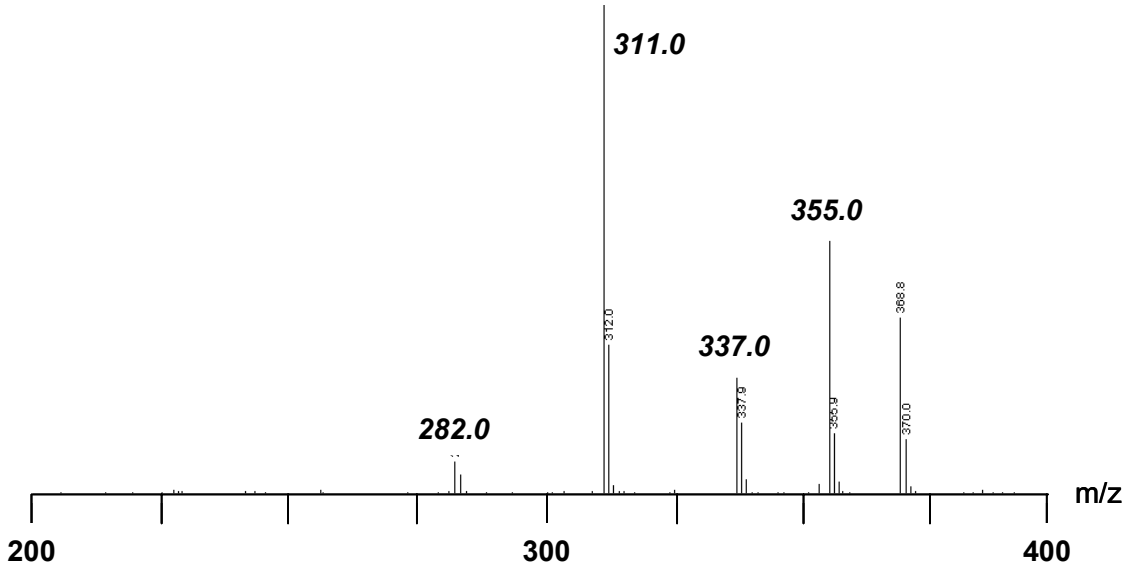

Figure 5.2: LC/MS spectrum for isolated CBNA as obtained using APCI mode with positive ionization.

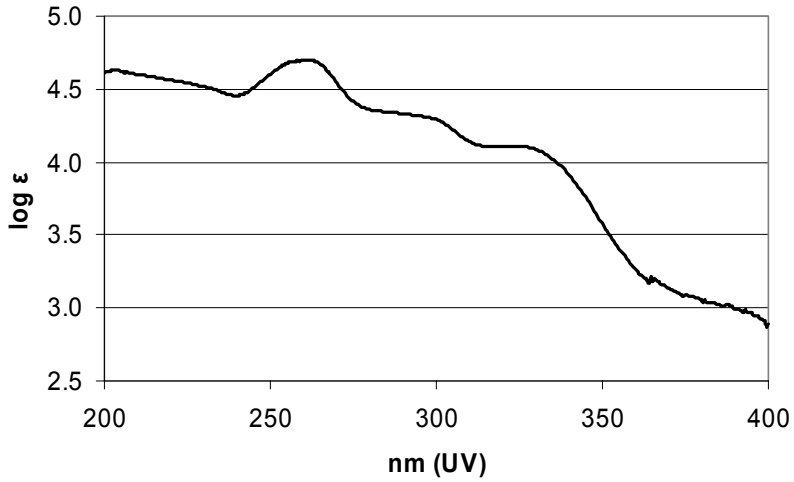

Figure 5.3: Extinction coefficients of CBNA (0.01 mg/ml) in the range of 200-400 nm in ethanol.

5.3 Results and discussion

We studied the production of CBNA by semi-synthesis from the structurally related and readily available THCA. Several methods have been reported for the aromatizing of alicyclic compounds bearing one or more double bonds, for example making use of dehydrogenating agents such as platinum or palladium [Ahmed, 1992; Monda, 2001]. However, the most efficient method reported so far for performing this reaction is using selenium dioxide mixed with trimethylsilylpolyphosphate (PPSE) as the catalyst in carbon tetrachloride [Lee, 1992].

We successfully applied this simple method for the production of CBNA. As a minor modification, we found that carbon tetrachloride could be replaced by the less toxic chloroform without effects on the final transformation yield.
The conversion rate under the applied conditions was about 10%. Reaction products other that the starting material (THCA) or the desired product (CBNA) were not further identified. Purification was achieved by centrifugal partition chromatography (CPC), a technique which permits easy upscaling and has been extensively described in chapter 3 of this thesis. Finally, a significant amount of 26 mg purified CBNA was obtained in a single experiment.
Analysis of the isolate by HPLC resulted in a single major peak, which was positively identified as CBNA based on its retention time, and UV- and MS-spectrum. Under the selected conditions for LC-MS analysis, isolated CBNA was mildly fragmented. The highest intensity was seen for the decarboxylated $[MH\text{-}CO_2]^+$ product, indicating the relative instability of the carboxylic group (figure 5.2).
Further confirmation was achieved on the basis of its conversion, through decarboxylation, to cannabinol, whose spectroscopic data has been published [Hazekamp, 2005]; injection of the isolate into GC resulted in a single peak that could be identified as CBN. Purity assayed by GC was found to be 96%, a large proportion of the impurity being THCA or THC. In fact, the heat of the GC results in the decarboxylation of CBNA into CBN, which can be prevented by derivatization (e.g. silylation). However, no derivatization was performed as this might obscure interpretation of the purity of the sample by formation of multiple derivatives of CBNA.

Table 5.1: ^{1}H-NMR data obtained at 300MHz for CBNA.

[a] Multiplicity, s: singlet; dd: double doublet; t: triplet; m: multiplet.

[b] Published ^{1}H-NMR data for CBN, obtained at 400MHz in $CDCl_3$ [Choi, 2004]

proton	signal (ppm)	# of protons, multiplicity [a]	CBN [b]
2	absent		6.29
4	6.40	1H, s	6.44
6α, 6β	1.60	6H, s	1.60
7, 8	7.11	2H, dd	7.07, 7.14
10	8.40	1H, s	8.16
11	2.38	3H, s	2.38
1’	2.96	2H, t	2.50
2’	2.15	2H, m	1.63
3’, 4'	1.32	4H, m	1.32
5’	0.83	3H, t	0.89

The isolate was further analyzed by TLC, in order to visualize impurities that can not be detected by GC or HPLC analysis (data not shown). A single major spot was observed for CBNA, with two minor spots being identified as CBN and THCA. The CBNA spot showed a very strong fluorescence under 366 nm UV-light and strong absorbance under 254 nm UV-light on the used fluorescent TLC plates. Compared to previously tested cannabinoids, CBNA showed a relatively poor staining with fast blue B dye, a preferred stain for cannabinoid detection [Corrigan, 1980].

When ^{1}H-NMR data of the isolated compound was compared to reported data on CBN, the signals of protons in ring A (H-7, H-8, H-10) were found to be identical, showing that the aromatization of the ring was successful (table 5.1). The absence of a signal for H-2 shows that the labile carboxyl-group is still intact after synthesis and isolation. The prominent difference in shift for H1' and H2' between CBN and CBNA is another indication the carboxyl group has been retained.

After performing quantitative NMR analysis, a quantified ethanolic solution of CBNA was obtained, allowing determination of the molar extinction coefficients of CBNA in the range of 200 to 400 nm. Furthermore, infrared spectroscopy has been a common tool for the identification and structure elucidation of cannabinoids and derivatives in isolation and synthesis experiments. As with molar extinction coefficients, IR-spectra are usually reported by presenting a few absorbance maxima only. However, consistent with our previously reported spectroscopic data of other cannabinoids, the full range of extinction coefficients (figure 5.3) and the IR-spectrum (figure 5.4) of CBNA are shown.

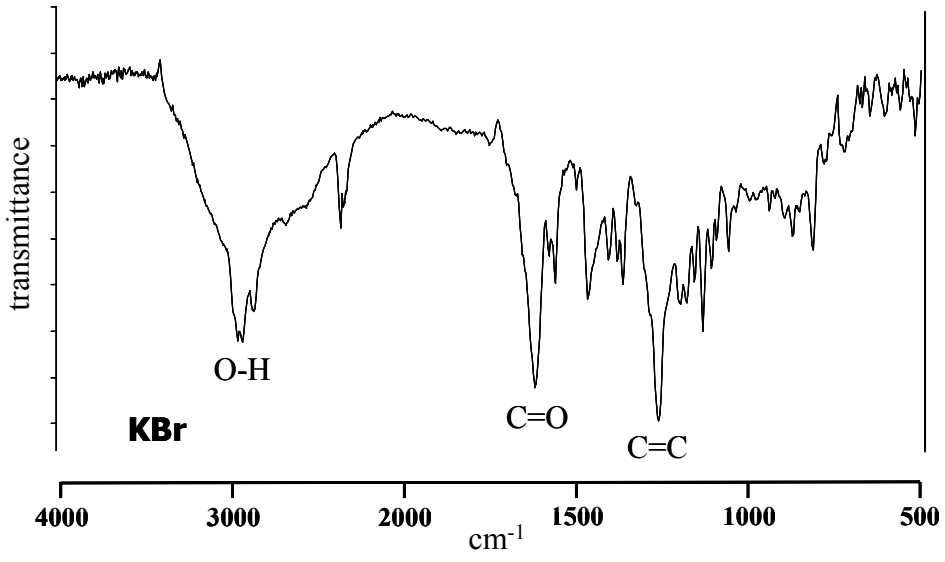

Figure 5.4: IR-spectrum of CBNA in the range of 500-4000 cm^{-1} obtained by Fourier-transform (FT)-IR spectrometry

5.4 Conclusion

In this study, the acidic cannabinoid CBNA was produced by dehydrogenation of THCA extract using a relatively simple synthesis. Final product was a highly pure (96% by GC analysis), quantified CBNA solution suitable for use as reference standard for further analytical studies. Unfortunately, the overall yield of the synthesis was found to be only around 10%. However, the described method for dehydrogenation is relatively simple and well described [Lee, 1992], and THCA is easy to obtain in large amounts from cannabis plant materials [Hazekamp, 2004a], making it feasible to scale up the procedure for production of larger amounts of CBNA.
Full spectroscopic data for CBNA (UV, fluorescence, IR, ^{1}H-NMR, MS) is now available, which should further facilitate studying the role of CBNA as a component of cannabis products. The spectroscopic and chromatographic data we obtained were published in a systematic manner, complementing the data that was earlier obtained on other natural cannabinoids [Hazekamp, 2005]. In total, we now published the spectroscopic and chromatographic data of 17 main cannabinoids occurring naturally in the cannabis plant.

CHAPTER 6

Chromatographic and spectroscopic data of cannabinoids from *Cannabis sativa* L.

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Abstract

Chromatographic and spectroscopic data was determined for 16 different major cannabinoids from *Cannabis sativa* plant material as well as 2 human metabolites of Δ^9-tetrahydrocannabinol. Spectroscopic analysis included UV absorbance, infrared-spectral analysis, (GC-) mass spectrometry and spectrophotometric analysis. Also the fluorescent properties of the cannabinoids are presented. Most of this data is available from literature but scattered over a large amount of scientific papers. In this case, analyses were carried out under standardised conditions for each tested cannabinoid so spectroscopic data can be directly compared. Different methods for the analysis of cannabis preparations were used and are discussed for their usefulness in the identification and determination of separate cannabinoids. Data on the retention of the cannabinoids in HPLC, GC and TLC are presented.

6.1 Introduction

In recent years a lot of research on the medical applications of *Cannabis sativa* L. has been initiated, as several, mostly European countries, move towards a more liberal view on the use of cannabis as a medicine [Baker, 2003]. Although more than 400 compounds have been identified in the cannabis plant [Turner, 1980], most studies have focused on the effects of the cannabinoids, in particular (-)-Δ^9-(*trans*)-tetrahydrocannabinol (Δ^9-THC). One reason is that the main pharmacological and psychoactive effects of cannabis have been attributed to Δ^9-THC. For instance, synthetic Δ^9-THC (dronabinol, Marinol™) has been shown to possess anti-emetic properties useful in cancer therapy. However, in several medical studies the effect of Δ^9-THC or dronabinol alone could not match the effects of a total cannabis preparation [Williamson, 2000], indicating there may be other active compounds present [Turner, 1985]. More than 60 cannabinoids [Mechoulam, 1970; Joyce, 1970; Nahas, 1973; Turner, 1980] have been identified in Cannabis, and occasionally new cannabinoids are being discovered [Ross, 1995]. The chemical structures of the main cannabinoids found in the cannabis plant are shown in figure 6.1 and their physicochemical properties are listed in table 6.1. Only a few of these cannabinoids have been studied in detail, although several of these have been shown to possess some biological activity (reviewed by Grotenhermen [2003]).

To extend the knowledge of the therapeutic properties to cannabinoids other than Δ^9-THC, large amounts of pure compounds must be available. Assessment of cannabinoids pharmacology is now almost restricted to the few that are commercially available (i.e.: Δ^9-THC, Δ^8-THC, CBD and CBN). Furthermore, pure cannabinoids must be available as reference compounds for their unequivocal identification and determination. For that purpose, chromatographic and spectroscopic methods and data are available from scientific literature. But although these data have been published for most known cannabinoids during isolation and identification experiments (see Turner *et al.* [1980] for an overview), they are scattered over a huge amount of scientific papers. Moreover, standardised data obtained under identical analytical conditions have not been reported yet. And as far as we know, the fluorescent properties of the cannabinoids remain largely unknown [Zoller, 2000].

This report lists the main chromatographic and spectroscopic data of 16 cannabinoids and of two of their human metabolites, all obtained under identical analytical conditions. Methods were kept as straightforward, simple and rapid as possible. The pros and cons of each method will also be discussed. All analyses were carried out for each cannabinoid as far as permitted by the amount of pure compound available to us.

6.2 Materials and methods

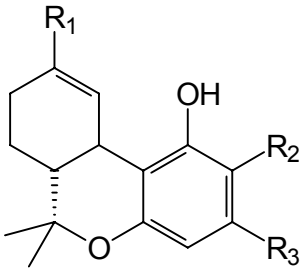
6.2.1 Standards and solvents

Reference compounds of Δ^9-THC, cannabinol (CBN), cannabidiol (CBD), cannabigerol (CBG), (-)-Δ^9-(*trans*)-tetrahydrocannabinolic acid A (THCA), cannabidiolic acid (CBDA)

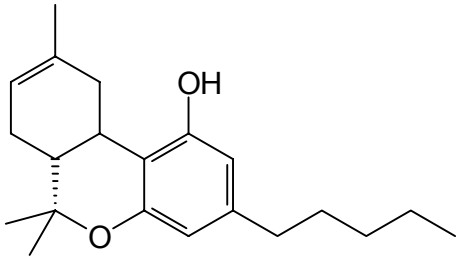
and cannabigerolic acid (CBGA) were isolated previously in our laboratory [Hazekamp, 2004a]. A quantitative ^{1}H-NMR method was developed for their quantitation [Hazekamp, 2004b]. (-)-Δ^{8}-tetrahydrocannabinol (Δ^{8}-THC) was obtained from Sigma. The main human metabolites of Δ^{9}-THC, i.e.: 11-hydroxy-THC (11-OH-THC) and 11-carboxy-THC (THC-COOH) were purchased from Cambridge isotope laboratories (CIL, Innerberg, Switzerland) and from Lipomed (Arlesheim, Switzerland) respectively. All these cannabinoids were available as certified and calibrated reference standards. The remaining cannabinoids used for this study (see table 6.1) were obtained by preparative HPLC on extracts of *Cannabis sativa* plant materials and identified by comparing their chromatographic and spectroscopic data with literature [Brenneisen, 1988; Harvey, 1992; Lehmann, 1995] and by a search in UV [Pragst, 2001] and mass spectra databases [Pfleger, 2000; Agilent technologies, 2000]. All organic solvents (analytical or HPLC reagent grade) were purchased from J.T. Baker (Deventer, The Netherlands) or from Fluka Chemie (Buchs, Switzerland).

Table 6.1: Physicochemical properties of the cannabinoids

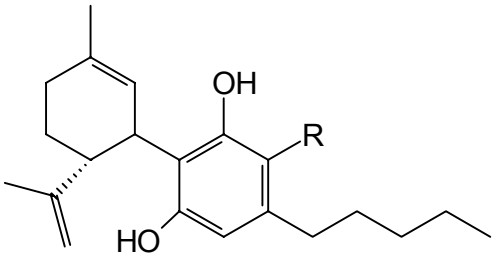
#	cannabinoid	full name	MW (calc.)	molecular formula		
				C	H	O
Neutral cannabinoids						
1	Δ^{9}-THC	*trans*-(-)-delta-9-tetrahydrocannabinol	314.472	21	30	2
2	Δ^{8}-THC	*trans*-(-)-delta-8-tetrahydrocannabinol	314.472	21	30	2
3	THV	*trans*-(-)-delta-9-tetrahydrocannabivarin	286.418	19	26	2
4	CBD	cannabidiol	314.472	21	30	2
5	CBN	cannabinol	310.440	21	26	2
6	CBG	cannabigerol	316.488	21	32	2
7	CBC	cannabichromene	314.472	21	30	2
8	CBL	cannabicyclol	314.472	21	30	2
Acidic cannabinoids						
9	THCA	*trans*-(-)-delta-9-tetrahydrocannabinolic acid A	358.482	22	30	4
10	THCA-C4	*trans*-(-)-delta-9-tetrahydrocannabinolic acid-C4	344.455	21	28	4
11	THVA	*trans*-(-)-delta-9-tetrahydrocannabivarinic acid	330.428	20	26	4
12	CBDA	cannabidiolic acid	358.482	22	30	4
13	CBNA	cannabinolic acid	354.450	22	26	4
14	CBGA	cannabigerolic acid	360.498	22	32	4
15	CBCA	cannabichromenic acid	358.482	22	30	4
16	CBLA	cannabicyclolic acid	358.482	22	30	4
Human THC-metabolites						
17	11-OH-THC	11-hydroxy-tetrahydrocannabinol	330.471	21	30	3
18	THC-COOH	11-carboxy-tetrahydrocannabinol	344.455	21	28	4



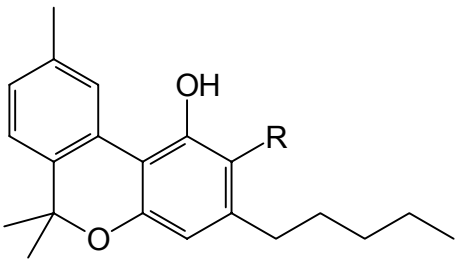
	R_1	R_2	R_3	
1:	*-CH_3*	*-H*	*-C_5H_{11}*	Δ^9-THC
3:	*-CH_3*	*-H*	*-C_3H_7*	THV
9:	*-CH_3*	*-COOH*	*-C_5H_{11}*	THCA
10:	*-CH_3*	*-COOH*	*-C_4H_9*	THCA-C_4
11:	*-CH_3*	*-COOH*	*-C_3H_7*	THVA
17:	*-CH_2OH*	*-H*	*-C_5H_{11}*	11-OH-THC
18:	*-COOH*	*-H*	*-C_5H_{11}*	THC-COOH



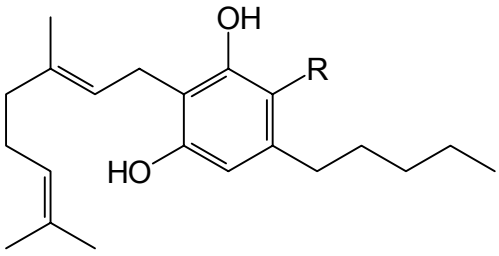
2: Δ^8-THC



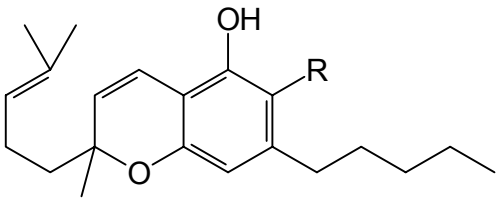
4: *R=H*; CBD
12: *R=COOH*; CBDA



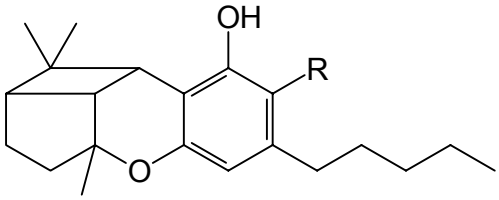
5: *R=H*; CBN
13: *R=COOH*; CBNA



6: *R=H*; CBG
14: *R=COOH*; CBGA



7: *R=H*; CBC
15: *R=COOH*; CBCA



8: *R=H*; CBL
16: *R=COOH*; CBLA

Figure 6.1: Structures of the studied cannabinoids.

6.2.2 Thin layer chromatography (TLC)

Samples in ethanol were spotted on 20x10 cm TLC plates. Two different TLC systems were used. For the non-polar system, reversed phase (C_{18}) silicagel plates F254 No. 105559 (Merck, Darmstadt, Germany) were used with methanol/5% acetic acid 19 : 1 (v/v) as the eluent. For the polar system, normal phase silicagel plates F254 No. 105554 (Merck, Darmstadt, Germany) were used with chloroform/methanol 19 : 1 (v/v) as the eluent.
Plates were developed in saturated normal chambers (saturation time 15 minutes). Absorption of chromatographic spots was evaluated under UV 254nm. General visualisation of compounds was done by spraying with modified anisaldehyde-sulphuric acid spray reagent [Stahl, 1967]. For selective visualisation of cannabinoids, the TLC plate was sprayed with 0.5% fast blue B salt (*o*-dianisidine-*bis*-(diazotized)-zinc double salt) (Sigma) in water, followed by 0.1 M NaOH [Corrigan, 1980].

6.2.3 Gas Chromatography-Mass Spectrometry (GC-MS)

To obtain GC retention times, molecular weights, and fragmentation spectra of cannabinoids, GC-MS analyses were performed on a Varian 3800 gas chromatograph, coupled to a Varian Saturn 2000 GC-MS apparatus. The system was controlled with Varian Saturn GC-MS workstation version 5.2 software. The GC was fitted with two different types of columns; a Durabond fused silica capillary column (30 m x 0.25 mm inner diameter) coated with DB-1 at a film thickness of 0.1 µm, and a similar column, coated with HP-50+ at a film thickness of 0.15 µm (J&W scientific Inc., Rancho Cordova, CA). The oven temperature was programmed from 100°C to 280°C at a rate of 10°C/min. The oven was then kept at 280°C until the end of a 30 min run time. The injector and detector port temperatures were maintained at 280°C and 290°C, respectively. Helium was used as the carrier gas at a pressure of 65 kPa. The injection split ratio was 1/50. Elution time of Δ^9-THC was used as internal reference to determine the relative retention times of all other cannabinoids.

6.2.4 High-Performance Liquid Chromatography (HPLC) with diode-array and fluorescence detection

The HPLC profiles were acquired on an Agilent 1100 series HPLC, consisting of a G1322A solvent degasser, a G1311A quaternary solvent pump, and a G1313A autosampler. The column was kept at constant temperature by using a G1316A column oven. The analytical column was a Waters XTerra MS C_{18} (2.1 x 150mm, 3.5µm) fitted with a XTerra MS C_{18} (2.1x10 mm, 3.5 µm) guard column. Light absorption and emission were detected by a G1315B UV-diode array detector (DAD) and a G1321A fluorescence detector (FLD). The system was controlled through a Vectra VL 420 DT computer equipped with Agilent A09.01 software. UV-spectra were measured on-line by DAD in the range of 195-400 nm with a slit of 2 nm. Fluorescence (FL) spectra were recorded on-line by the FLD in the range of 280-650 nm

with a step of 5 nm after excitation at 222 nm. Retention times were expressed as relative to Δ^9-THC.
DAD and FLD data of cannabinoids were recorded under acidic conditions, with a mobile phase consisting of a mixture of methanol-water containing 25 mM of formic acid (pH ±3). The proportion of methanol was linearly increased from 65 to 100 % over 25 minutes, and then kept constant for 3 minutes. Analysis under basic conditions was obtained with a mobile phase consisting of a mixture of acetonitrile-phosphate buffer (10 mM, pH 7.5). The acetonitrile concentration was increased from 40 to 100 % in 25 minutes, and then kept constant for 3 minutes. In both HPLC systems, the column was re-equilibrated under initial conditions for 10 minutes, the flow rate was 0.3 ml/min, and the total run time was 38 minutes. All determinations were carried out at 30°C.

6.2.5 Spectrophotometric analysis (extinction coefficients)

Cannabinoids that were available as calibrated certified standards were diluted to a concentration of 0.01 mg/ml in ethanol to determine molar extinction coefficients in the range of 200 to 400 nm. A blank measurement was obtained with ethanol. UV-spectra were recorded using a Varian Cary 1 Bio UV-Visible spectrophotometer controlled by Cary 1/3E system software, version 3.02. A sample cell of 10 mm was used for all measurements.

6.2.6 Infrared Spectroscopy (IR)

Infrared spectra of cannabinoids that were available in sufficient amounts were measured using a Perkin Elmer paragon 1000PC FT-IR instrument, which was controlled by Perkin Elmer spectrum IR V2.00 software. Concentrated ethanolic solutions of the cannabinoids (25 µl) were mixed with finely ground KBr (Merck, IR-grade), and ethanol was evaporated under vacuum for 10 minutes. After proper calibration of the apparatus, IR-spectra were measured as an average of 4 scans in the wavenumber range of 500 to 4000 cm^{-1}. After acquisition, the spectra were smoothened by using the software.

6.3 Results and discussion

Spectroscopic and chromatographic data is shown for 14 different cannabinoids that were available to us. However, not all cannabinoids were available in large enough quantities to obtain exploitable data in all analyses that were carried out. Therefore the presented data is not complete for all cannabinoids.

6.3.1 Thin Layer Chromatography

By using two TLC-systems (polar and non-polar system) in combination with fast blue B spray reagent, it was possible to detect and distinguish all tested compounds. The Rf-values of

the cannabinoids in both TLC-systems and their spot colour after spraying with fast blue B are shown in table 6.2. The use of fast blue B as a selective detection reagent for cannabinoids [Corrigan, 1980] results in differently coloured spots for some compounds. Unfortunately, these colours also depend on the concentration of the substance and on the presence of interfering compounds. The results therefore must be considered with caution. Nevertheless, we found that fast blue B was more sensitive for detection of cannabinoid spots than UV-detection at 254nm. For example, the detection limit for Δ^9-THC was about 0.5 mg/mlL (2 µL spotted) with UV-detection under 254 nm, and around 0.002 mg/ml with fast blue B detection.

Table 6.2: Relative retention (Rf) values of the cannabinoids in a polar (silica-gel) and non-polar (C_{18}) TLC-system. The colours of chromatographic spots after spraying with the cannabinoid-selective spray reagent fast blue B (FBB) are indicated.

Non-polar TLC system (RP-18)			*Polar TLC system (silica)*	
Cannabinoid	**Rf-value**	**Color FBB**	**Cannabinoid**	**Rf-value**
CBDA	0.68	red	Δ^9-THC	0.65
CBGA	0.67	brown	Δ^8-THC	0.65
CBG	0.59	orange-brown	CBD	0.64
CBD	0.58	red-brown	CBN	0.62
CBN	0.48	purple	CBG	0.61
Δ^9-THC	0.44	red	CBC	0.58
Δ^8-THC	0.43	red	THCA	0.39
THCA	0.40	red	CBDA	0.37
CBC	0.37	purple	CBGA	0.31
CBCA	0.35	purple	CBCA	0.25

The main advantages of TLC are its ability to detect all spotted compounds, while analysing several samples simultaneously under identical conditions within a short timeframe. Lack in selectivity can sometimes be overcome by the use of selective detection reagents. However, in the case of cannabinoids it does not seem possible to obtain a good separation with positive identification of all cannabinoids when complex mixtures (e.g. plant extracts) are analysed. Several TLC systems are therefore needed for tentative identification. For instance, CBDA and CBGA, or CBD and CBG which were not separated in the non-polar system could be distinguished when using silica as stationary phase. On the other hand, Δ^8THC and Δ^9THC were found to co-elute on both systems (see table 6.2). In conclusion, TLC is very useful to rapidly screen many samples for the presence of cannabinoids in crude plant extracts, or in

eluted fractions collected during preparative chromatography. However, reproducibility of TLC depends on several parameters, such as relative humidity and temperature. Compared to other separation methods, the performance of TLC performances is relatively low. Consequently, unequivocal identification of cannabinoid spots requires further methods.

6.3.2 GC-MS

Two different capillary column phases were used for GC analysis (HP-50+ and DB-1). The HP-50+ column was a medium-polar column, resulting in relatively longer retention times compared to the non-polar DB-1 column. Simultaneous injection on both columns enables the distinction of all tested cannabinoids. Retention times (relative to Δ^9-THC) of the analysed cannabinoids are shown in table 6.3. All cannabinoids eluted well after other major cannabis components such as the terpenoids.

Because no derivatization was used in our case, the mass-spectra obtained by GC-MS (figure 6.2) are similar for the acidic cannabinoids and their corresponding neutral cannabinoids (e.g. THCA and Δ^9-THC). Although CBD is structurally quite distinct from CBC and CBL, these three cannabinoids nonetheless show similar MS spectra (compare spectra of figure 6.2), with identical base peak (m/z = 231) and molecular ion (m/z = 314). Also their retention times in GC were quite similar (table 6.3), but their separation is good enough to distinguish them. Cannabidiol differs from CBC and CBL with one significant fragment at m/z=246. A retro-Diels-Alder reaction accounts for the formation of the minor ion at m/z = 246. Subsequent loss of a methyl fragment results in a contribution to the ion at m/z=231 [Harvey, 1992]. As can be seen in figure 6.2, the base peak of all tested cannabinoids (except Δ^8-THC) does not correspond to the molecular ion, but to a fragment, indicating that these cannabinoids are easily fragmented by GC-MS.

In the absence of derivatization, the high temperature that is applied in GC causes the decarboxylation of acidic cannabinoids to their corresponding neutral form [Raharjo, 2004]. Since the cannabis plant mainly contains the (carboxylic-) acidic forms of cannabinoids [Shoyama, 1975], GC analysis is not the method of choice to establish the metabolic profile of a cannabinoid sample. To avoid decarboxylation, the acids must be derivatized, e.g. by silylation or formation of the alkylboronates [Harvey, 1977]. However, a 100 % derivatization yield is difficult to obtain. Moreover, we believe that thermo-degradation (oxidation, isomerization) of cannabinoids in the injector port and column may also occur. In the case of Δ^9-THC, low but significant amounts of Δ^8-THC and CBN were detected in the GC-chromatogram, whereas other analyses (HPLC, NMR, TLC) did not show these compounds which are known degradation products of Δ^9-THC (data not shown). Despite these problems associated with GC, it remains a very useful method for the analysis of cannabinoids [Raharjo, 2004].

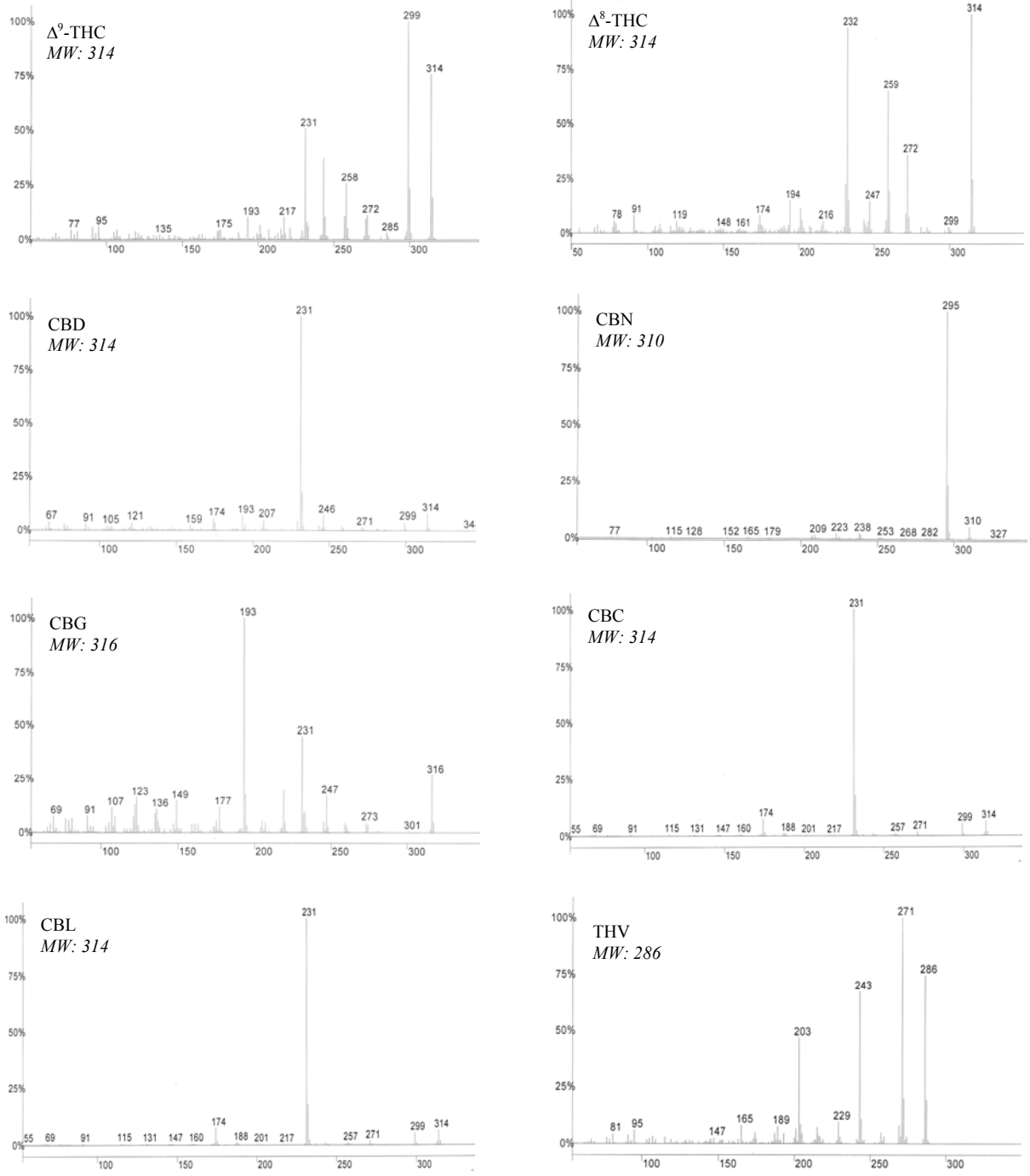


Figure 6.2: Mass-spectra in the range of *m/z* 50-335 obtained by GC-MS.

Table 6.3: Relative retention time (RRT, relative to THC) of cannabinoids in GC using a non-polar (DB-1) and medium-polar (HP-50) column.

	GC column type	
	DB-1	*HP-50*
Cannabinoid	**RRT**	**RRT**
THV	0.885	0.902
CBL	0.922	0.907
CBD	0.942	0.935
THC-C4	0.942	0.948
CBC	0.956	0.924
Δ^8-THC	0.988	0.981
Δ^9-THC	1	1
CBG	1.026	1.012
CBN	1.033	1.046

6.3.3 HPLC with UV/FLD detection

With gradient-elution, most cannabinoids were base-line separated as sharp peaks with excellent peak purity level, yielding fully exploitable UV and fluorescence spectra. The retention times of cannabinoids (relative to Δ^9-THC) are shown in table 6.4. It is interesting to note that the relative elution time of the acidic cannabinoids can be influenced by changing the pH of the eluent, while the order of elution for the neutral cannabinoids remains the same [Turner, 1982]. In this way overlap between chromatographic peaks of acid and neutral cannabinoids can be decreased by changing the elution pH. Notwithstanding these pH differences, the elution order of THCCOOH (also an acidic cannabinoid) and THC was not modified.

Although the UV-spectra of the analysed cannabinoids (figure 6.3a) were left unchanged when the pH was changed from 3.0 to 7.5, the FL-spectra differ drastically (figure 6.3b). Acidic cannabinoids completely lose their fluorescence under acidic conditions, while CBC has no fluorescence under basic conditions and CBN has no fluorescent properties at all. The fluorescent properties of the other analysed cannabinoids are not influenced by pH. The UV absorption and FL yield in figure 6.3a and b cannot be directly compared, because no standardised concentrations of the cannabinoids were used. Standardised UV-spectra were obtained using a spectrophotometer (see below).

In some cases, partially unresolved peaks could not be identified because their UV and fluorescence spectra were identical. This can be seen with table 6.4 and on figures 6.3a and 6.3b in the case of CBD and CBG, or Δ^8-THC and Δ^9-THC, which are characterised by very close retention times and identical UV and fluorescence spectra.

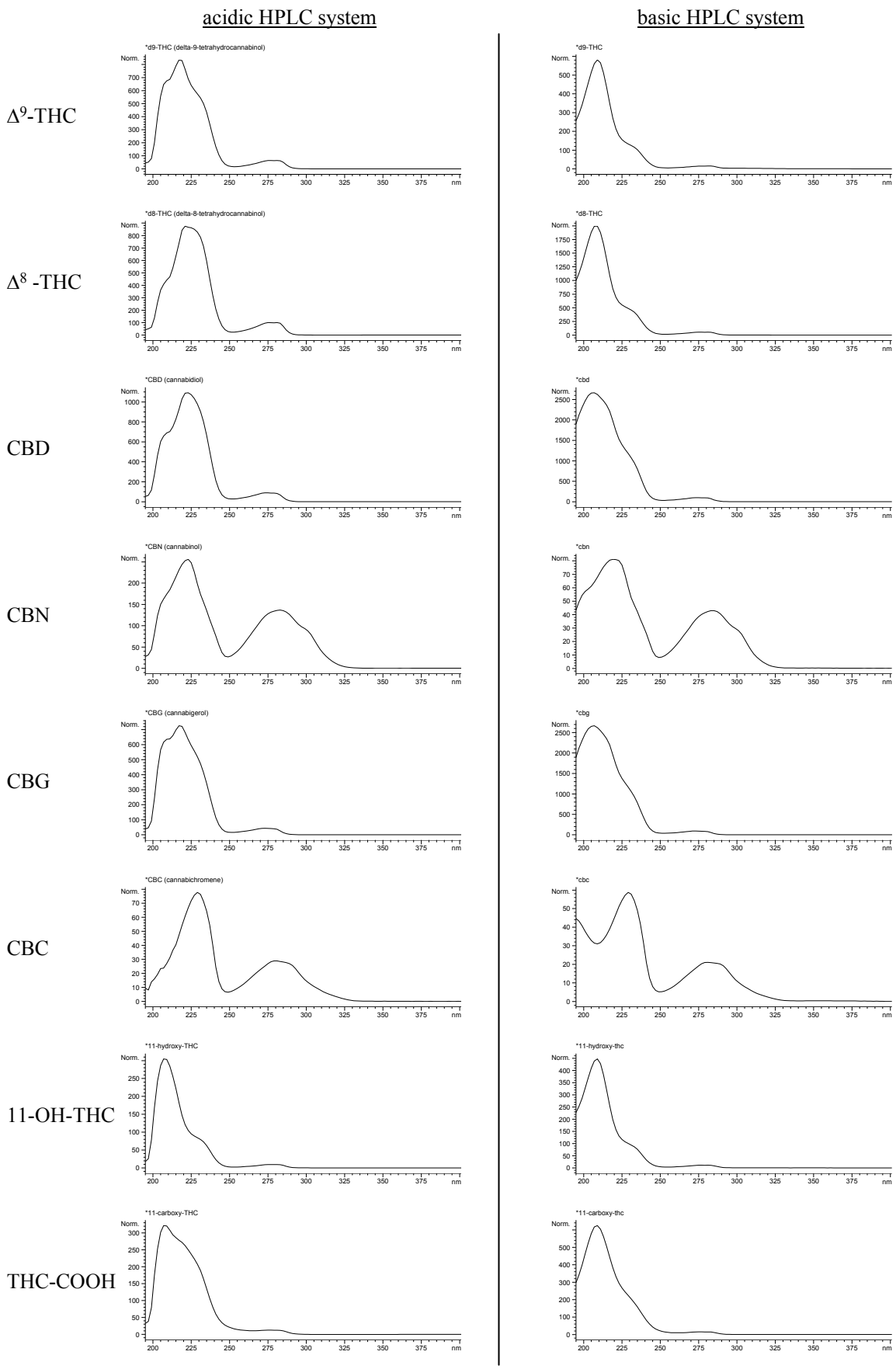


Figure 6.3a: UV-spectra in the range of 190-400 nm obtained in two HPLC-systems (acidic and basic pH).

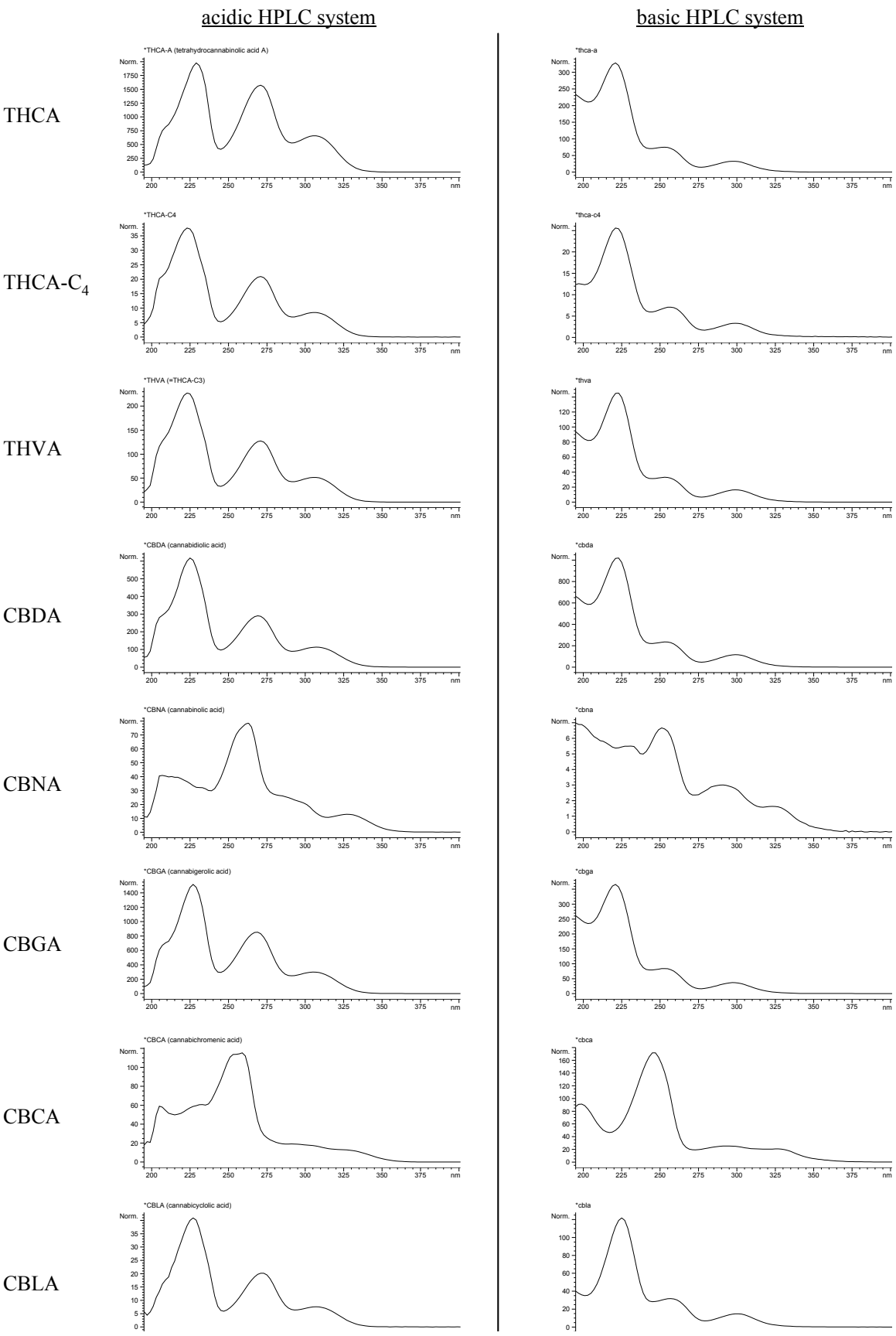


Figure 6.3a: Continued

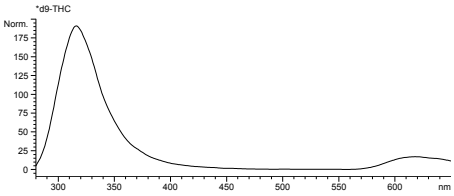
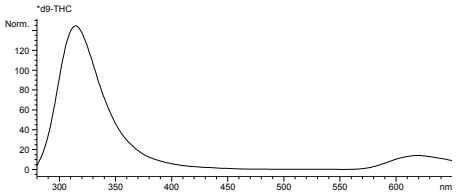
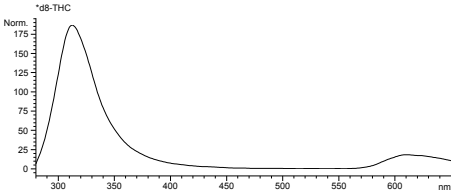
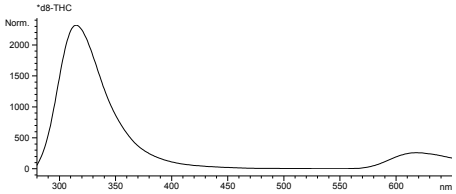
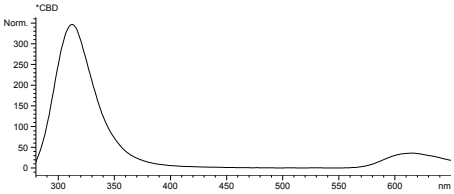
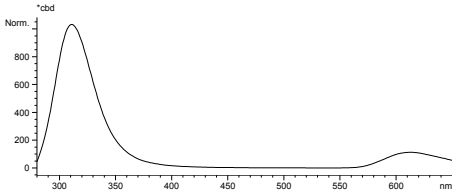
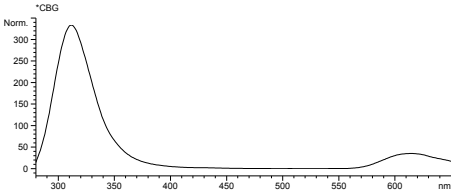
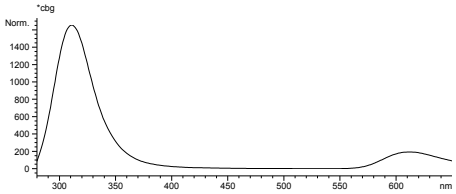
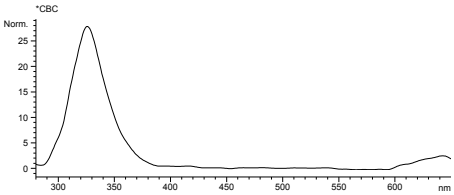
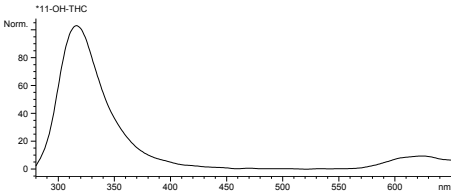
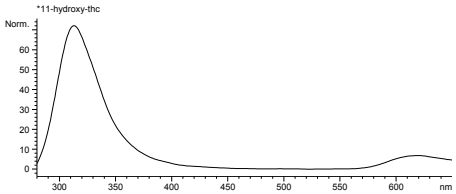
	acidic HPLC system	basic HPLC system
Δ^9-THC		
Δ^8 -THC		
CBD		
CBN	No spectrum	No spectrum
CBG		
CBC		No spectrum
11-OH-THC		
THC-COOH	No spectrum	No spectrum

Figure 6.3b: Fluorescence spectra in the range of 280-650 nm obtained in two HPLC-systems (acidic and basic pH).

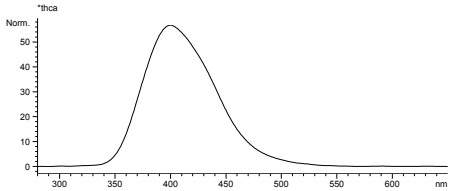
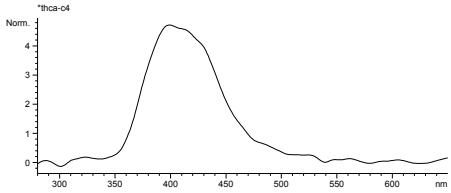
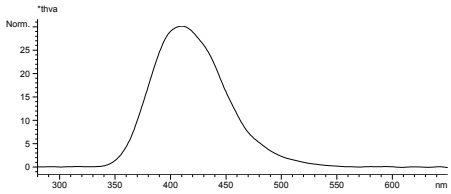
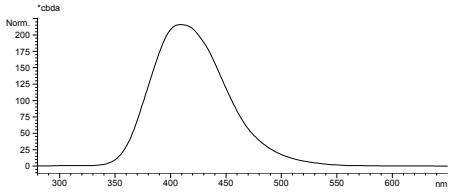
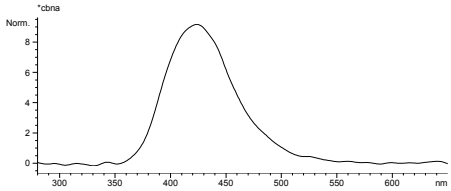
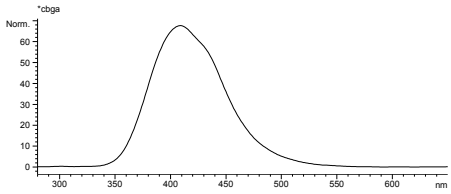
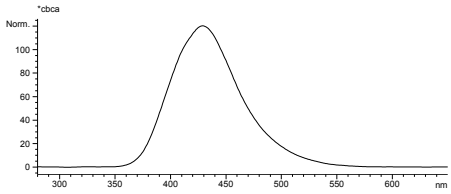
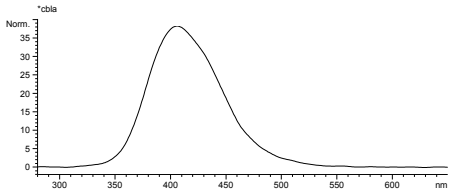
	acidic HPLC system	basic HPLC system
THCA	No spectrum for all acidic cannabinoids	
THCA-C_4		
THVA		
CBDA		
CBNA		
CBGA		
CBCA		
CBLA		

Figure 6.3b: Continued

Table 6.4: Relative retention time (RRT, relative to THC) of cannabinoids in HPLC using a reversed phase column (C_{18}) and a slightly basic (pH 7.5) or acidic (pH 3) eluent

Acidic HPLC system		*Basic HPLC system*	
Cannabinoid	**RRT**	**Cannabinoid**	**RRT**
11-OH-THC	0.70	THC-COOH	0.26
THC-COOH	0.76	CBDA	0.34
CBD	0.76	THVA	0.36
THV	0.77	CBGA	0.40
CBG	0.78	THCA-C4	0.42
CBDA	0.82	CBNA	0.50
CBGA	0.92	THCA-A	0.51
CBN	0.93	CBLA	0.53
Δ9-THC	1.00	CBCA	0.61
Δ8-THC	1.03	CBD	0.83
THVA	1.04	CBG	0.83
CBC	1.12	CBN	0.95
THCA-C4	1.13	Δ9-THC	1.00
CBNA	1.21	Δ8-THC	1.01
THCA-A	1.25	CBC	1.08
CBLA	1.32	11-OH-THC	1.31
CBCA	1.34		

The chromophore of the cannabinoids corresponds to its substituted phenolic ring, as this is a common structural element among the tested cannabinoids. The UV spectrum of Δ^9-THC is identical to that of olivetol, which shows the same phenolic ring structure and is the precursor of Δ^9-THC and the other cannabinoids. The alkyl-sidechain does not influence the UV-absorbance, as there is no difference between THCA (C_5 –sidechain) and THVA (C_3 –sidechain). The cyclization of the non-phenolic part of the cannabinoids also has no influence on the absorbance, except when another aromatic ring (CBN, CBNA) or a conjugated double bond (CBC, CBCA) is introduced.

In the case of HPLC peak overlap the use of MS-detection in the form of LC-MS or LC-MS-MS can provide better clues about cannabinoid structure and identity. In the acid system (pH3), formic acid was used as the acidifying agent to make the eluent compatible with mass spectrometry. In contrast to HPLC-DAD or Fl which are carried out at room temperature, LC-MS with ionspray ionisation at relatively high temperature (e.g. 300°C) may result in partial thermal decomposition of acid cannabinoids. An example of an LC-MS separation of a range of THC metabolites in body fluids at a concentration of 50 ng/ml is shown on figure 6.4. For separation, a Waters XTerra C_8 microbore column was used. In contrast to GC-MS operating in the EI mode, the mass spectra are very simple with one prominent $[MH]^+$ or

[M-H]$^-$ pseudo-molecular ion and very little fragmentation. For better sensitivity, the data were recorded in the Selected Ion Monitoring (SIM) mode. Except THC ([MH]$^+$ = 315.2), all cannabinoids were measured in the negative ionisation mode. The monohydroxylated (8β-OH- and 11-OH-THC) and dihydroxylated (8β-11-diOH-THC) metabolites were well resolved from the acid inactive metabolite (THCCOOH) and its conjugated derivative (THCCOOH-glucuronide) in a single analytical run.

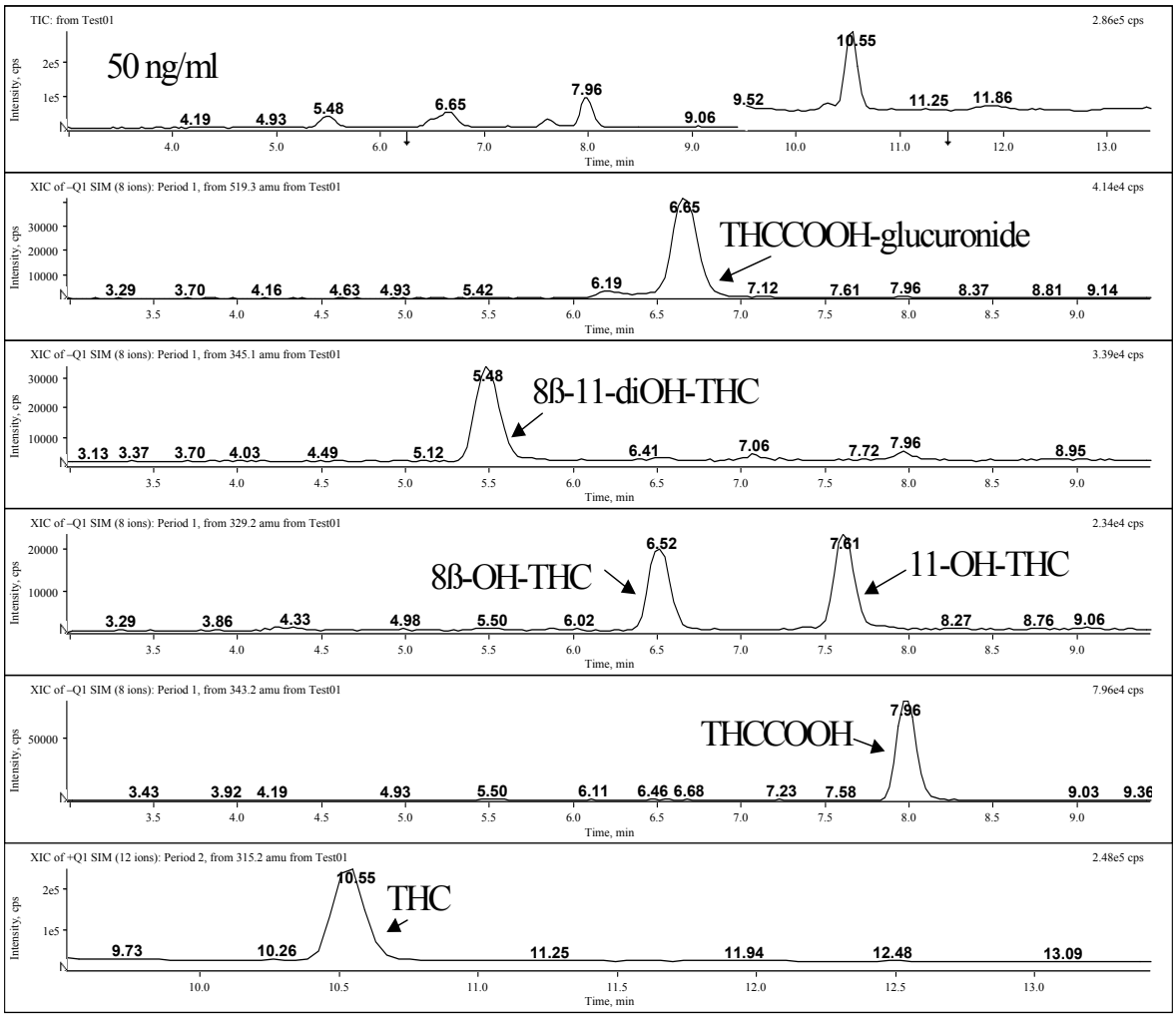


Figure 6.4: Chromatogram of a separation and identification of cannabinoid metabolites from human blood in a single chromatographic run, by using LC-MS. All cannabinoids can be identified because of the high selectivity of the mass-detector.

6.3.4 Spectrophotometric analysis (extinction coefficients)

Very few UV-absorption spectra of purified cannabinoids are shown in the scientific literature [Pragst, 2001]. They are generally characterised by a few parameters (maxima and minima, shoulders of the UV spectra). The extinction coefficients are very seldom presented. Because

most cannabinoids differ in their UV with several absorption peaks, many wavelengths can be selected for quantification. Figure 6.5 shows that absorption generally decreases with increasing wavelength. So while a better sensitivity can be obtained in the low 200-210 nm range, selecting a higher wavelength will increase the selectivity by diminishing the risk of measuring interfering compounds. The use of the extinction coefficient provides the possibility of a quick quantification of cannabinoid solutions. In order to perform such rough quantification at a large range of selected wavelengths, the UV spectrum measured at 0.01 mg/ml between 200 and 400 nm is presented for 7 major cannabinoids (figure 6.5). The extinction coefficients (ε) at 3 different maxima are also indicated.

6.3.5 Infrared Spectroscopy (IR)

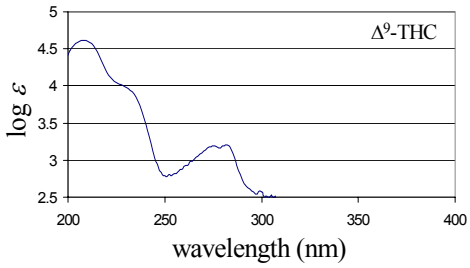
Infrared Spectroscopy has been a common tool for the identification and structure elucidation of cannabinoids and derivatives in isolation and synthesis experiments. As with UV-spectra, usually IR-spectra are reported by presenting a few maximum absorbance peaks only. Obviously, reported IR-spectra have been measured with a large variety of IR-spectrometers. In this report (figure 6.6) we present the full IR-spectra of 8 common natural cannabinoids measured on a single modern FT-IR-spectrometer.

6.4 Conclusion

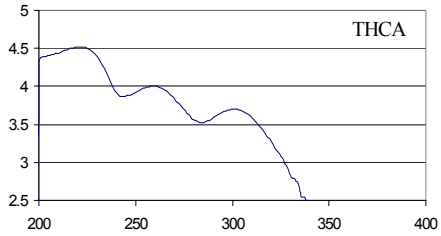
A growing interest in Cannabis as a source of medicinal compounds has emerged during the last few years. Several crude preparations or synthetic drugs derived from Cannabis are under development, or in the clinical pipeline for introduction on the market. In order to carry out these investigations, pharmacologically pure cannabinoids must be available in large quantities. Reference compounds for analytical research must also be present. Chromatographic and spectroscopic data are, therefore, a prerequisite for their determination and identification.

The analytical data presented here makes it possible to positively identify the major cannabinoids found in the cannabis plant. Presenting all analytical parameters measured under standardised conditions should facilitate the identification of cannabinoids isolated from or present in cannabis preparations. Unequivocal identification of cannabinoids cannot totally rely on only one of the tested methods because confusion of some common cannabinoids always remains possible. However, we believe that the use of LC-MS, and especially LC-MS-MS, should make it possible to identify all tested cannabinoids in one single analysis even in the low ng/ml concentration range.

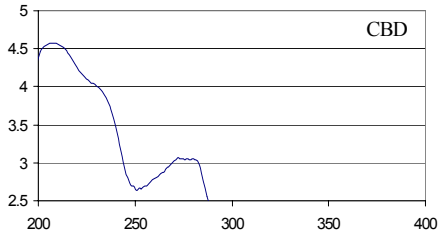
	λ_{max}	*log ε*
Δ^9-THC:	208	*4.62*
	275	*3.19*
	282	*3.20*



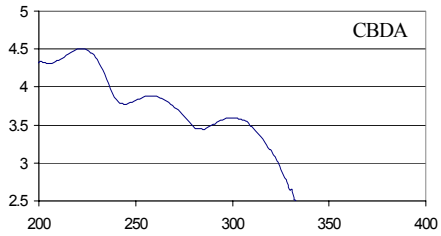
THCA:	222	*4.52*
	258	*4.00*
	301	*3.70*



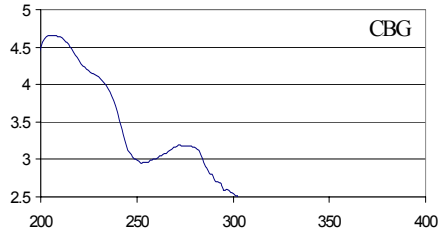
CBD:	207	*4.57*
	272	*3.06*
	280	*3.05*



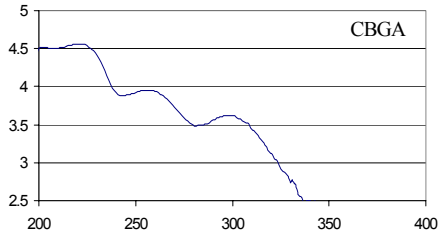
CBDA:	222	*4.50*
	258	*3.88*
	299	*3.59*



CBG:	206	*4.66*
	271	*3.18*
	278	*3.18*



CBGA:	221	*4.56*
	257	*3.95*
	298	*3.62*



CBN:	217	*4.81*
	283	*4.50*
	299	*4.34*

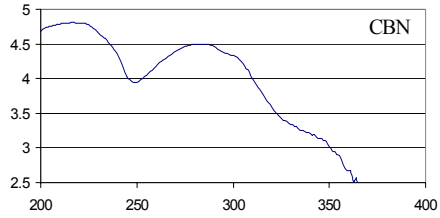


Figure 6.5: Extinction coefficients in the range of 200-400 nm at a concentration of 0.01 mg/ml in ethanol. Absorption values at maxima or shoulders are indicated

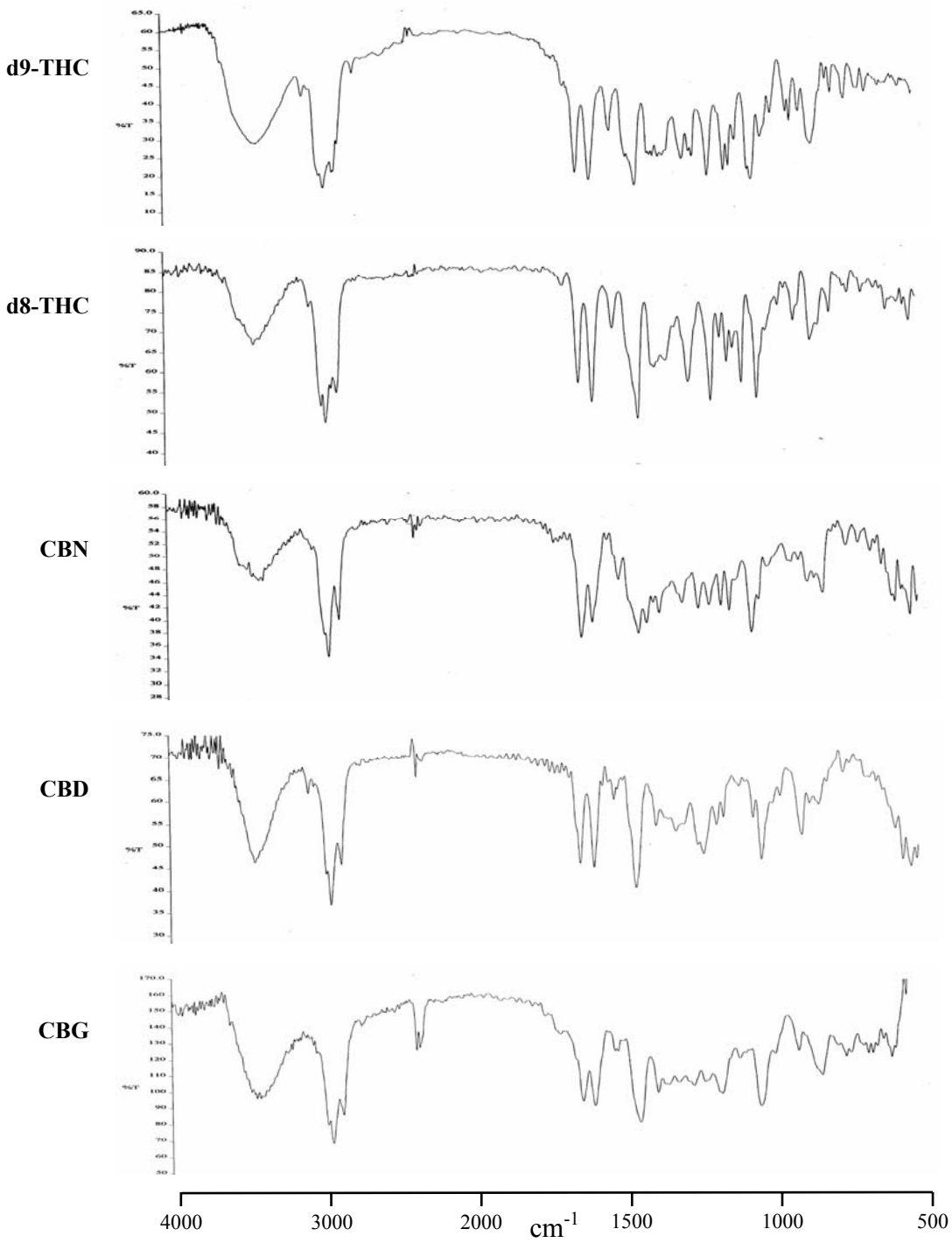


Figure 6.6: IR-spectra in the range of 500-4000 cm^{-1} obtained by fourier-transform (FT)-IR spectrometry.

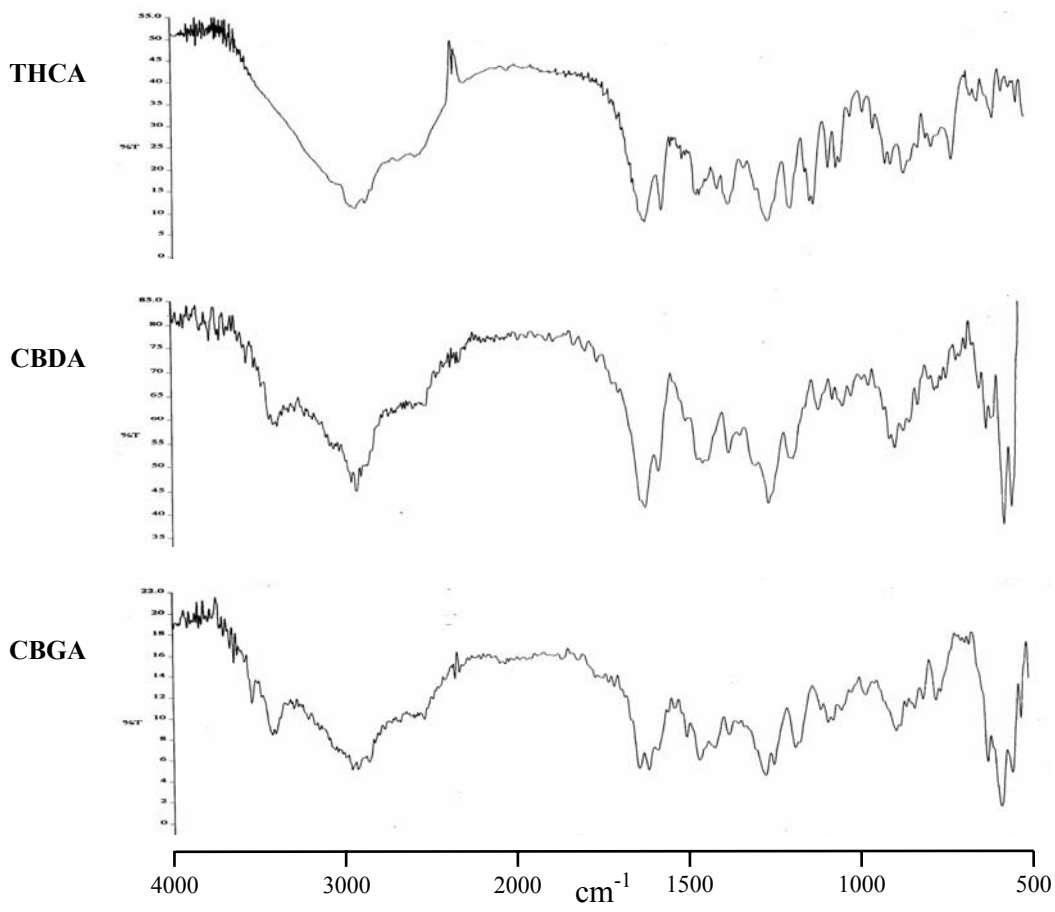


Figure 6.6: Continued.

6.5 Acknowledgements

Mr Pascal Cardinal, chemist, University of Alberta, Canada, is thanked for fruitful discussion. The grower of certified cannabis plants, Bedrocan BV, The Netherlands, is acknowledged for providing cannabis plant material.

CHAPTER 7

Development and validation of an HPLC method for the determination of major cannabinoids from medicinal grade *Cannabis sativa* plant material

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Abstract

After decades of severe legal restrictions on cannabis research, herbal cannabis and its constituents, the natural cannabinoids, are again under intensive study for their medicinal properties. As a result, there is a need for analytical methods for qualitative as well as quantitative analysis of cannabis plant materials. However, most of the methods described are not suitable for the analysis of the acidic cannabinoids, such as tetrahydrocannabinolic acid (THCA), the carboxylic acid precursor of tetrahydrocannabinol (THC). Other methods have not been properly validated for their used in pharmaceutical research. As a result, currently no simple, fully validated method exists for analysis of the authentic composition of cannabis plant materials.

In this study an HPLC method was developed for the analysis of the major cannabinoids present in a high-potency cannabis plant. The method was fully validated according to ICH guidelines by making use of pure cannabinoid standards. HPLC analysis was combined with a secondary analysis by gas chromatography, which made it possible to quantitatively analyze the tested cannabinoids over a wide range of concentrations. Finally, the application of the method was tested on cannabis flowertops. The validated method is routinely used for the analysis of medicinal grade cannabis, as provided through pharmacies in the Netherlands.

7.1 Introduction

The cannabis plant (*Cannabis sativa* L.) is intensively studied for its medicinal effects. The constituents that are thought to be responsible for most of the claimed bio-activities of cannabis are the cannabinoids [Grotenhermen, 2002; Mechoulam, 2005]. The naturally occurring cannabinoids form a complex group of closely related compounds of which currently about 66 are known [Turner, 1980; Ross, 1995]. An important distinction that can be made within the group of cannabinoids is between acidic and neutral cannabinoids; cannabinoids are produced by the metabolism of the plant in the form of carboxylic acids (acidic cannabinoids) [Shoyama, 1975] which can be converted into the decarboxylated (neutral) cannabinoids under the influence of storage, light and heat, by losing the relatively unstable carboxyl-group in the form of CO_2 [Veress, 1990]. The most common types of acidic cannabinoids found in a typical drug-type cannabis plant are tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), cannabigerolic acid (CBGA) and cannabichromenic acid (CBCA). These acids can be converted to their neutral counterparts by decarboxylation to form delta-9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabigerol (CBG) and cannabichromene (CBC), respectively. Degradation of THC results in formation of cannabinol (CBN) and delta-8-tetrahydrocannabinol (delta-8-THC), while THCA can further degrade into cannabinolic acid (CBNA) [Turner, 1980]. Structures and interrelatedness of the cannabinoids are shown in chapter 1 of this thesis.

A few of the pure cannabinoids, and predominantly THC, have been tested for pharmacological and clinical activities. However, it has been repeatedly pointed out that the effects of THC or other single cannabinoids are not equal to that of whole cannabis preparations [Williamson, 2000; Russo, 2003]. Therefore increasingly studies are being conducted with whole cannabis preparations, either as raw plant materials (flower tops) or as extracts [Perras, 2005; Nadulski 2005; Ben Amar, 2006; Holdcroft, 2006]. The bio-activities found for such preparations are possibly the result of the acidic cannabinoids [Verhoeckx, 2006], and consequently a method must be available to identify and quantify neutral as well as acidic cannabinoids present in the plant materials used.

In our studies we routinely work with medicinal grade cannabis of high potency. For these studies we have a clear need to analyze the authentic composition of the plant material. However, analysis of all major cannabinoids in a typical cannabis extract is not easily achieved, because of the complex composition resulting in chromatographic overlap of peaks. Although HPLC coupled to mass-detection (LC-MS) is capable of resolving all cannabinoid peaks in a single analytical run [Stolker, 2004; Hazekamp, 2005], this method is not routinely available to most laboratories. Instead, the most commonly used method for analysis of cannabinoids is gas chromatography [Raharjo, 2004]. But because this method is based on heating of sample components, it converts acidic cannabinoids present in the sample into their decarboxylated counterparts. Therefore, GC analysis is not suitable for the determination of the authentic composition of the cannabinoids in the plant.

The preferred way to analyze cannabis extracts should be by direct analysis, without prior conversion of the acidic cannabinoids. In contrast to GC, no decomposition of the cannabinoids occurs during analysis by HPLC, and hence the acidic cannabinoids may be analyzed directly for phenotypic determination. A good review of HPLC methods developed for cannabinoid analysis was recently given by Raharjo [2004]. However, to our knowledge, none of these methods have been validated according to ICH guidelines; the pharmaceutical standards for adequate validation of analytical methods. Validated HPLC methods do exist for the analysis of cannabinoids according to the American USP or German DAC guidelines. However, these were specifically developed for the analysis of highly pure preparations of THC, either derived from synthetic (USP) or natural source (DAC). They were not intended, and hence not validated, for use with whole cannabis plant materials. Moreover, until recently no calibrated standards were commercially available for the acidic cannabinoids, including THCA, the main acidic cannabinoid found in the drug-type variety of cannabis [Hazekamp, 2004a,b].

Occasionally, new methods are explored for the analysis of cannabinoids, such as capillary electrochromatography [Lurie, 1998] or supercritical fluid chromatography [Backstrom, 1997], but with limited success. Consequently, to our knowledge, there currently is no validated method available for the reliable analysis of authentic herbal cannabis samples.

In this study, an HPLC method is described that was developed for this purpose. The method was focused on the analysis of the cannabinoids that are thought to be mainly responsible for the bioactivity of the cannabis plant. The analysis of these cannabinoids was fully validated for its intended use, according to USP guidelines and in conformity with the current ICH Guideline on Method Validation Methodology [ICH, 2006].

Because the full analysis of a complete cannabis extract with a single HPLC method proved to be difficult, as a result of chromatographic overlap, the HPLC method was routinely combined with a secondary analysis by GC. By combining these two simple techniques of analysis, all major cannabinoids could be effectively identified and quantified. The intended application for this method is the quantification of cannabinoids present in a typical drug-type cannabis cultivar. The method was specifically developed for analysis of the cannabis variety (type “Bedrocan”) that was routinely used by our lab, which means that the ranges of tested concentrations are adapted to the levels found in this plant type. However, only limited additional validation testing should be necessary to adapt the developed method for analysis of broader ranges of concentrations and, hence, other cannabis varieties.

7.2 Materials and methods

7.2.1 Materials

Standards for THC, THCA, CBN, CBD, CBDA, CBG and CBGA were prepared from plant material as previously described [Hazekamp, 2004a]. Pure CBNA was produced by semi-synthesis [chapter 5]. CBC and CBCA were isolated from cannabis hexane extract by

preparative HPLC on a C_{18}-column using methanol/water/0.1% formic acid as the eluent. A standard for delta-8-THC (1.0 mg/ml in methanol) was obtained from Sigma. All standards had a purity of ≥98% as assayed by HPLC and GC, and quantified solutions were prepared by using a previously developed ^{1}H-NMR-method [Hazekamp, 2004b].
Plant material of *Cannabis sativa* L. (variety 'Bedrocan') was obtained from Bedrocan B.V., Veendam, The Netherlands, and was cultivated under standardized conditions according to Good Agricultural Practice (GAP) regulations. Only female flower tops were used and this plant material will be referred to as 'Cannabis Flos'. After harvest, the plant material was air-dried in the dark under constant temperature and humidity for 1 week. For calculation of cannabinoid levels, the weight of the cannabis samples was corrected for water content (typically 5-10%), which was determined by loss on drying. The cannabinoid composition of the used cannabis material, as well as the selected 100% levels for the tested range, are listed in table 7.1. Specifications for the cannabinoid levels were taken from the official Dutch monography on medicinal cannabis [OMC, 2006].
Organic solvents were analytical or HPLC grade (Merck Biosolve Ltd. Valkenswaard, The Netherlands). Water was purified and de-ionized to 18MΩcm^{-1} with a Millipore milli-Q plus water purification system.

7.2.2 HPLC equipment and chromatographic conditions

All chromatographic runs were carried out using a ThermoFinnigan (Waltham, MA) HPLC System, consisting of a P4000 pump, an SCM1000 solvent degasser, an AS3000 autosampler and a UV1000 UV-detector. For specificity testing, full spectra were recorded in the range of 200-400nm using a UV6000LP photodiode-array (PDA) detector. Chromatographic separation was achieved using a GraceVydac C_{18} analytical column (type 218MS54, 5µm, 4.6x250 mm), protected by a Phenomenex C_{18} guard column (3x4 mm). Equipment control, data acquisition and integration were performed with Chromeleon version 6.60 software (Dionex).
The mobile phase consisted of methanol and water, acidified with 25mM of formic acid. Initial setting was 65% methanol (v/v), which was linearly increased to 100% methanol over 25 minutes. After maintaining this condition for 3 minutes, the column was re-equilibrated under initial conditions for 4 minutes, so total runtime was 32 minutes. Flow-rate was set to 1.5 ml/min, the injection volume was 10µL, and detection wavelength was 228 nm. All experiments were carried out at a column temperature of 30°C.

7.2.3 Selection of analytes

The tested cannabinoids are all naturally occurring components of *Cannabis sativa* plant material. The cannabinoids that were used for complete validation (i.e.: the major analytes) were: THCA, THC, delta-8-THC, CBD and CBN. The other cannabinoids were used only for specificity/selectivity testing (i.e.: the minor analytes): CBG, CBGA, CBDA, CBNA, CBC and

CBCA. The selection of major and minor analytes was based on their content in cannabis plant materials and on availability of sufficient amounts of pure reference standards at the time of this study.
The analytes selected for method development represent the majority of cannabinoids which currently are of potential interest to the medicinal cannabis research community. However, the reported HPLC method should allow quantitative determination of additional analytes with little or no modification.

7.2.4 Preparation and stability of standard solutions

Standard solutions for the major analytes were prepared in ethanol at 10, 50, 80, 100, 120, 140, 175% of the concentrations specified in table 7.1. Primary stock solutions were accurately prepared followed by rigorous dilution with ethanol to give secondary standard solutions.
The minor analytes were used only for selectivity testing at a concentration of 0.1 mg/ml. All standard solutions were kept at -20°C until analysis.
Stability of the major analytes was tested by storing analytical solutions in HPLC vials on a laboratory bench under normal lighting conditions for 20 h at ambient temperature. Vials were subsequently analyzed, and compared with the same solutions analyzed immediately.

Table 7.1: Specifications for cannabinoid levels in cannabis plant material (according to monography), and cannabinoid concentrations used as 100% level for this study. Values are based on dry weight plant material. Specification for THCA and THC is based on total THC after heating of plant material to convert THCA into THC.

Analyte	Specifications (official)	Specified 100% level for this study	Equivalent concentration in ethanol extract
THC	18% (after heating)	4.0%	0.20 mg/ml
THCA		16.0%	0.80 mg/ml
CBD	0.1-1.5%	5.0%	0.25 mg/ml
CBN	<1.0%	1.0%	0.05 mg/ml
delta-8-THC	n.s.	0.5%	0.025 mg/ml

7.2.5 Validation of the method

The HPLC method was validated for the quantitative analysis of the major analytes in agreement with International Conference on Harmonization guidelines (ICH, 2006), using the following analytical parameters: range & linearity, precision (repeatability), accuracy (recovery of spiked solutions), specificity, lower limit of quantification, and robustness.
Linearity was evaluated by calculation of a regression line using the least squares method. Calibration curves were obtained from 7 different concentrations analyzed 3 times on 4 different days.

Precision was assessed by analyzing the full range of the standard solutions 3 times in the same day (intra-day precision, repeatability) and by analyzing these same standards on 4 different days (inter-day or intermediate precision).
Accuracy was tested by determining the recovery of spiked cannabinoids at three different concentrations and by calculation of the relative standard deviation (RSD) of the recovery.
Specificity was determined by confirming authenticity of peaks before and after stressing plant extract in order to induce formation of degradation products.
Quantification limits were determined based on the standard deviation of the response compared to the allowed (≤5%) inter-assay precision at the specified 100% concentration.
Robustness was evaluated by applying the method on cannabis plant material while introducing variations in sample amount, and furthermore by having the calibration curves prepared by two different technicians.

7.2.6 Application of the method

Cannabis plant material (60 grams; in order to obtain a representative sample) was ground to fine pieces (<1mm) using a mechanical grinder (blender). Sample extracts were prepared by mechanically shaking 500 mg of ground Cannabis Flos for 10 minutes with 40 ml of ethanol. Sample was centrifuged and clear supernatant was transferred to a 100 ml volumetric flask. The procedure was repeated twice more with 30 ml of ethanol, then solutions were combined and filled up to 100 ml with ethanol. Finally the solution was filtered through a 0.45µm PTFE syringe filter. The first 10 ml were discarded because of cannabinoids that might absorb to the filter. Filtrate was stored at -20°C until analysis.
Positive identification of sample components in extracts was accomplished by analysis of peaks using photodiode array (PDA-) and mass (MS)-detection, and comparing spectra and retention time with reference compounds. Acceptability criteria for peak identification required that the retention time for a given analyte be within ±2% of the average retention time for the respective standard (see table 7.2). Repeatability of the analysis of Cannabis Flos was established by analysis of six individually prepared sample extracts.
Results of HPLC analysis were finally expressed as weight % of each analyzed cannabinoid, relative to dry weight of the cannabis sample.

7.2.7 Gas chromatography (GC) analysis of unresolved peaks

The developed HPLC method was not able to fully separate the peaks for CBGA/CBN. To a lesser extent the peaks for CBD/CBG were also unresolved. Therefore, in order to perform a full analysis of the plant material used throughout our studies, HPLC data was always complemented by GC analysis. The GC-FID profiles were generated with a Chrompack (Middelburg, The Netherlands) CP9000 gas chromatograph, fitted with a Durabond fused silica capillary column (30 m x 0.25 mm inner diameter) coated with DB-1 (J&W scientific Inc., Rancho Cordova, CA) at a film thickness of 0.1 µm. The oven temperature was

Table 7.2: Retention time, LDR, recovery and LLOD for studied cannabinoids.

	Analyte	Abbreviation used	Average retention time (min) [a]	LDR (mg/ml) [b]	Linearity (r^2)	Recovery (%) [c]	LLOQ (mg/ml)
Major analytes							
1	Delta-9-tetrahydrocannabinol	THC	11.51	0.02 - 0.35	0.9996	99.8 ± 3.7	0.02
2	Tetrahydrocannabinolic acid	THCA	15.39	0.08 - 1.4	0.9992	98.8 ± 5.8	0.08
3	Cannabidiol	CBD	7.93	0.025 - 0.44	0.9990	95.4 ± 2.9	0.025
4	Cannabinol	CBN	10.44	0.005 - 0.087	0.9973	97.5 ± 4.0	0.005
5	Delta-8-tetrahydrocannabinol	Delta-8-THC	12.20	0.0025 - 0.044	0.9994	97.9 ± 3.7 [d]	0.0025
Minor analytes							
6	Cannabidiolic acid	CBDA	8.99				
7	Cannabigerol	CBG	8.27				
8	Cannabigerolic acid	CBGA	10.34				
9	Cannabinolic acid	CBNA	14.69				
10	Cannabichromene	CBC	13.66				
11	Cannabichromenic acid	CBCA	16.96				

[a] based on calibration curves

[b] investigated linear dynamic range (LDR)

[c] average analyte recovery for spiked plant material at specified concentration +20, +40 and +60%

[d] average analyte recovery for spiked plant material at 50, 100 and 150% of specified specification

programmed from 100°C to 280°C at a rate of 10°C/min. The oven was then kept at 280°C until the end of a 25 min. run time. The injector and detector port temperatures were maintained at 280°C and 290°C, respectively. Helium was used as the carrier gas at a pressure of 65 kPa. Injection volume was 5 µL, with a split ratio of 1/50. Detection of analytes was performed by flame ionization detection (FID). The FID-signal was recorded on a Shimadzu (Kyoto, Japan) CR3A integrator. The same GC method is described in chapter 6 for analysis of cannabinoids based on their retention time [Hazekamp, 2005]. The chromatographic peaks for the major analytes THC, delta-8-THC, CBD and CBN were separated satisfactorily by this method.

7.2.8 HPLC-MS analysis

After stressing extracts of Cannabis Flos under different conditions, peaks in the chromatograms were analyzed by an Agilent (Amstelveen, the Netherlands) single-quadrupole mass-spectrometer for positive identification. The mass-spectrometer was controlled by Agilent LC/MSD Chemstation A.10.02 software. The HPLC solvent system and column conditions were as described above. The settings of the mass spectrometer (MS) were as follows: APCI mode; positive ionization; fragmenter voltage, 100 and 240 V; gas temperature, 350°C; vaporizer temperature, 400°C; drying gas (N_2) flow rate, 4 liters min^{-1}; nebulizer gas pressure, 45 psig (lb/in^2); capillary voltage, 4000 V; corona current, 4.0 µA. Cannabinoids were mildly fragmented under these conditions. Ions were detected in the range of 50-600u.

7.3 Results and discussion

7.3.1 Optimization of the chromatographic method

A reversed-phase HPLC method for the quantification of cannabinoids in authentic cannabis plant material has been proposed, providing a simple procedure, without significant sample preparation. The result is an analytical method which permits the analysis of a wide range of cannabinoids in authentic cannabis plant material, while avoiding the necessity of a decarboxylation or derivatization step prior to analysis. Application of the proposed gradient elution profile results in separation, with a resolution (R_s) of not less than 1.5, of the cannabinoids: CBDA, THC, delta-8-THC, CBC, THCA and CBCA, in a runtime of only 25 minutes (32 minutes including re-equilibration). Chromatographic peaks of CBD/CBG were partially overlapping, as well as the peaks for CBGA/CBN. The choice of 228nm as detection wavelength enabled a high sensitivity for all cannabinoids without too much interference of the eluent. A typical chromatogram obtained with the proposed method is shown in figure 7.1.

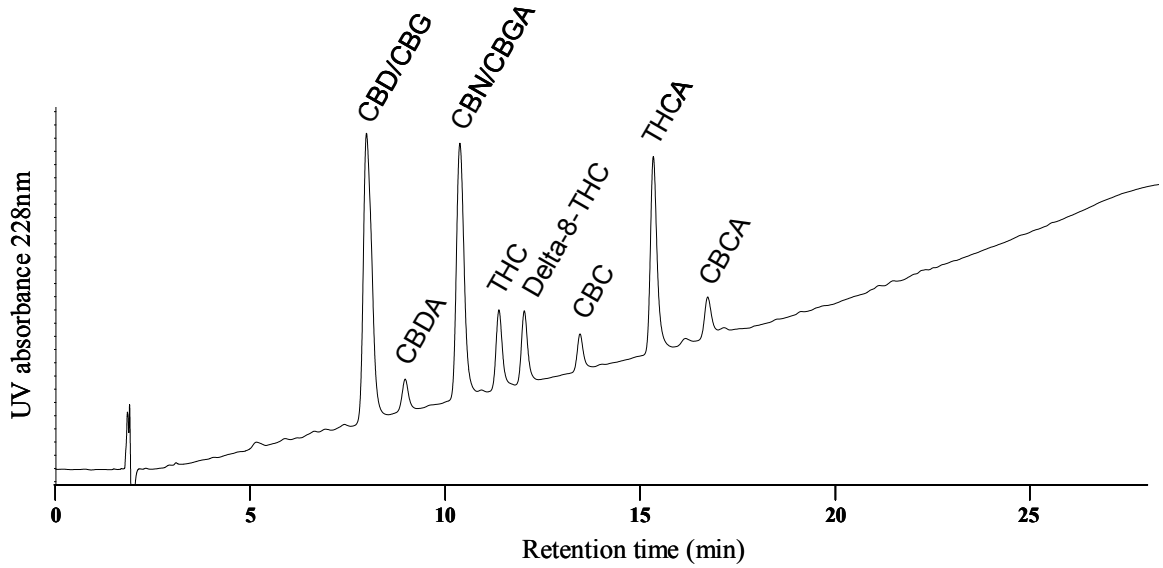

Figure 7.1: A typical HPLC chromatogram (228nm) obtained by applying the developed method. Baseline rises because no baseline correction was applied.

7.3.2 Stability of standards

By comparing peak areas of standards injected immediately with standards that were stored for 20h, it was found that the stability of all cannabinoids tested was very good, being within the range of 100% ± 3%. No peaks from possible degradation products, or any change in peak area was observed, indicating that degradation of the compounds is not a critical factor during the period of analysis. Results are shown in table 7.3. The values (peak area after storage / peak area without storage) were slightly higher than 100%, which might be caused by slight evaporation of solvent (ethanol) during period of storage, resulting in concentrating of the sample components.

Although it is possible that degradation could be promoted by heating or excessive light conditions, these conditions were not evaluated for the standard solutions used in the validation of this method.

Table 7.3: Stability of cannabinoid standards after 20h of storage. RSD indicates relative standard deviation of the mean.

Analyte	Stability (mean)	RSD
THC	100.0%	1.0%
CBD	102.6%	3.9%
CBN	101.4%	5.0%
THCA	100.3%	2.2%
delta-8-THC	102.2%	4.9%

7.3.3 Method validation

Linearity Each standard solution was analyzed a total of 12 times (3 determinations, each on 4 different days). Linearity was evaluated from this data by plotting the peak area versus injected amount. Regression lines were calculated using the least squares method, and linearity was expressed by the r^2-value. A good linearity was obtained in the range studied for each analyte. Regression coefficients are listed in table 7.2. With the exception of CBN, the average r^2-value obtained was higher than 0.999 in all cases, indicating a good linearity in the proposed range (Épshtein, 2004). The r^2-value obtained for CBN (0.9973) was slightly lower, but still very well acceptable. The obtained calibration curves were subsequently used to determine analyte concentrations in all further experiments.

Precision Precision of the assay was determined by analyzing all standard solutions 12 times. For the assessment of the intra-day variation samples were analyzed 3 times (n=3) in the same day; for the inter-day variation the same samples were analyzed on 4 different days (n=4). Analysis for inter-assay precision was performed by 2 different technicians, and this data was also used for investigation of robustness (see below). Results are shown in table 7.4. The pre-set values for acceptance were an RSD of ≤5% for intra-day and ≤10% for inter-day precision. The obtained results were all within these specifications, indicating good precision of the analytical method within the tested range.

Table 7.4: Results of precision tests for the determination of cannabinoids standards (inter- and intra-day) and cannabis extract (inter-day).

	Plant extract	Standards	
Analyte	**Inter (n=6)**	**Intra (n=3)**	**Inter (n=4)**
THC	2.2%	0.5%	3.1%
THCA	2.5%	0.2%	3.0%
CBN	2.6% [a]	2.7%	3.2%
CBD	2.5% [b]	1.3%	1.8%
delta-8-THC	c	4.7%	4.8%

[a] overlap with CBGA
[b] overlap with CBG
[c] not detected in extract

Accuracy (recovery) The accuracy characterizes the proximity between the obtained experimental results and the theoretical results. It was assessed by the determination of the recovery of known amounts of the cannaboids. Each recovery experiment was performed in duplicate at three different concentrations, so mean recovery and relative standard deviation (RSD) were calculated from 6 determinations for each cannabinoid.
Because placebo cannabis (free of the major analytes THC, CBD, CBN and THCA) was not available at the time of this study, recovery study for these cannabinoids was performed by

standard addition (spiking) at 3 different concentrations; 120%, 140%, 160% of specified concentrations, which means an addition of 20, 40, 60% of the authentic levels present in the plant material. Because no significant amount of delta-8-THC was found in authentic extract, this compound was spiked at 50%, 100% and 150% of the specified amount (table 7.1).
Table 7.2 shows the obtained recoveries for the different standards. A mean recovery ranging from 95.4% (CBD) to 99.8% (THC) was found, showing that the recovery of all the analytes met the evaluation criterion for accuracy (100% ± 5%) over the tested range. Thus, it can be emphasized that this method is accurate. Because all recoveries were >95%, no correction factor was used in further calculations.

Specificity The specificity of peak identification in Cannabis Flos extract was determined by investigating the authenticity of the peaks. Each quantified peak was therefore correlated to the UV-spectrum and MS spectrum of the pure reference compound. From the MS-data it was possible to distinguish even (UV-)overlapping peaks.
Specificity was further determined by stressing Cannabis Flos samples at various conditions (i.e.: treatment with acid, base and heating) to obtain chromatograms of decomposition products. HPLC peaks of decomposition products had to match with the reference standards in terms of retention time, UV-spectrum and MS-spectrum. The data showed that all relevant peaks were positively identified, and that no previously unknown degradation peaks were formed.

Robustness The evaluation of robustness was based on the linearity of peak area obtained after using cannabis samples of different weight. This approach shows the vulnerability of the specified method for variations in sample size, but also in sample homogeneity. In case the ground cannabis material is non-homogenous, fluctuations in the calculated cannabinoid levels are expected, specifically at the smaller sample sizes.
Cannabis samples of 150, 250, 500 and 750 mg (30-150% of specified sample size) were analyzed, and cannabinoid contents were evaluated. An extract without addition of cannabis Flos was prepared to evaluate for background peaks, but it was found that no such peaks were observed. By plotting the sample size against the observed peak area, the linearity was determined. Because of chromatographic overlap, peaks for CBD/CBG, and peaks for CBN/CBGA were evaluated together.
A linearity (r^2) of more than 0.999 was observed for all peaks, indicating that this analytical procedure is robust with respect to sample size and allows its use for further cannabinoid determinations from cannabis plant materials (data not shown).
Another factor tested as part of robustness was the inter-assay variation as a result of different technicians performing the analysis. Therefore, of the 4 runs performed for evaluation of the inter-assay variation, 2 different technicians each performed 2 different runs. Because inter-assay variation was significantly lower than the maximally allowed specification of 10% (see table 7.4), it was concluded that the method is robust.

Lower limit of quantification Lower limit of quantification (LLOQ) was determined for THCA, THC, delta-8-THC, CBD and CBN with the aid of the linearity data. The LLOQ was defined as the lowest analyzed concentration at which the intra-assay precision (%RSD) is not more than 10% (being twice the acceptance criterion for precision of ≤5%). LLOQ for HPLC detection were found to be at the lowest concentration tested for each cannabinoid (see table 7.2). For THC and THCA, the lowest tested concentration was relatively high, because of their high content in the studied plant material. The lower limit of quantification for these cannabinoids is probably much lower that the lowest concentration tested in this study, but this was not further evaluated.

7.3.4 Application of the method

The proposed method was finally used for the quantitative analysis of the major analytes present in Cannabis Flos material. It is a simple method because no complex pre-treatment of the sample is necessary before analysis. Instead, the ethanol extract can be immediately analyzed. Only a simple filtration step was required to protect the HPLC column from contamination and to prevent pre-column obstruction.
By performing the extraction procedure in the absence of Cannabis Flos, it was demonstrated that none of the solvents or materials used during preparation of the extract resulted in formation of interfering peaks in the HPLC chromatogram. Subsequently, repeatability of the analysis of Flos was established by preparation of an extract solution six times. The highest variability (RSD) found was 2.6% for the peak of CBN/CBGA, which is well within the pre-set specifications of 5% for acceptable precision.
Delta-8-THC and CBN, which are considered the major degradation products of THC and THCA in cannabis plant materials, could only be detected at very low levels in the extracts studied. The absence of these degradation products reflects the extreme care that is taken during growing and processing of the medicinal grade cannabis. The analyte CBD is associated with fiber type cannabis, and was therefore present in the studied (drug-type) Cannabis plant only at low levels (below 0.2%).
Although they were not fully evaluated in this study, the peaks for CBC, CBCA and CBNA were all baseline separated from other cannabinoids peaks. These components are usually present at low levels in fresh plant materials. It seems likely that they could be quantitatively analyzed with the proposed method once sufficient amounts of a calibrated standard become commercially available.

7.3.5 Analysis by GC and HPLC-MS

During GC analysis, acidic cannabinoids are decarboxylated by the heat of the injector, resulting in formation of their neutral counterparts. Therefore, only the total content of each cannabinoid (the sum of its acidic and neutral form) can be determined. We believe that the conversion of acidic cannabinoids into neutral ones by decarboxylation in GC is complete:

injection of pure standards of each of the acidic cannabinoids resulted in a single peak only, which corresponded to the decarboxylated product. No degradation was observed for any of the studied cannabinoids. So even though acidic cannabinoids could not be analyzed, GC analysis helped in the interpretation of overlapping peaks in the HPLC chromatogram.
Of course, the GC-method was not validated, as was the case for the HPLC method, and therefore it can not be used directly in the quantification of cannabinoids. However, by using quantified cannabinoid standards, the GC data was helpful in the interpretation of the HPLC data by providing information about the ratio in which certain cannabinoids are present in the extract. A GC chromatogram showing the separation of the major cannabinoids is shown in figure 7.2. By combining data obtained by GC and HPLC, all major analytes could be quantitatively analyzed.
For definitive identification of cannabinoid peaks (as part of specificity testing), HPLC-MS data was used. It was found that the developed HPLC method was specific and that all cannabinoid peaks were positively identified. Overlapping peaks could easily be resolved because of the differences in molecular weight of the components: the overlapping cannabinoids CBD and CBG have molecular weights of 314 Da and 316 Da, respectively, while overlapping CBN and CBGA have molecular weights of 310 Da and 360 Da, respectively [Hazekamp, 2005]. But although LC-MS is a great tool for analysis of cannabinoids present in plant materials, its use is still not widespread or common enough to be considered an easy and accessible alternative to HPLC-UV analysis.

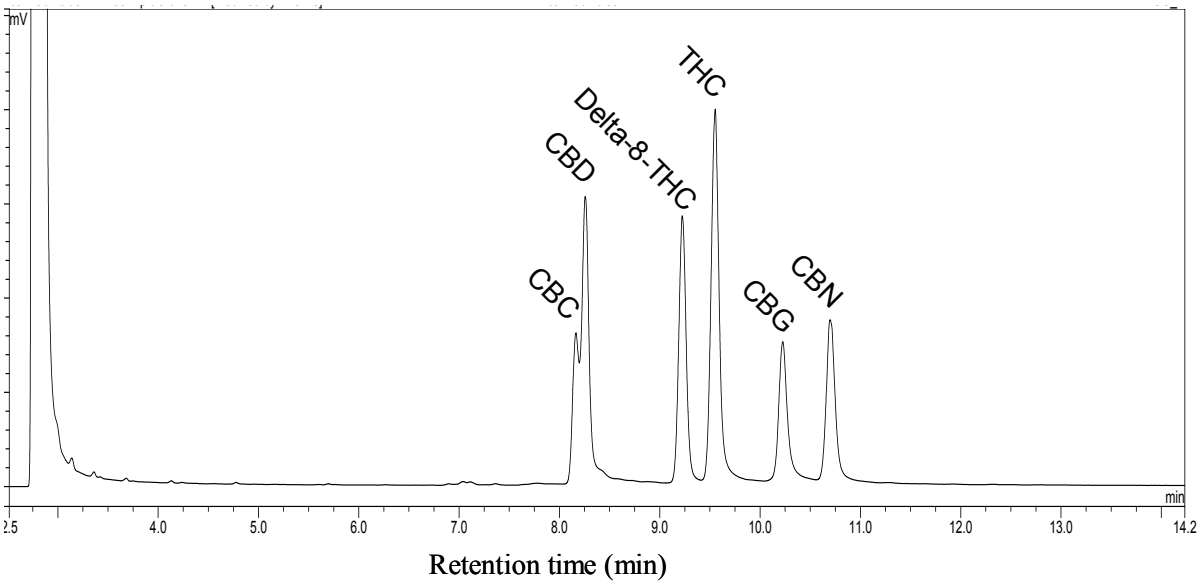

Figure 7.2: A typical GC chromatogram (FID-detection) obtained by applying the described method. GC data is used as secondary data to assist in the interpretation of HPLC data.

7.4 Conclusion

This study describes a simple and validated RP-HPLC method for the determination of cannabinoids according to ICH guidelines. All tested parameters were within the limits proposed by those guidelines for pharmaceutical testing, indicating that this method is highly linear, precise, accurate and robust. The analysis should be performed within 20 hours after extraction. Although the lower limit of quantification that was determined for most of the analytes was relatively high, it is acceptable for the intended purpose of the method, being the quantitative analysis of highly potent cannabis cultivars. The linear range tested for the most important cannabinoids THC and THCA is relatively wide (THC, up to 7%; THCA up to 28% of dry weight) and should cover the levels found in almost any drug type cannabis plant.
The proposed method was used for quantitative analysis of cannabinoids in authentic cannabis plant material. Because some peaks of interest showed chromatographic overlap in HPLC, a secondary analysis using GC was performed for the analysis of cannabinoids in the extract. The combined data of these two determinations makes it possible to fully analyze the content of major cannabinoids in the cannabis plant material used in this study and throughout this thesis. The validated method is part of the official Dutch monography which is routinely used for the analysis of medicinal grade cannabis as provided through pharmacies in the Netherlands. Quantitative results are described in the form of a Certificate of Analysis (CofA) as shown in figure 7.3. Standard water determination by loss on drying is used for correction of dry weight, and cannabinoid levels are finally shown as % content of dry weight.
The validation performed here demonstrates that the analytical procedure described is suitable for its intended purpose. In contrast to many other methods of analysis, it is also applicable for the acidic cannabinoids. However, separation of CBN and CBGA remains the main challenge for this system. Although it is possible to selectively shift the retention time of acidic cannabinoids by changing pH of the eluent [Turner, 1982] we had the experience that this usually lead to other, even more challenging overlap of peaks.
In order to achieve full separation of all mentioned cannabinoids, we are recently studying the use of the most current development in liquid chromatography: Ultra Performance Liquid Chromatography (UPLC, by Waters Chromatography). Due to higher selectivity of the stationary phases used in this type of chromatography (i.e. C_{18} column with particle size of 1.7 μm), full separation of all mentioned cannabinoids (and more) was found to be possible in a 10 minutes chromatographic run. The knowledge gained in the course of this study is currently exploited for validation of an UPLC method.

	Specification	Result
Identification		
Appearance	Dirty green clustered flowers with characteristic scent	conform
Texture	conform	conform
Foreign material	absent	absent
Identity		
test A: microscopy	conform	conform
test B: TLC	conform	conform
Contamination		
Microbiological contamination		
total aerobic contamination	< 100 cfu/g	< 100 cfu/g
yeasts and fungi	< 10 cfu/g	< 10 cfu/g
enterobacteriaceae en gram-neg. bact	absent	absent
Pseudomonas aeruginosa ≤ 0 cfu/g	absent	absent
Staphylococcus aureus ≤ 0 cfu/g	absent	absent
Pesticides	conform EP 2.8.13	conform
Heavy metals		
Lead (Pb)	≤ 20.0 ppm	≤ 20.0 ppm
Mercury (Hg)	≤ 0.5 ppm	≤ 0.5 ppm
Cadmium (Cd)	≤ 0.5 ppm	≤ 0.5 ppm
Composition		
Assay HPLC/GC		
THC (after heating)	15.5 – 21.0 %	18.40%
THCA (before heating)	n.s.	21.70%
CBN	< 1.0%	0.10%
CBD	0.1 – 1.5 %	0.90%
delta-8-THC	n.s.	0.03%
Fingerprint cannabinoids (HPLC)	conform	conform
Loss on drying	≤ 10.0%	6.80%

Figure 7.3: Example of a Certificate of Analysis (CofA) that is prepared for each batch of cannabis plant material that is used in our studies.

CHAPTER 8

Cannabis tea revisited: a systematic evaluation of the cannabinoid composition of cannabis tea

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Abstract

Cannabis is one of the oldest known medicinal plants, and a large variety of biological activities have been described. The main constituents, the cannabinoids, are thought to be most important for these activities. Although smoking of cannabis is by far the most common way of consumption, a significant part of medicinal users consume it in the form of a tea. However, not much is known about the composition of cannabis tea, or the effect of different parameters during preparation, handling or storage. In this study we used the high-grade medicinal cannabis available in Dutch pharmacies to study the cannabinoid composition of tea under standardized and quantitative conditions. Experimental conditions were systematically varied in order to mimic the possible variations made by medicinal users. During analysis there was a specific focus on the cannabinoid tetrahydrocannabinol and its acidic precursor, tetrahydrocannabinolic acid. Also the role of non-psychoactive cannabinoids as components of cannabis tea are discussed. The results obtained in this study provide a clear quantitative insight in the phytochemistry of cannabis tea preparation and can contribute to a better appreciation of this mode of cannabis administration.

8.1 Introduction

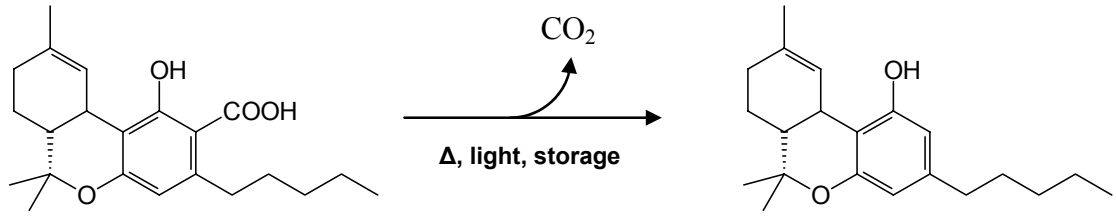
The cannabis plant (*Cannabis sativa* L.) has a long history as herbal medicine, and contains a large variety of pharmacologically interesting constituents. Most important among these are the cannabinoids [Turner, 1980a], which are unique to the cannabis plant. They are produced by the metabolism of the plant in the form of carboxylic acids [Shoyama, 1975], which can be converted into their decarboxylated (neutral) analogs under the influence of light, heat or prolonged storage, by losing the relatively unstable carboxyl-group in the form of CO_2 [Veress, 1990]. Cannabis can be consumed in a variety of ways, such as smoking, vaporizing, preparing cannabis tea and using it in baked products. A common factor of all administration forms is a heating step, which is essential for conversion of the acidic cannabinoids into the pharmacologically more active neutral ones. The most important conversion that takes place is that of tetrahydrocannabinolic acid (THCA) into delta-9-tetrahydrocannabinol (THC), which is the main bioactive component of cannabis (see figure 8.1).

One popular way to undergo the effects of cannabis is by consuming it in the form of a decoction, which will be referred to in this manuscript as 'cannabis tea'. In Jamaica, which is sometimes quoted as the country with the highest consumption of cannabis, the different uses of cannabis have been thoroughly studied [Rubin and Comitas, 1975]. Although cannabis, which is locally known as ganja, is mostly consumed by smoking, drinking of ganja tea is common among non-smokers [Boekhout van Solinge, 1996] and is consumed even by young children and the elderly. The tea is attributed various therapeutic and prophylactic qualities and is used as a remedy for fever, cold and stress.

Also around Europe, hemp containing foods, including leaves for tea preparation, are widely available. Often these products are associated with health. Although it is legally not permitted, herbal hemp leaves used for tea have been found to contain high THC levels (1020-5000mg/kg) and significant concentrations were determined in the corresponding tea infusions (1.0-2.4 mg/L) [Giroud 1997; Zoller 2000]. Potentially, any health claims based on the consumption of such teas might therefore be attributable to its content of THC. After all, positive drug tests for cannabis use as well as intoxication have been reported after ingestion of such products [Struempler, 1997], and analytical methods have been developed for the forensic screening of THC in these products [Lachenmeier, 2004].

In contrast, other (non-psychoactive) cannabinoids usually go undetected and might be present in any concentration in officially allowed hemp products, including tea. For example, the major cannabinoids cannabidiol (CBD) and cannabinol (CBN) can be found in most cannabis cultivars, and both have reported biological effects, such as antibacterial and anti-inflammatory activity, and modulation of immune responses [Grotenhermen and Russo, 2002]. The potent immuno-modulating properties of the major cannabinoid THCA have only recently been discovered (Verhoeckx, 2006). These effects clearly make the non-psychotropic cannabinoids potential candidates for any medicinal claims attributed to the consumption of cannabis tea.

However, with few exceptions [Steinagle, 1999; De Jong, 2005] virtually no standardized studies have been performed with tea preparations of cannabis. The single large scale field study which includes the use of cannabis tea [Rubin and Comitas, 1975] lacks a focus on analytical data, such as chemical composition and potency of cannabis used, making it difficult to understand the effects or reliability of this administration form. Clearly, there is a need for a better understanding of the composition of cannabis tea prepared under varying conditions, before further conclusions can be made on its effects or reliability.
Recently, the introduction of high grade cannabis for medicinal use in The Netherlands has provided a good opportunity to study the composition of cannabis tea. The detailed conditions of this introduction, through the Dutch Office of Medicinal Cannabis (OMC), have been previously described [Hazekamp, 2006a]. Under the Dutch regime, patients essentially are able to freely choose their manner of cannabis consumption. Based primarily on health implications, the OMC advices to consume medicinal cannabis preferably by vaporizing or in the form of a tea. Indeed, polls under medicinal cannabis users in The Netherlands have indicated tea preparation to be a popular way of consuming cannabis [Janse, 2004].
Considering these developments, a systematic study on the composition of cannabis tea would be very interesting. We performed this phytochemical study on the preparation and handling of cannabis tea, in particular on the parameters that can have an effect on the composition of the tea, such as boiling time, volume of tea prepared, and duration of storage. To understand the magnitude of such effects, parameters were systematically varied in order to determine their effect on the cannabinoids present in the tea, with a particular focus on the main cannabinoids THC and THCA. To improve the observed poor stability of tea during refrigerated storage, we evaluated the use of solubilizers. Finally, we discuss the potential role that the non-psychoactive cannabinoids may play in the effects attributed to cannabis tea.



Tetrahydrocannabinolic acid (THCA)

Delta-9-tetrahydrocannabinol (THC)

Figure 8.1: Conversion of THCA into THC, as it is taking place during the preparation of tea. The same conversion also takes place, more slowly, as the result of storage and aging.

8.2 Materials and methods

8.2.1 Materials

Cannabis plant material used in this study was of the variety 'Bedrocan' and was obtained from Bedrocan BV (Veendam, the Netherlands) where it was cultivated under standardized conditions according to the requirements of Good Agricultural Practice (GAP) [Hazekamp, 2006a]. Only female flower tops were used ('Cannabis Flos'). After harvest, the plant material was air-dried in the dark under constant temperature and humidity for 1 week. The same cannabis material is officially dispensed through Dutch pharmacies under the Dutch medicinal cannabis program, supervised by the Office of Medicinal Cannabis (OMC). This cultivar is of the drug-type [Fetterman, 1971b] and at the time of use it had a THCA content of 191 mg/gram (19.1%), and a THC content of 6 mg/gram (0.6%) of dry weight plant material.
Pure ethanolic standards for THC and THCA were produced as previously described [Hazekamp, 2004a,b]. Randomly-methylated (RM)-beta-cyclodextrin was obtained from Wacker Chemie GmbH (Burghausen, Germany) and was used as received.
All organic solvents were HPLC or analytical grade and were purchased from Biosolve (Valkenswaard, The Netherlands). Water used for tea preparation was regular tap-water.

8.2.2 Preparation of tea samples

The users of medicinal cannabis in the Netherlands are advised by the OMC to prepare cannabis tea according to the following standard protocol: "Add 1.0 gram of cannabis to 1.0 litre of boiling water and let simmer for 15 minutes. Filter out solid parts by using a common tea-sieve. Tea can be consumed immediately, or stored in a closed bottle in a refrigerator for up to 5 days" [OMC, 2006]. Throughout this study, tea prepared according to this protocol is referred to as 'standard tea', and it is the reference material for all performed tests.
Tea was prepared in 2 L glass Erlenmeyer flasks on an electronic heating plate. For each experiment, three separate preparations were made, unless stated otherwise. Samples for analysis were taken after the tea was allowed to cool down to a temperature of about 55°C. After shortly stirring up the tea, samples of 30 ml were collected by pouring the liquid through a common metal tea-sieve into a calibrated measuring cylinder. Samples were lyophilized to complete dryness and reconstituted in ethanol for analysis.

8.2.3 Determination of cannabinoids

Cannabinoid content of the tea samples was determined by high pressure liquid chromatography (HPLC), as described before [Hazekamp, 2004a]. The HPLC method was validated according to recent ICH guidelines [ICH, 2006] for the quantitative analysis of

cannabinoids in extracts of herbal cannabis. Pure ethanolic standards of the cannabinoids were used for quantitation.

8.2.4 Stability and recovery of THCA and THC standards

Stability and recovery of the main cannabinoids THC and THCA during preparation of samples for analysis was studied by standard addition (spiking) of pure cannabinoids to boiling water, in concentrations that were similar to those found in standard tea. Water with added standards was processed as described for regular tea samples. For THCA, its conversion rate into THC was determined.

8.2.5 Variability of standard tea

The variability in the composition of standard tea was determined by analyzing 6 different preparations of standard tea (1 L) and calculation of relative standard deviation (%STD) of cannabinoid levels. Because the levels of THC and THCA are commonly considered most important for bioactivity, these cannabinoids were analyzed quantitatively. Other cannabinoids were analyzed only qualitatively, based on HPLC peak area, so without the use of calibrated standards.
The herbal cannabis material that remained after tea preparation (residue after filtering by the sieve) was extracted with ethanol in order to determine its cannabinoid composition by HPLC analysis. Obtained data was used to determine the mass balance for the distribution of THC and THCA before and after preparation of standard tea.

8.2.6 Effect of preparation parameters on tea composition

Changing the preparation parameters may have an effect on the composition of the tea, both on the absolute concentration and on the relative ratio of cannabinoids that are found in the tea. We tested the effect of systematically changing each of the parameters described below. Effects were statistically evaluated by using the independent Student's t-test with 2-tailed distribution.

- *Volume:* Tea was prepared with 250 mg of cannabis in 250 ml of water versus 1.0 gram in 1.0 L of water. The 250 ml preparations were made in 500 ml glass Erlenmeyer flasks.
- *Amount of cannabis:* Tea was prepared using 0.5, 1.0 and 1.5 gram of cannabis.
- *Boiling time:* Tea was prepared by boiling for 10, 20 and 30 minutes. The influence of evaporation of water during boiling was not evaluated in this study, but this factor was kept to a minimum by loosely covering the opening of the flask.

8.2.7 Storage and stability

Based on microbial spoiling, it is claimed that medicinal cannabis tea can be stored in a refrigerator for a maximum of 5 days [OMC, 2006]. To test the effect of storage on the THC and THCA concentration of standard tea, multiple samples of 50 ml were taken from a single preparation of tea, and stored in a refrigerator (+4°C - +7°C) for periods of 1, 3, 5 and 12 days. After this period, samples were gently stirred, and 30 ml was removed for analysis.
Samples that had been stored for 3 days were used for analysis of the precipitate that had formed. Samples were gently stirred and subsequently the water phase was poured off. Residue that remained in the storage tube was dissolved in ethanol for quantitative analysis of THC and THCA. Obtained data was used to determine the mass balance for the distribution of THC and THCA before and after storage of standard tea.

8.2.8 Effect of solubilizers

An important drawback of tea preparation, is the very limited solubility of cannabinoids in water [Garrett, 1974; Hazekamp, 2006b]. In order to stabilize the composition of cannabis tea, the addition of solubilizers was evaluated. Previous studies have shown that the addition of cyclodextrins is a promising way to increase the water solubility of several cannabinoids, including THC and THCA [Mannila, 2005; Hazekamp, 2006b], suggesting that addition of cyclodextrins can stabilize the levels of THC and THCA during storage. Therefore, the addition of 1% and 3% (w/v) of randomly methylated beta-cyclodextrin (RAMEB) to standard tea was evaluated. Other common types of cyclodextrins were previously shown to be ineffective in improving the aqueous solubility of THC [Hazekamp, 2006b]. Addition was done directly after preparation and tea (200 ml) was stored in a refrigerator for 5 days.
Another solubilizer tested was coffee creamer powder, which was added to cannabis tea (one standard package per cup; ± 2.5 grams per 200 ml) while still warm. Tea was stirred until powder was completely dissolved, before refrigerated storage for 5 days.

8.3 Results and discussion

8.3.1 Behaviour of pure cannabinoids in boiling water

In order to understand the composition of cannabis tea, initially some studies were done with pure cannabinoid standards. Recovery of THC and THCA during sample preparation for HPLC analysis (i.e.: lyophilization and reconstitution) was found to be 79.8% (±4.5%) for THC and 94.8% (±0.5%) for THCA. All subsequent measurements were corrected for these values.
When pure THC was added to boiling water, only about 17% was recovered after 15 minutes of boiling. A THC precipitate was clearly visible on the surface of the glass flask used for boiling the water, indicating that a saturated solution had formed. Spiking of pure THCA

resulted in a much higher recovery of about 63%. A small part of added THCA could be recovered from the water phase in the form of THC (6.6%), the remaining part was found as a precipitate on the glass container used for boiling.
These results indicate that conversion of THCA into THC is limited in boiling water. Furthermore it is suggested that a saturated THC solution forms in boiling water, implicating that addition of extra THC will probably not increase its water concentration. Similar observations were made when analyzing cannabis tea samples (see below).
Boiling of the standards did not results in the formation of degradation products such as CBN or delta-8-THC, indicating that degradation of these major bioactive cannabinoids is not a significant factor during tea preparation.

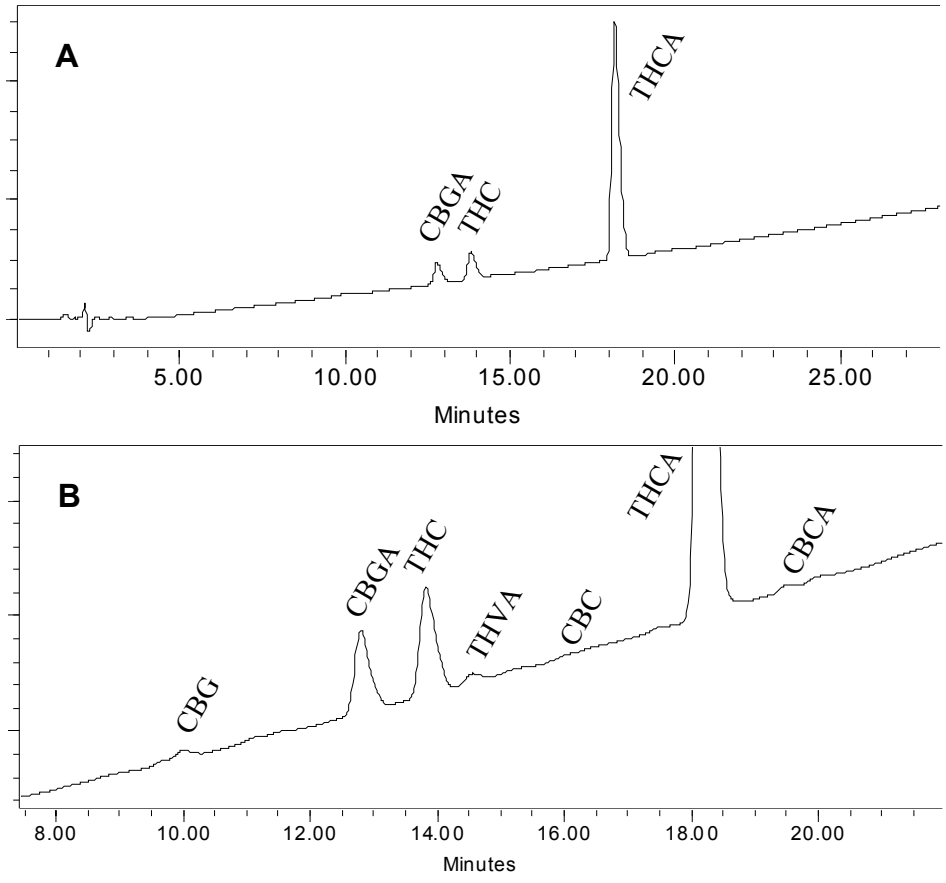


Figure 8.2: Typical HPLC chromatogram (228 nm) obtained by analysis of standard cannabis tea according to the method described.
a) whole chromatogram; b) enlargement of the cannabinoid peaks

8.3.2 Composition of standard tea

Analysis of 6 different batches of standard tea showed that the variability in the composition of standard tea is relatively low for a preparation method that is essentially very crude: variability for the content of THC (mean: 0.010 mg/ml) was 15% while for THCA (mean: 0.043 mg/ml) it was only 12%. Other cannabinoids visible in the HPLC chromatogram were analyzed only qualitatively (based on relative HPLC peak area). A typical HPLC chromatogram obtained during analysis of standard tea is shown in figure 8.2. Variability was found to be in the range of 8.4-17.4% for all cannabinoids.

8.3.3 Mass balance of THC and THCA

By calculation of total THC (sum of THC and THCA, taking into consideration the difference in molecular weight) present, the mass balance of THC before and after tea preparation was determined. It was found that no net loss of THC occurred during tea preparation: total THC present in the plant material before preparation (174 mg) was found to be equal to the amount present (in water phase plus in residual plant material) directly after preparation (176 mg). These results indicate that loss of THC by degradation does not play a significant role. Indeed, no degradation products of THC were observed during the experiments with pure cannabinoids, as described above.

8.3.4 Effect of preparation parameters

Studying the effects of changing the basic parameters of tea preparations gave a good insight into the behaviour of THCA and THC during the preparation process. Results are summarized in figure 8.3. Differences with a significance of $p<0.05$ are indicated.

- **Volume:** No significant differences were found between tea prepared in a volume of 1 L or 250 ml; THC and THCA levels, as well as general profile of cannabinoids were similar directly after preparation of the tea, and also after 5 days of refrigerated storage (data not shown). These results indicate that downscaling of the volume of tea does not influence the composition of the final product.
- **Amount of cannabis:** The use of a higher than usual amount of cannabis (1.5 gram) did not significantly increase the aqueous concentration of THC or THCA, compared to the use of 1.0 gram, suggesting that a saturated solution forms. In contrast, the use of half the usual amount (0.5 gram) of cannabis significantly decreased the water concentration of both THC and THCA to about half the concentration found for standard tea.
- **Boiling time:** Variation in boiling time in the range of 10-30 minutes had only a slight effect on the level of THCA; levels found were similar at all tested boiling times. In contrast, the level of THC was found to be dependant on boiling time, as increased

boiling time resulted in significantly higher levels of THC. However, THC levels remained much lower than THCA levels found in these preparations.

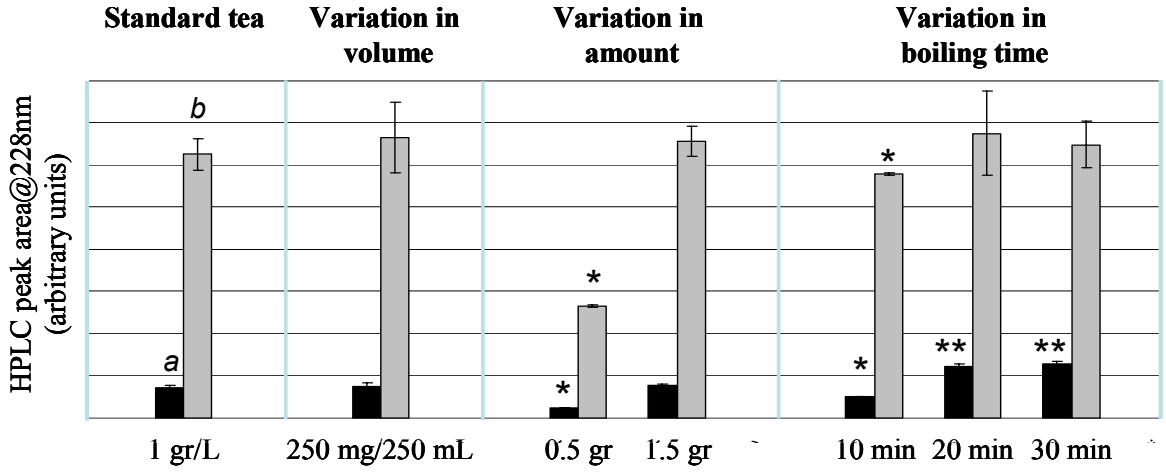


Figure 8.3: Effect of variations in water volume, amount of cannabis, and boiling time used in the preparation of cannabis tea. The levels of THC and THCA are expressed in units of peak area (HPLC at 228 nm).
a: bar corresponds to a THC level of 0.010 mg/ml; *b*: bar corresponds to a THCA level of 0.043 mg/ml.
*: significantly lower than standard tea; **: significantly higher than standard tea; p<0.05

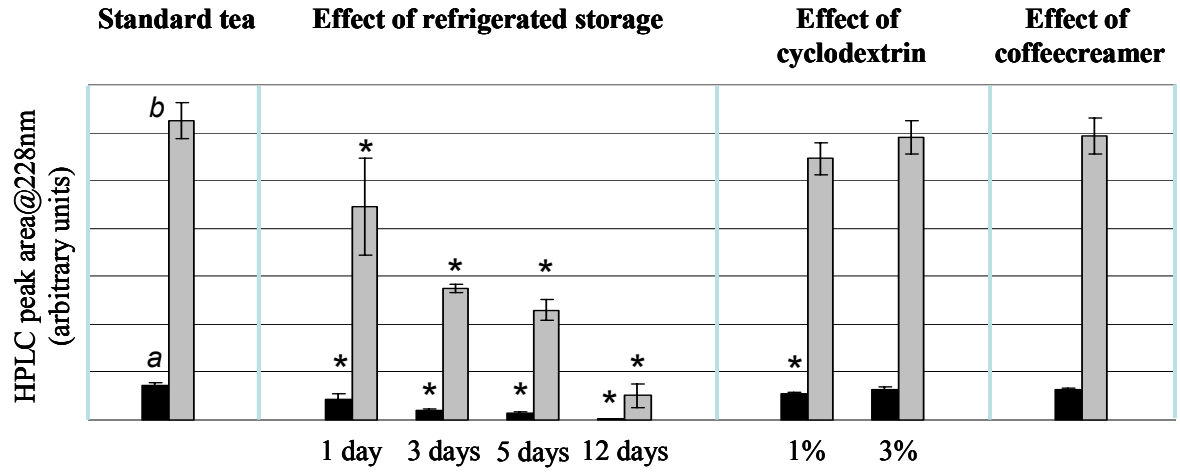


Figure 8.4: Effect of prolonged refrigerated storage of standard cannabis tea, without and with addition of solubilizers. The levels of THC and THCA are expressed in units of peak area (HPLC at 228 nm).
a: bar corresponds to a THC level of 0.010 mg/ml; *b*: bar corresponds to a THCA level of 0.043 mg/ml.
*: significantly lower than standard tea; p<0.05

8.3.5 Effect of storage and solubilizers

Refrigerated storage resulted in steady decrease of cannabinoid levels (figure 8.4). Even after a single day of storage, concentrations of THC and THCA had significantly decreased to 60% and 71% of initial levels, respectively. After 12 days of storage, these values had decreased further to only 6% and 8% of initial values, respectively. After preparation, when the tea cools off, the liquid is observed to turn from clear to opaque, indicating formation of a precipitate. Analysis of this precipitated matter after 3 days of storage showed that the amount of THC and THCA recovered from the precipitate was equivalent to the amount lost from solution. However, the relative cannabinoid composition did not change very much during the same period, meaning that all cannabinoids present precipitated roughly to the same extent. In other words, the potency decreased while the qualitative composition remaind the same.
It was found that addition of cyclodextrin as well as coffeecreamer was effective in stabilizing cannabis tea during refrigerated storage (see figure 8.4). After 5 days the levels of THC and THCA in the tea were found to be virtually unchanged. Addition of 3% of RAMEB had a slightly better stabilizing effect than addition of 1% of this compound.

8.4 Conclusion

Cannabis tea can be considered as a contemporary example of a widely used, but poorly understood herbal medicine. A major concern with the medicinal use of cannabis is the risk of (accidental) overdosing of THC, which could lead to psychotropic effects. However, our results show that moderate changes in the standard preparation protocol for cannabis tea do not result in dramatic changes in the composition of the tea, neither quantitatively nor qualitatively. Rather, the results indicate that cannabis tea has only limited potency, and that probably a saturated solution of THC forms.
By performing a series of experiments, we systematically discovered the effect of different parameters on the cannabinoid composition of medicinal cannabis tea. The study of pure standards in boiling water provided detailed insight into the behaviour of THC and THCA during the tea preparation process. Relatively more THCA was solubilized in boiling water than THC, which probably can be understood by the relatively higher water solubility of THCA compared to THC [Hazekamp, 2006b]. Interestingly, although the amount of THC in the used amount of cannabis (1 gram) is potentially very high (about 174 mg, as sum of THC and THCA), the whole volume of standard tea contains only a fraction of this (about 10 mg THC per liter) in the water phase. This relatively low concentration is probably the result of saturation of the water phase with THC, in combination with a moderate conversion of THCA into THC, as was also suggested by the experiments performed with pure standards.
In case storage of cannabis tea is required, the addition of a solubilizer was found stabilize the THC and THCA levels of the preparation for a period of at least 5 days. Although addition of the cyclodextrin RAMEB clearly improved the stability of cannabis tea, its oral use has not yet been fully validated and its common use in medicinal preparations might still take several

years to be established. However, the addition of coffee creamer can be an easy and safe alternative for medicinal consumers of cannabis tea to stabilize their preparation during short term storage.

Finally, some attention should be given to the unique composition of cannabis tea, compared to other forms of administration, where heating of the material is typically performed at much higher temperatures (e.g. smoking, vaporizing or baking), resulting in a virtual complete conversion of acidic into neutral cannabinoids. This is the reason that, during studies into the medicinal effects of cannabis preparations, the attention is commonly focussed on THC alone. However, in the cannabis tea studied, a significant proportion of THCA was found. The recently described immuno-modulating properties of THCA (Verhoeckx, 2006) may contribute to the effects that certain groups of medical users claim after consumption of cannabis tea. Furthermore, a variety of other acidic cannabinoids were found by HPLC analysis, such as cannabigerolic acid (CBGA) and tetrahydrocannabivarinic acid (THVA). Although the biological activities of these compounds have hardly been explored, their presence makes cannabis tea a unique administration form that should not be considered as simply a vehicle for THC.

In conclusion, cannabis tea is already consumed by a large number of patients on a daily base, and their medical claims may certainly be compatible with the unique composition of the tea. The results obtained in this study can contribute to a better understanding of cannabis tea, resulting in a better appreciation of this popular form of cannabis administration.

CHAPTER 9

Structure elucidation of the tetrahydrocannabinol complex with randomly methylated β-cyclodextrin

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Abstract

The low aqueous solubility of the bioactive cannabinoid tetrahydrocannabinol (THC) is a serious obstacle for the development of more efficient administration forms. In this study the aqueous solubility of THC was tested in the presence of α-, β- and γ-CD, and randomly methylated β-CD (RAMEB). It was found that only RAMEB was able to increase the aqueous solubility of THC to a significant level. A THC concentration of about 14 mg/ml was reached by using a 24% (187mM) RAMEB solution, which means an increase in solubility of 4 orders of magnitude. The resulting THC/RAMEB complex was investigated through phase-solubility analysis, complemented by ^{1}H-NMR, NOESY- and UV-studies in order to obtain details on the stoichiometry, geometry and thermodynamics of the complexation. The binding ratio of THC to CD was found to be 2:1, with the second THC molecule bound by non-inclusion interactions. Based on the obtained results a model for the complex structure is presented. Stability of the complex under laboratory room conditions was tested up to 8 weeks. Results show that complexation with RAMEB may be promising for the development of water-based THC formulations.

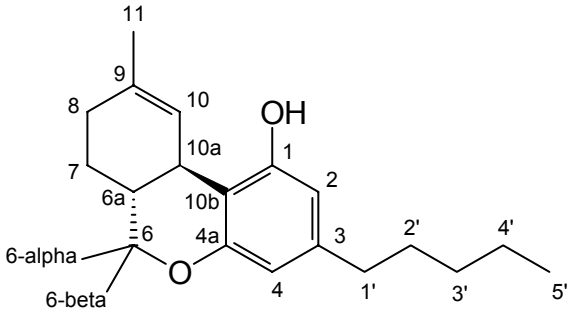
9.1 Introduction

The Cannabis plant (*Cannabis sativa* L.) has a long history of medicinal use and the main constituents, the cannabinoids, are under intensive study [Grotenhermen, 2002]. At present a number of medicines based on the biological activities of the cannabinoids are available, such as Marinol® and Nabilone, and several more are expected to be introduced in the near future. Among them are Rimonabant, for treatment of obesity [van Gaal, 2005], and the potent analgesic ajulemic acid [Burstein, 2004]. It seems clear that the Cannabis plant still has highly relevant potential for medicinal development.
The main psychoactive cannabinoid, Δ^9-tetrahydrocannabinol (THC, figure 9.1a), has been shown to be clinically useful for a large diversity of indications, including nausea and weight-loss associated with chemotherapy and HIV/AIDS, spasms in multiple sclerosis, chronic neuropathic pain and glaucoma [Grotenhermen, 2002]. However, the reduced bioavailability of orally administered THC, due to low absorption and high first-pass metabolism [Brenneisen, 1996], prompts the development of more reliable administration forms, such as aqueous THC solutions for inhalation, sublingual or injection purposes. However, the solubility of THC was reported to be only 1-2 μg/ml in a 0.9% NaCl solution [Garrett, 1974]. Recently a water-based preparation of cannabis-extract has been developed for sub-lingual use (Sativex ®). However, it contains ethanol and propyleneglycol as solubilizing agents, resulting in frequent irritation of the administration site (Sativex product monograph, Bayer Healthcare Canada). Clearly there still is a need for the development of a more optimal preparation of aqueous THC.
Cyclodextrins (CDs) are natural cyclic oligosaccharides constituted by six (α-CD), seven (β-CD) or eight (γ-CD) D-glucose units (figure 9.1b). The three-dimensional structure of the CD-ring is a truncated cone, with each of the α-, β-, and γ-CDs having a different cavity volume. They can form inclusion complexes with lipophilic guest molecules, thereby improving their aqueous solubility, increasing stability and bioavailability, and reducing side effects [Martin Del Valle, 2004]. Various modifications of the natural CDs have been developed, such as the randomly methylated β-CD (RAMEB) and hydroxypropyl (HP)-β-CD.
The use of cyclodextrins for the development of aqueous THC preparations seems to be promising. In a study by Jarho et al. [1998], THC could be solubilized up to about 1 mg/ml, using a 40% HP-β-CD solution with addition of the polymer hydroxypropylmethylcellulose. However, no further details were reported on the chemical structure, stability or kinetics of the complex. In another study, complexation with β-CD was found to improve the chemical stability of THC [Shoyama, 1983]. Recently, Mannila et al. [2005] demonstrated that complexation with RAMEB increases both the aqueous solubility and dissolution rate of THC as well as the related compound cannabidiol (CBD). The same study also showed that the sublingual administration of a THC/RAMEB complex substantially increased the bioavailability of THC in rabbits. Based on phase-solubility data a binding ratio of 1:2 (guest:CD) was suggested for the complex, but no further elucidation of the structure was performed.

However, there is growing evidence that the stoichiometry of drug/cyclodextrin complexes cannot be derived exclusively from simple phase-solubility studies, as it becomes increasingly clear that these are highly oversimplified descriptions that ignore important aspects of the formation of cyclodextrin complexes. Cyclodextrins are able to form both inclusion and non-inclusion complexes. Self-association of surface active drugs, lipophilic drug molecules, and drug/cyclodextrin complexes, as well as drug solubilization through non-inclusion interactions with the drug/cyclodextrin complex, will influence both the shapes and mathematical interpretation of phase-solubility diagrams [Loftsson, 2002, 2004]. In several cases a different stoichiometry was obtained when using the phase-solubility studies compared to the more reliable construction of a continuous variation (Job's) plot using techniques such as NMR, UV or potentiometry (reviewed by Loftsson et al., 2004). Therefore, such techniques, preferably in combination with theoretical computer simulated modelling, are important complementary data for determination of stoichiometry.

In this study the aqueous solubility of THC was tested in the presence of α-, β-, γ-CD and RAMEB, and the most efficient CD-type was selected for further study. The resulting complex of THC with RAMEB was investigated through phase-solubility analysis, complemented by ^{1}H-NMR, NOESY and UV studies in order to obtain details on the stoichiometry, geometry and thermodynamics of the complexation. Based on the obtained results a model for the complex structure is presented. Stability of the complex under laboratory room conditions was tested up to eight weeks.

a)



b)

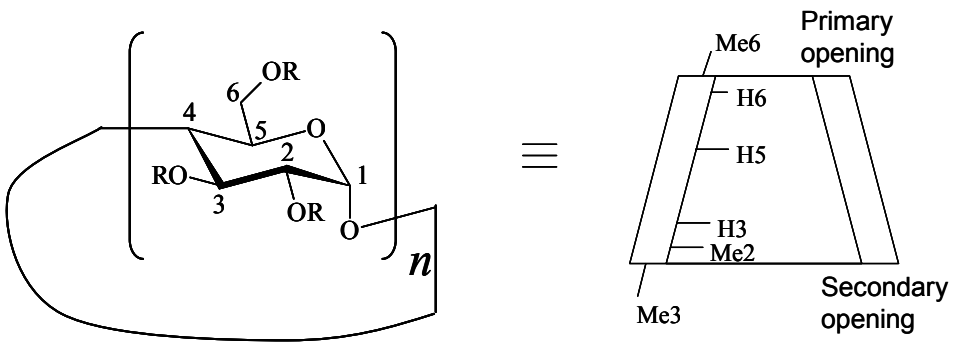


Figure 9.1: a) Structure of delta-9-tetrahydrocannabinol (THC). The lettering of the rings is indicated.
b) General structure of the cyclodextrins; alpha-CD (n=6), beta-CD (n=7), gamma-CD (n=8). In randomly methylated-beta-CD a proportion of hydroxyl-groups is substituted for methoxy-groups.

9.2 Materials and methods

9.2.1 Materials and chemicals

All solvents were analytical or HPLC-grade and were obtained from Biosolve (Valkenswaard, The Netherlands). Deuterated solvents for NMR studies were from Eurisotop (Gif-sur-Yvette, France). Cyclodextrins; alpha-, beta-, gamma- and randomly methylated beta-CD (RAMEB) were purchased from Wacker Chemie GmbH (Burghausen, Germany) and were used as received. RAMEB was of pharmaceutical grade (Cavasol W7 M Pharma) and had a degree of substitution of 1.7. The cannabinoids used in this study were isolated and quantified according to a method developed by our laboratory [Hazekamp, 2004a,b]. Stock solutions of cannabinoids and CDs were prepared in ethanol. Water was of Millipore quality.

9.2.2 Assay of THC

THC concentrations were assayed by an HPLC-method. The HPLC profiles were acquired on a Waters (Milford, MA, USA) HPLC system consisting of a 626 pump, a 717plus autosampler and a 2996 diodearray detector (DAD), controlled by Waters Millennium 3.2 software. Ten-microliter samples were injected on a Vydac column (Hesperia, CA, USA) C_{18}, type 218MS54 (4.6x250 mm, 5 μm) fitted with a Waters Bondapak C_{18} (2x20 mm, 50 μm) guard column. The mobile phase consisted of a mixture of methanol-water containing 25 mM of formic acid in gradient mode from 65 to 100% methanol over 25 minutes. Flow rate was adjusted to 1.5 ml/min. All samples were analysed in duplicate or triplicate at 228nm.
This method was successfully validated and showed good linearity, reproducibility and accuracy between 10 μg/ml and 1 mg/ml. The method is suitable for evaluating the stability of cannabinoids.

9.2.3 General procedure for preparation of complexes

For preparation of complexes, ethanolic stock solutions of CD and THC were mixed in appropriate ratios and samples were evaporated to dryness under vacuum. Dried samples were resuspended in unbuffered water, or methanol/water (for some of the NMR studies) by ultrasonication [Lyng, 2004], then left to equilibrate for 72 hours in the dark at room temperature under constant agitation.
For the phase-solubility study an excess amount of THC was added. After equilibration, undissolved THC was removed from the suspensions by centrifugation. Intrinsic solubility (S_0) of THC in pure water was determined by following the same protocol, but without addition of cyclodextrin. After equilibration, the water phase was lyophilized and reconstituted in a small quantity of ethanol for quantification of dissolved THC by HPLC.

9.2.4 Phase solubility study

Effects on the aqueous solubility of THC were studied using the phase-solubility method [Higuchi and Conners, 1965]. Excess amounts of THC were mixed with ever increasing concentrations of CD. The tested CDs were α-CD (4-50 mM), β-CD (4-16 mM), γ-CD (4-40 mM), and RAMEB (8-187 mM). Complex was prepared as described above and the solutions were assayed for THC content by HPLC.

9.2.5 Job's plots

The Job's (continuous variation) plot of THC was determined from ^{1}H-NMR and UV data, according to the continuous variation method [Job, 1928; Chankvetadze, 1998].
The NMR experiment was carried out as described below with solutions of THC and RM-β-CD in unbuffered D_2O/MeOD (1:1, v/v). The total molar concentration of the two components concentrations was kept constant at 6.36 mM, but the mole fraction of RAMEB {i.e., [RAMEB]/([RAMEB]+[THC])} varied from 0.1 to 0.9. Chemical shift of proton signals was observed for preparation of the plot.
Solutions of the same composition, but in unbuffered water only, were used for UV-spectrophotometric determination of the stoichiometry using the same method. The shift of λ_{max} around 275 nm of the UV-spectrum of THC was observed to prepare the Job's plot. Spectra were obtained with a Shimadzu UV-Vis 1240-mini spectrophotometer (0.1 nm resolution). Each complex solution was measured in triplicate.

9.2.6 NMR-study of the THC-RAMEB interaction

The ^{1}H-NMR spectrum of pure THC in D_2O could not be determined due to its very low aqueous solubility. Therefore ^{1}H-NMR signal assignments for THC were performed in D_2O/MeOD (1:1). Also the Job's plot was determined in D_2O/MeOD (1:1) in order to have enough signal strength at low RAMEB concentration.
All spectra were recorded on a Bruker DPX-300 spectrometer operating at 300 MHz for protons. Temperature was set at 30°C. The peak of residual water (H_2O) was used as internal reference at 4.80 ppm. For proton (^{1}H)-NMR, 128 scans were recorded with the following parameters: 32K data points, pulse width of 4.0 μs and relaxation delay of 1 second. FID's were Fourier transformed with LB of 0.5 Hz.
For two-dimensional (2D) Nuclear Oberhauser Effect spectroscopy (NOESY)-experiments, measurements were performed in D_2O with 8 number of scans, 2K data points in F2, relaxation delay 1 s and mixing time 1 s.
In order to avoid confusion in discussing the results of NMR, protons of THC are referred to in normal font type (H4), while protons of CD are referred to in italic (*H3*).

9.2.7 Stability during storage

Solutions of the THC / RM-β-CD complex in unbuffered water were stored at ambient temperature in tightly closed, clear glass vials while exposed to natural light conditions in the laboratory room. Initial THC concentration was 1 mg/ml. After 1, 2, 4 and 8 weeks of storage, duplicate samples were taken and analyzed by HPLC for signs of decomposition.

9.3 Results and discussion

9.3.1 Complexation and phase solubility studies

It is most common to perform complexation studies such as described here, in buffered aqueous solutions. However, it has been shown that, in most cases, ionic strength has a negligible effect on the binding of neutral molecules to CDs [Zia, 2001]. Furthermore, we found that pH changes in the range of 5-9 had no effect on the solubilizing of THC by RAMEB. We therefore concluded that it was possible to perform our complexation studies in unbuffered pure water. Although treatment of a THC/hydroxypropyl-β-CD complex with an ultrasonic bath was reported to result in some minor degradation of THC [Jarho, 1998], such degradation was not observed in our study after ultrasonication.
Testing of four different cyclodextrins showed that only the use of RAMEB results in significant levels of solubilized THC. At their highest tested concentrations, α-CD (50 mM) and β-CD (16 mM) had a very slight solubilizing effect in the order of 0.1mM THC, but whether this was the result of inclusion or some other mechanism was not further determined. Practically no THC was solubilized with the use of γ-CD (40 mM). At the maximal RAMEB concentration tested (24%; 187 mM) a THC concentration of 45 mM (14 mg/ml) was reached, which means an increase of aqueous solubility of THC of about 4 orders of magnitude. The phase-solubility diagram is shown in figure 9.2.

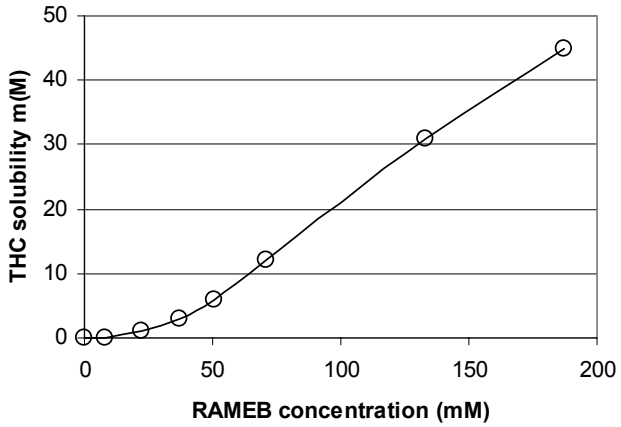


Figure 9.2: Phase solubility diagram for THC in the presence of RAMEB at 298K. Datapoints are average values of duplicate measurements.

An A_p-type phase-solubility diagram was obtained, which suggests formation of a higher-order complex with respect to cyclodextrin (i.e. 1:2 complex). Based on similar data, Mannila et al. [2005] concluded earlier that THC forms a complex with RAMEB in a 1:2 stoichiometric ratio. However, complementary data obtained in our study by preparing the Job's plot of THC/RAMEB showed the stoichiometry to be a 2:1 ratio of THC to RAMEB (discussed below).
The intrinsic solubility (S_0) of THC in unbuffered water at 20°C was determined to be 2.3 μM (0.7 μg/ml). This value was used to calculate the apparent stability constant from the initial linear part of the phase-solubility diagram according to Higuchi and Connors [1965]:

$$K_{1:1} = \text{Slope} /([S_0] (1 - \text{Slope})) M^{-1}$$

The value of $K_{1:1}$ was found to be 15900 M^{-1}, which is in accordance with the value (19600 M^{-1}) reported earlier for this complex based on phase-solubility study [Manilla, 2005]. The 2:1 binding constant can not be determined from this type of diagram.
At higher concentrations of CD the diagram slightly curves off. As pointed out by Higushi and Connors [1965], negative curvature diagrams reflect an alteration in the effective nature of the solvent in the presence of high concentrations of the host molecule (i.e. viscous and "non ideal" charasteristic of the solution), leading to a change in the complex formation constant. Alternatively, it is possible that the formation of 2:1 complexes results in subsequent formation of micellar-like structures. Such structures could precipitate from solution, thereby lowering the THC concentration.

9.3.2 Determination of the stoichiometry

Two independent techniques were used for preparation of a continuous variation plot in order to determine the stoichiometry of the inclusion complex. Thus, the ratio of CD and THC was varied while the sum of their concentrations was kept constant, and a continuous variation plot was prepared. Using this method the value for Δδ reaches a maximum at the stoichiometric point. The NMR results were obtained for most of the THC peaks but for only some CD peaks (*Me2, Me6*), mainly because of spectral overcrowding. The plot for the NMR-peaks of THC undergoing the largest shifts is shown in figure 9.3a. Results for the NMR-determination of CD are not shown, but all results yielded 2:1 stoichiometry of THC to CD. In a single, stable complex, the plot usually has a triangular form with a maximum, while the formation of weak complexes results in curved plots. The shape of the plot in figure 9.3a therefore suggests that the studied complex is indeed not of the single (1:1 stoichiometry) stable kind. For all ratios of THC:CD only a single set of peaks was observed for THC, indicating a fast exchange regime.
The very low solubility in water did not allow NMR studies of the guest in pure water. Instead, some studies had to be carried out in a methanol/water mixture. Although it must be noted

that the addition of methanol possibly changes the nature of the complex., the stoichiometry of 2:1 was confirmed by the results of the UV determination, which was performed in water only (figure 9.3b).

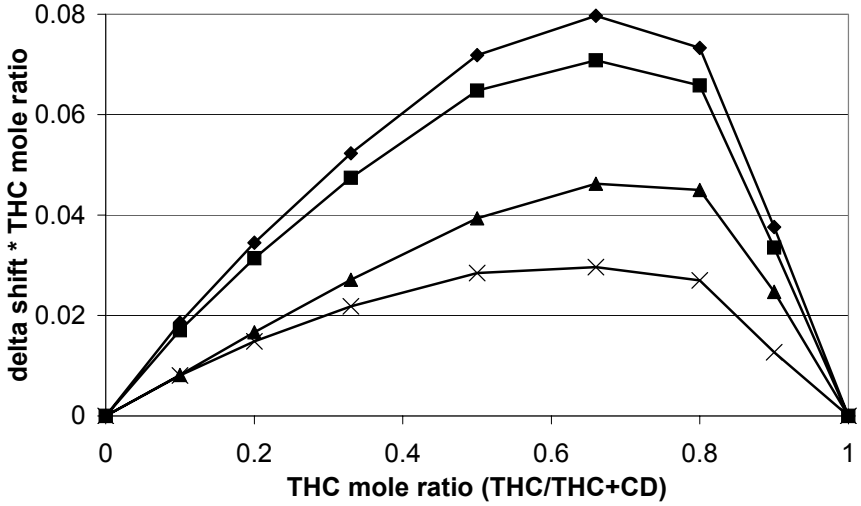


Figure 9.3a: Continuous variation plot for THC obtained from the chemically induced shift displacement (CID) of selected NMR proton signals of THC; H2 (♦), H4 (■), H5' (▲), H1' (x).

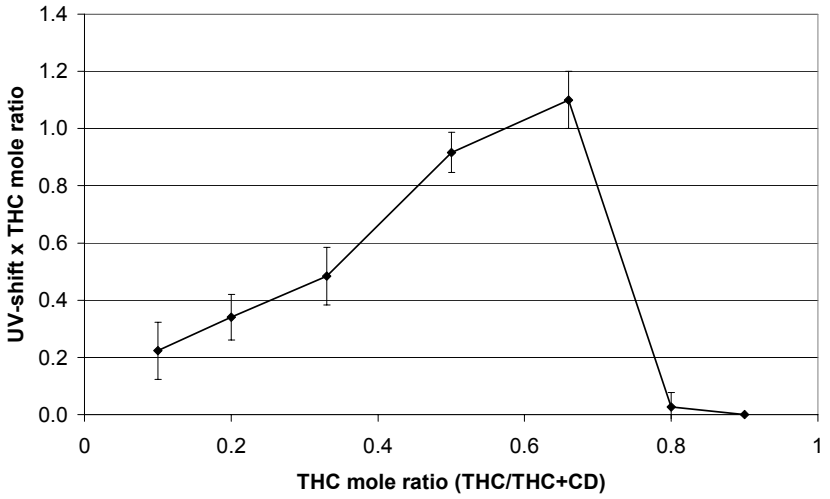


Figure 9.3b: Continuous variation plot for THC obtained from UV investigations. Datapoints are average values of triplicate measurements. Error bars indicate standard error.

9.3.3 Chemically induced shift displacements (CID) study of the complex

An updated assignment of signals for THC was recently published by Choi et al. [2004]. The signals in the obtained ^{1}H-NMR spectrum of THC were well separated from the signals of

RAMEB, with the exception of the H10a signal. Moreover, the signal of H6α was obscured by the signal of residual water in the deuteriated solvent.
Peak assignment for RAMEB was performed by using published data on RAMEB and DM-β-CD [Ravichandran, 1998; Correia, 2002] in combination with the obtained results of ^{1}H- and NOESY-NMR. NMR studies on RAMEB are difficult because it is not a single pure compound, but rather a mixture of β-CD molecules, each methylated in a random fashion. As a result only some of the NMR-signals for RAMEB could be unambiguously identified: *Me2*, *Me6*, and *H1*. Other signals were uncertain and could not be used for interpretation.
A definite increase of the water-solubility was observed for THC in the presence of RAMEB, and addition of RAMEB to a solution of THC (in D_2O/MeOD) resulted in modification of the ^{1}H-NMR spectrum of THC. These changes of the NMR spectra of THC can be understood in terms of the formation of inclusion complexes, where a molecule of THC is positioned inside the hydrophobic cavity. Examination of the observed chemically induced shift displacements (CID, shown in table 9.1) provided information of the nature of guest-CD interaction because protons that undergo the largest shift upon complexation are considered to be most strongly involved in the interactions leading to complexation.
The THC signals of H2, H4, H5' and H1' were most affected, while those of H7, H8 and H11 underwent almost no displacement. This indicates an inclusion of ring A and the alkyl side chain of THC into the CD cavity, while ring C is not, or only partially, included. It should be noted that H2, H4 and H1' all undergo an expected upfield shift upon inclusion, while the H5' signal showed a shift downfield. A potential explanation is that the alkyl side chain completely enters the CD cavity and protrudes from the opposite opening, thereby exposing H5' to the solvent. The moderate downfield shift that is observed for H6β may be explained by a change in the orientation of the surrounding shell of water molecules upon inclusion, or possibly by conformational changes in a non-included part of the molecule. In general, the relatively small Δδ values observed for all signals indicate a relatively weak association.
Regarding the NMR-spectrum of RAMEB, the presence of THC is related to an upfield shift of *Me2*, which seems to suggest its involvement in complexation. The associated small upfield shift for *H1*, located on the outside of the CD-ring, is possibly due to conformational changes in the CD-ring structure upon complexation. Data on *Me6* was inconclusive. Shift of any other signal could not be observed due to spectral overcrowding in the NMR-spectrum, so based on these data alone, only limited conclusions can be made on the involvement of CD-protons in complexation. It is possible that more conclusive data could be derived by studying THC complexation with the chemically more well-defined DM-β-CD, but such study was not performed as part of this work. Moreover there is the possibility that substituting RAMEB with DM-β-CD might alter the nature of the complex.

Table 9.1: ^{1}H-NMR chemical shift values for free and complexed THC with RM-β-CD (equimolar ratio, total conc. = 6.36mM). a :not clear

Proton signal	Chemical shift (ppm)		Δ Shift (ppm)
	Free	*Complex*	
H2	6.14	6.00	-0.14
H4	6.27	6.14	-0.13
H-6alpha	1.41	1.44	+0,03
H-6beta	1.09	1.10	+0,01
H7	1.90	1.90	0
H8	2.16	2.16	0
H10	6.31	6.27	-0.04
H11	1.68	1.67	-0.01
H1'	2.42	2.36	-0.06
H2'	1.55	a	-
H3'/H4'	1.29	a	-
H5'	0.87	0.95	+0,08

9.3.4 NOESY-experiments

The NOESY spectrum of the complex dissolved in D_2O (figure 9.4) showed a variety of interactions between THC and CD protons, which confirmed the inclusion of at least one THC molecule inside the cavity of RAMEB. Two signals of RAMEB could be clearly identified (*Me2* and *Me6*) and this proved to provide enough information to elucidate the complex structure. The *H1*-signal (not shown) could be identified also, but it shows no crosspeaks at all as this proton is present at the outside of the CD ring.

When it is assumed that a THC molecule is positioned inside the cavity, two general orientations along the long axis of THC are possible. A strong interaction between H3'-, H4'- and H5'-signals of the pentyl side chain of THC and *Me6* of CD indicate that the side chain protrudes through the primary opening. This orientation of THC would bring H11 and H6β into proximity of *Me2*, which is indeed confirmed by the presence of the expected crosspeaks. A notable absence of crosspeaks is observed for H7 and H8, while only very weak interactions are observed for H6a and H10a (not indicated in figure 9.4). This suggests that ring C remains at least partially outside the CD-cavity and this is in agreement with the analysis of the observed chemical shift displacements discussed above.

Based on the obtained NMR data a model for the inclusion of THC into the RAMEB cavity can be suggested. The proposed structure of the 1:1 complex can be understood from figure 9.5. Although the THC sidechain is included inside the complex, there is a notable absence of crosspeaks between H1' and H2' of THC with CD-protons. Likely, the presence of the more bulky phenolic ring restricts the movement of the alkyl chain and physically prevents the H1'

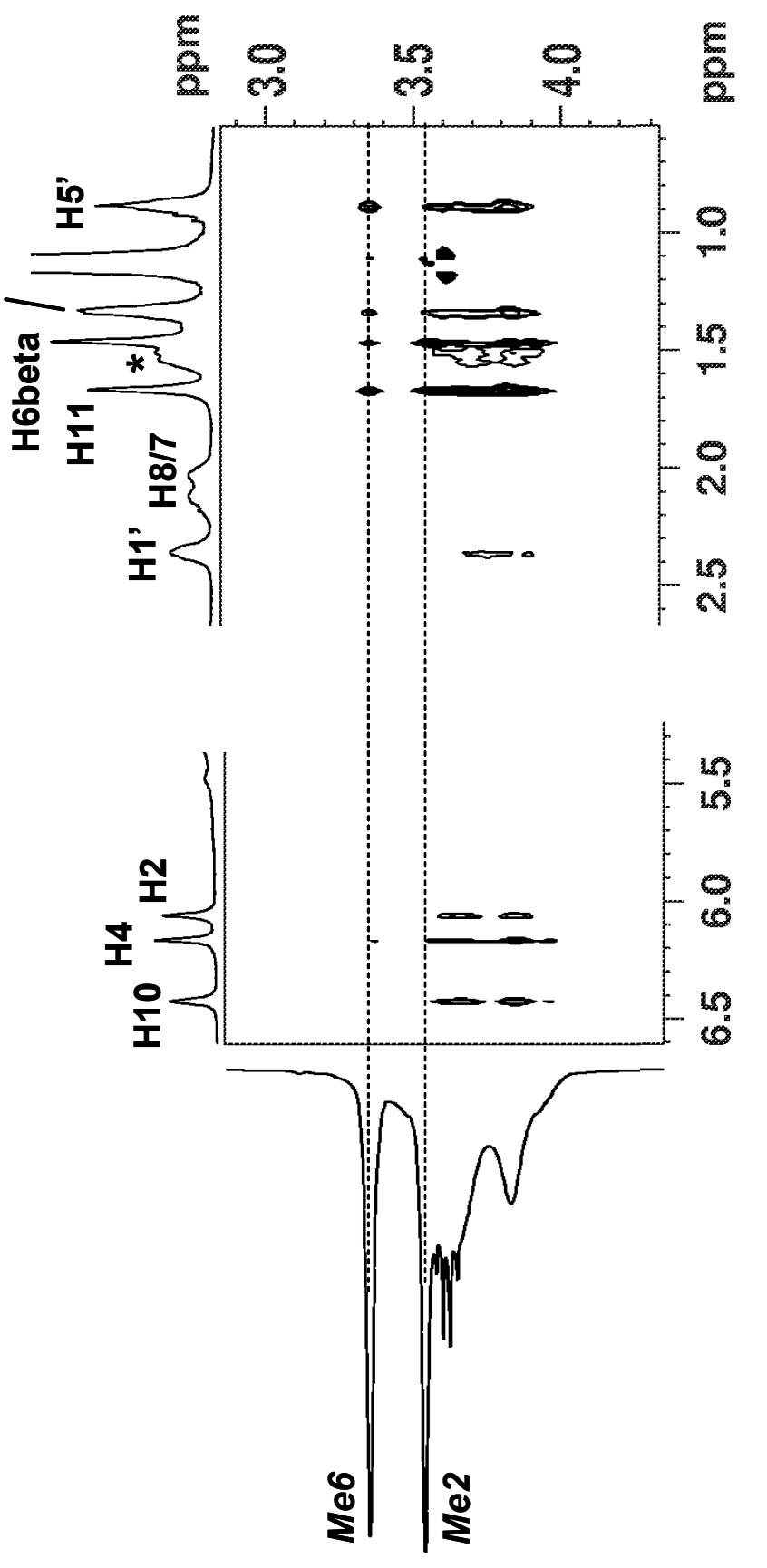


Figure 9.4: Partial contour plot of a NOESY spectrum of the THC complex with RM-β-CD. Peaks of THC are identified on the top of the figure, while peaks of CD are marked on the left. *: position of H2'-signal

and H2' protons to come into proximity of the CD protons on the inside of the cavity. A similar result was obtained for complexes of γ-CD with fusidate and helvolate, which contain a side chain attached to a rigid (ring)structure [Jover, 2003]. The proposed structure also corresponds with the suggestion, based on the study of chemically induced shift displacements (CID), that H5' is exposed to the solvent.
Because we propose a 2:1 binding of THC to CD, based on the reults of the Job's plot, a second THC molecule must be bound to the complex. This binding is thought to be the result of non-inclusion interactions. It was discussed above that an inclusion interaction exists between H11 and H6β, and *Me6* of RAMEB. However, at the same time H11 and H6β show a clear interaction with *Me2*, which is positioned at the other end of the CD cavity. This seemingly incompatible data can be explained by the presence of the second THC molecule at the primary opening of CD as shown in figure 9.5. A non-inclusion interaction between protruding methyl groups from both THC and RAMEB seems very plausible.
The proposed structure allows interaction between H5' of included THC with the free THC, possibly providing an alternative explanation for the observed positive CID value for H5'. However, no such crosspeaks were observed in the NOESY spectrum, indicating this interaction, if present, must be very weak.

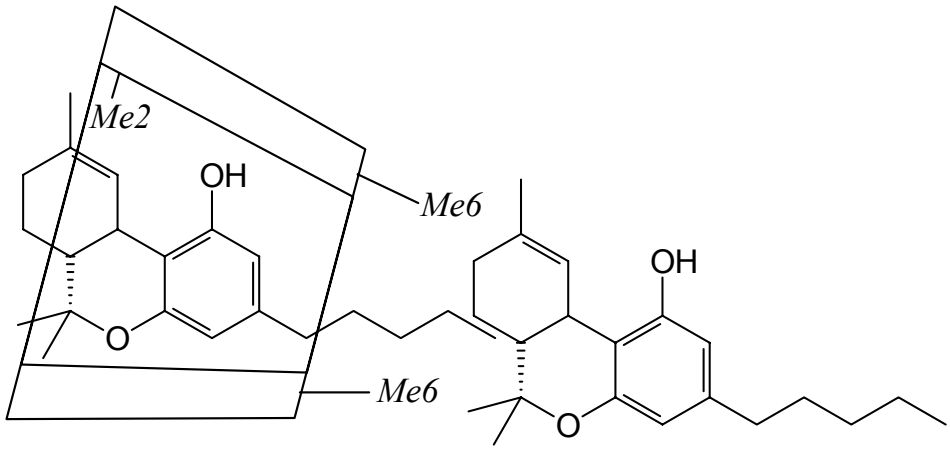


Figure 9.5: Proposed structure of the THC-RAMEB complex. Figure is not on scale.

9.3.5 Stability during storage

A solution of THC in ethanol will rapidly degrade under the influence of light and air, resulting in formation of degradation products delta-8-THC and cannabinol (CBN) [Fairbairn, 1976]. However, storage of the complex dissolved in unbuffered water under standard laboratory room conditions (artificial light, temperature ± 22°C) did not result in

any significant degradation of THC during the test period of 8 weeks. Furthermore, the THC concentration remained constant.
In general, stability studies are performed in buffered solutions to get the most reliable results. However, in our case we were interested in the behaviour of complex in unbuffered water, as our research is focussed on the future preparation of purely aqueous THC solutions with a minimum of additives. For this reason water was not buffered in the stability test. We believe this is possible because THC and CD have no effect on pH upon dissolving in water, and we found that complex formation was not influenced by pH in the range of pH 5 to 9.

9.4 Conclusion

In this study it was found that, out of four different types of cyclodextrins tested, only randomly methylated-β-cyclodextrin was able to increase the aqueous solubility of THC to a significant level. A concentration of THC of about 14 mg/ml was reached by using a 24% (187mM) RAMEB solution. The binding ratio of THC to CD was found to be 2:1 by using both an NMR- and a spectrophotometric method. However, such a complexation theoretically should result in a linear phase-solubility diagram while in fact an A_p-type was observed [Mannila, 2005; this study]. The cavity of RAMEB has a diameter that is somewhat smaller than that of natural β-CD (6 Å) and this would allow inclusion of THC no further than ring B. Based on spatial restrictions it seems unlikely that RAMEB could accommodate 2 molecules of THC. This seemingly incompatible data could be plausibly explained by assuming the formation of a 1:1 inclusion complex with non-inclusion interaction leading to a 2:1 complex. A similar structure was recently found for the complexation of ketoprofen with β-CD [Rozou, 2005].
It has been suggested that 1:1 drug/cyclodextrin inclusion complexes form water-soluble non-inclusion complexes with additional drug molecules to give rise to A_p-type phase-solubility diagrams [Loftsson, 2002]. This has been shown with acridine/DM-β-CD [Correia, 2002], where it was concluded that a real 1:1 inclusion complex was formed, while a second molecule of acridine probably interacts with the DM-β-CD, but it remains outside the cavity. We speculate that this is also the case for the THC/RAMEB complex.
From the obtained NMR data it was concluded that THC forms a complex through inclusion of ring A and B, with the pentyl sidechain partly protruding from the primary opening of RAMEB. Ring C seems to be only partially included due to steric hindrance presented by the methyl-groups in positions 6β and 11. In order to even better allow the proposed inclusion of THC inside the CD cavity, the side-chain may adopt a folded conformation inside the β-CD cavity. A similar folded configuration was found for the flexible side-chain of bile-salts [Ramos, 1999, 2003]. In several studies it was shown that alkyl sidechains, because of their lipophilic character, are the preferred substituent of the guest molecule for inclusion into the cavity, provided they are accessible for interaction with the CD molecule [Ravichandran, 1998; Ramos, 1999; Zhang, 2002; Ramos, 2003].

The formation of a 2:1 complex by binding of a second THC molecule to the 1:1 complex through non-inclusion interactions was supported by NMR data. A weak binding between THC and RAMEB was suggested by the obtained data (CID-values, shape of the Job's plot of NMR data). However, the apparent 1:1 stability constant was relatively high. This supports the idea of a second THC molecule, strengthening or stabilizing binding of the included molecule. Unfortunately, because of the A_p-type phase solubility diagram, the binding constant of the 2:1 complex could not be calculated from the obtained data.
Although the use of RAMEB highly increased aqueous solubility of THC, only a very weak solubilization was observed when THC was mixed with unsubstituted β-CD. Apparently the presence of methyl groups is needed for inclusion of THC in the cavity, which is a further indication that complexation leading to formation of the 2:1 complex is mostly due to hydrophobic interactions between THC and these non-polar methylgroups.
The water concentration of THC that can be achieved by the use of CDs is in a suitable range for possible clinical or analytical applications. In a preliminary study we found that several other major cannabinoids could be solubilized as well in the presence of RAMEB. Studied cannabinoids included delta-9-tetrahydrocannabinolic acid (THCA), cannabinol (CBN), cannabidiol (CBD) and cannabigerol (CBG). Without CDs present, all of these compounds were practically insoluble in pure water. However, real inclusion could not be proven by these experiments and complementary studies have to be performed. Nevertheless, the CD complexation of THC and possibly other cannabinoids seems to be a promising way for producing water based solutions of cannabinoids without the need for addition of other solubilizers or organic solvents. Hopefully the results obtained in this study will be a contribution to the further development of cyclodextrin studies with the cannabinoids.

9.5 Acknowledgements

The authors are grateful to Farmalyse BV, The Netherlands for providing the high quality THC and other cannabinoids that were needed for our study. The van Leersum fund, The Netherlands, is acknowledged for providing us with the funds for obtaining the spectrophotometer.

CHAPTER 10

Evaluation of a vaporizing device (Volcano®) for the pulmonary administration of tetrahydrocannabinol

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Abstract

What is currently needed for optimal use of medicinal cannabinoids is a feasible, nonsmoked, rapid-onset delivery system. Cannabis "vaporization" is a technique aimed at suppressing harmful respiratory toxins by heating cannabis to a temperature where active cannabinoid vapors form, but below the point of combustion where smoke and associated pyrolytic products are released. The goal of this study was to evaluate the performance of a vaporizer of the brand 'Volcano' as a novel method for the clinical administration of THC. Performance was evaluated in terms of reproducible delivery of the bioactive cannabinoid tetrahydrocannabinol (THC) by using pure cannabinoid preparations, so that it could be used in a clinical trial. By changing parameters such as temperature setting, type of evaporation sample and balloon volume, the vaporization of THC was systematically improved to its maximum, while preventing the formation of breakdown products of THC, such as cannabinol or delta-8-THC. Inter- and intra-device variability was tested as well as relationship between loaded- and delivered dose. It was found that an average of 54% of loaded THC was delivered into the balloon of the vaporizer, in a reproducible manner. When the vaporizer was used for clinical administration of inhaled THC, it was found that on average 35% of inhaled THC was directly exhaled again. Our results show that with the Volcano a safe and effective cannabinoid delivery system seems to be available to patients. The final pulmonal uptake of THC is comparable to the smoking of cannabis, while avoiding the respiratory disadvantages of smoking.

10.1 Introduction

Cannabis (*Cannabis sativa* L.) has a long history as a recreational drug and as part of traditional medicine in many cultures of the world. Nowadays, cannabis is used medically by patients suffering from diseases varying from cancer and HIV/AIDS to multiple sclerosis, frequently in the form of unprescribed self-medication [Page, 2003; Furler, 2004]. Marinol®, an oral form of the main psychoactive constituent of cannabis, delta-9-tetrahydrocannabinol (THC), has been developed for some indications. However, oral THC is notoriously unreliable in its effects [Grinspoon, 1997]. Drawbacks of Marinol® include its slow onset of action, large variability in bioavailability and extensive first pass metabolism. Moreover there is the inconvenience of taking oral medication in case of nausea or vomiting. Therefore, for many patients the demand for more effective cannabinoid based medications persists. For this group of patients cannabis smoking is a more convenient method of administration, allowing self-titration of the desired effects. However, inhalation of toxic compounds during cannabis smoking poses a serious hazard. This risk is not thought to be due to cannabinoids, but rather to noxious pyrolytic byproducts [Hiller, 1984; Matthias, 1997]. Consequently, the shortcomings of smoked cannabis have been widely viewed as a major obstacle for approval of crude cannabis as a medicine by public health authorities [Institute of medicine, 1999].

Cannabis "vaporization" or "volatilization" is a technique aimed at suppressing irritating respiratory toxins by heating cannabis to a temperature where active cannabinoid vapors are formed, but below the point of combustion where pyrolytic toxic compounds are made. Vaporization offers patients who use medicinal cannabis the advantages of the pulmonary routes of administration, i.e.: rapid delivery into the bloodstream, ease of self-titration and concomitant minimizing the risk of over- and under-dosing, while avoiding the respiratory disadvantages of smoking.

In a series of studies the vaporizing of cannabis samples was systematically tested to show its advantage over smoking. When a variety of smoking devices (including water-pipes) were compared, specifically examining THC and solid smoke tars, it was found that only vaporizers were capable of achieving reductions in tar relative to THC when compared to direct smoking of cannabis [Gieringer, 1996; McPartland, 1997]. A follow-up study tested a vaporizer that was found to deliver THC while completely eliminating three specific toxins (naphthalene, benzene and toluene) in the solid phase of the vapor [Chemic Laboratories, 2000]. The study also detected a ≥56% reduction in tars and a qualitative reduction in carbon monoxide, but did not test for any other chemicals [Gieringer, 2001]. In a more recent study [Gieringer, 2004] GC-mass-spectrometry was used to analyze the gas phase of vaporized cannabis for a wide range of toxins, particularly concentrating on the highly carcinogenic polynuclear aromatic hydrocarbons (PAHs). The vaporizer that was used was the Volcano® [Storz & Bickel website]. It consists of a heater, a ventilator, a filling chamber, a valve, and a balloon. During operation the balloon is inflated with hot air and cannabinoid vapors. Using cannabis plant material as the sample, vapors were found to consist overwhelmingly of cannabinoids,

while the combusted control contained over one hundred additional chemicals, including several known PAHs.
Although a large variety of vaporizing devices is available on the market, the Volcano is one of the few devices that have been tested scientifically to some extent. It is a herbal vaporizer, intended for the vaporization of whole cannabis plant materials (i.e. flowertops), but numerous unexplored variables could affect the efficiency and output of vaporization. These parameters are variations in temperature; differences in specimen density, weight, content of water and essential oils, and consistency of material in the filling chamber; differences in the variety and potency of cannabis used; and use of different preparations such as crude flowertops, hashish, hash oil, etc. Because of the paucity of data it has so far been difficult to show that the Volcano vaporizer can be used as a reliable tool for the reproducible administration of THC or other cannabinoids. A solution to this would be in the use of pure cannabinoid preparations of known concentration to guarantee an exact and reproducible loading of cannabinoids.
In this study the Volcano vaporizer was evaluated as a novel method for the administration of THC. Pure cannabinoid preparations were used in order to obtain quantitative results in terms of efficiency and reproducibility of THC delivery into the balloon of the Volcano. By changing parameters such as temperature setting, type of evaporation sample, and balloon volume, the vaporization of THC was systematically improved to its maximum yield, while preventing the formation of degradation products. Factors that resulted in loss of THC by condensation, that is, storage time of the balloon and influence of the filling chamber, were evaluated. The inter-device reproducibility of THC vaporization under optimized conditions was determined. Finally, the results of this study were used for the clinical administration of THC by vaporizing. The amount of exhaled THC was determined and compared to the dose, which was inhaled through the Volcano.
Our results indicate that the Volcano is a convenient device for the administration of THC by inhalation.

10.2 Materials and methods

10.2.1 Materials

All organic solvents were HPLC or analytical grade, and were purchased from J.T. Baker (Deventer, The Netherlands). Glass fiber filters (Cambridge type, borosilicate glass, 92 mm diameter) and tightly fitting filter holders for vapor extraction were obtained from Borgwaldt Technik GmbH (Hamburg, Germany).
Cannabis plant material (female flowertops) was medical grade and obtained from Bedrocan BV (The Netherlands). It had a water content of about 8%, a THCA content of about 12% and virtually no free THC.

Purified THC and THCA (purity ≥98%) were produced and quantified as reported earlier [Hazekamp, 2004a,b]. THC was of pharmaceutical grade. The cannabinoids were stored as ethanolic solutions at -20°C at a concentration of 50 mg/ml.

Figure 10.1a: The Volcano vaporizer.

10.2.2 The Volcano device

The Volcano® was obtained from Storz & Bickel GmbH&Co. (Tuttlingen, Germany) and was used according to the manual as provided by the manufacturer. It is a vaporizer or evaporator that can evaporate the active substances or aromas from plant material by using a hot air flow (figure 10.1a). Depending on the type of filling chamber used, whole plant material or liquid samples (e.g. aromatic oil, extract or pure compounds in solution) can be used. Evaporated compounds are collected in a detachable plastic balloon (figure 10.1b), which can be removed and fitted with a mouthpiece for inhalation. Volume of the balloon can be varied. Unless stated otherwise, a balloon length of 55 cm (around 8 L content) was used, as recommended by the manufacturer. The temperature control ranges from setting 1 to 9, corresponding to

temperatures of 130°C to 226°C (see table 10.1). Before each new set of experiments, the whole device was thoroughly cleaned with ethanol. At the start of each evaporation the Volcano was pre-heated until the indicator light showed that the target temperature was reached. The balloon, connected to the filling chamber, was then immediately placed onto the Volcano and the ventilation was started. When the balloon was completely inflated, ventilation was stopped and the content of the balloon was processed for analysis within 5 minutes, unless stated otherwise.
All laboratory experiments were carried out in a standard laboratory fume hood under constant ventilation with an ambient room temperature of about 22°C and a humidity of 40-60%.

Figure 10.1b: The balloon construction of the Volcano, fitted with mouthpiece.

10.2.3 Use of the liquid pad

The pure cannabinoids THC or THCA were used as ethanolic solutions. For these liquid samples an adapted filling chamber was used, containing a removable disc made of tightly packed stainless steel wire mesh (liquid pad), obtained from the manufacturer of the Volcano. For each experiment the appropriate amount of the cannabinoid was dissolved in a final volume of 200 µl of ethanol for application onto the liquid pad and ethanol was allowed to evaporate for 10 minutes under ambient conditions. A new liquid pad was used for each experiment.

10.2.4 Extraction of THC from the vapor and the liquid pad

Cannabinoids were recovered from the vapor phase inside the balloon by condensation onto glass fiber filters, designed to capture particles > 0.1 microns. Vapor was slowly aspired through the glass-fiber filter, which was then extracted twice with 15 ml of methanol/chloroform (9:1, v/v) under ultrasonication. After evaporating the extraction solvent, samples were reconstituted in 5 ml of ethanol for analysis by HPLC or NMR. These ethanolic samples will be further referred to as vapor extracts.
Residual THC on the liquid pad was recovered by extracting the liquid pad twice using methanol/chloroform (9:1, v/v) under ultrasonication. Extracts were further handled as described above for the vapor extracts. Recovery was determined by spiking filters or liquid pads with THC (2 mg) and performing the described extraction procedure.
To assess the efficiency of condensation of cannabinoids onto the glass fiber filter, a washbottle filled with ethanol was placed after the filter. The escaping gases were led through this liquid which was thereafter analyzed by HPLC to measure cannabinoids untrapped by the filter.

10.2.5 Quantitative ^{1}H-Nuclear Magnetic Resonance spectroscopy (NMR)

Quantification of THC in the extracts was done by quantitative ^{1}H-NMR using a Bruker 300 MHz NMR apparatus as described by Hazekamp *et al.* [2004b]. In short, an exact volume of the sample was mixed with 1.0 mg of anthracene as internal standard for quantification. The sample was then evaporated to dryness under vacuum and reconstituted in chloroform (deuterated) for ^{1}H-NMR analysis.

10.2.6 High pressure liquid chromatography (HPLC)

HPLC was used for both qualitative and quantitative analysis of the obtained extracts. The HPLC profiles were acquired on a Waters (Milford, MA, USA) HPLC system consisting of a 626 pump, a 717plus autosampler and a 2996 diode array detector (DAD), controlled by Waters Millennium 3.2 software. Full spectra were recorded in the range of 200-400 nm. The analytical column was a Vydac (Hesperia, CA, USA) C_{18}, type 218MS54 (4.6x250 mm, 5 µm), with a Waters Bondapak C_{18} (2x20 mm, 50 µm) guard column. The mobile phase consisted of a mixture of methanol-water containing 25 mM of formic acid in gradient mode; methanol: water in ratios from 65:35 to 100:0 over 25 minutes, then isocratic to 28 minutes. The column was re-equilibrated under initial conditions for 4 minutes. Flow-rate was 1.5 ml/min and total runtime was 32 minutes. All determinations were carried out at ambient temperature. The main neutral and acidic cannabinoids were well separated with this method [Hazekamp, 2005]. Analyzed concentrations were well above the limit of quantification of the used method.

10.2.7 Evaluation of temperature control

Temperature control was evaluated at setting 1, 3, 5, 7 and 9 (see table 10.1). Time needed to reach target temperature, and accuracy and stability of target temperature were determined using an electronic thermometer (response time; 250 msec). Temperature was measured in the middle of the filling chamber, on top of the liquid pad. Each measurement was started by turning on the air-flow, directly after the indicator light of the heater had switched off (meaning the heater inside the apparatus had reached its target temperature). Inter-device variability for the same parameters was tested for four different Volcano devices. All experiments were repeated three times.

Temperature setting	Temperature in 'C
1	130
3	154
5	178
7	202
9	226

Table 10.1: Temperature (°C) corresponding to the different temperature settings of the Volcano

10.2.8 Optimization of vaporizing parameters

Temperature: cannabis plant material, and pure cannabinoids THCA and THC were vaporized at temperature settings 1, 3, 5, 7 and 9 in order to determine the delivery of THC into the balloon, as well as the formation of degradation products. Vapor extracts were qualitatively analyzed by HPLC for detection of degradation products, while quantitative analysis by NMR was used for determination of delivery.

Heating time: in order to determine the minimal time that is needed to reach maximal evaporation of THC, the following experiment was performed: THC (2 mg) was applied onto the liquid pad and the ventilation was activated for a duration ranging from 10 to 300 seconds, without balloon attached to the device so THC could evaporate freely. Subsequently, residual THC was extracted from the liquid pads and extracts were quantitatively analyzed by NMR.

10.2.9 Relationship between loaded dose and delivery

The relationship between quantity of THC loaded onto the filling chamber and delivery into the balloon was determined in the range of 2-8 mg of THC. Vapor extracts were analyzed by NMR and HPLC and each experiment was performed threefold.

10.2.10 Inter-device variability

Using the optimized parameters as determined in this study, four Volcano devices were finally evaluated for inter-device variability of THC delivery. Samples of 4 mg of THC were used for vaporizing and each Volcano was tested on 5 occasions. Vapor extracts were analyzed by NMR.

10.2.11 Condensation of THC onto the balloon and filling chamber

The effect of storage time of the balloons on condensation of THC was determined by storage of the balloon at room temperature for a duration of up to 180 minutes after vaporizing 2 mg of THC. The vapor extract was then collected for analysis. Each experiment was performed threefold.
Throughout this study balloons were always processed within 5 minutes after vaporizing. Therefore it was determined more exactly how much THC was lost due to condensation onto the walls of the balloon after 5 minutes of storage by carefully cutting the balloon (n=5) into pieces and extracting twice with ethanol under ultrasonication.
In order to determine the amount of THC that condensated onto the filling chamber (excluding liquid pad) and valve, these parts were extracted twice with ethanol under ultrasonication. Finally, extracts were concentrated and THC was quantified by NMR.

10.2.12 Clinical application of the Volcano

At the Centre for Human Drug Research (CHDR, Leiden, The Netherlands) a methodology study was performed to study the effects of THC administration using the Volcano vaporizer. The study was approved by the Medical Ethical Committee of Leiden University, The Netherlands. Preliminary results of this study were published recently [Zuurman, 2004], and full results will be published in the near future. In short, during two separate occasions, twelve subjects received a rising dose of 2, 4, 6 and 8 mg THC (loading dose in filling chamber) or placebo (ethanol only) administered via the Volcano, using the optimized parameters as determined in this study. Administrations were given with 1.5 hour intervals. The balloon (8 L) had to be inhaled through the mouth within 3 min and breath was held for 10 s after each inhalation. Following each inhalation, subjects were asked to exhale through a filter of the same type as used for vapor extraction. Filters were subsequently extracted as mentioned above, and the quantity of exhaled THC was determined by NMR.

10.3 Results

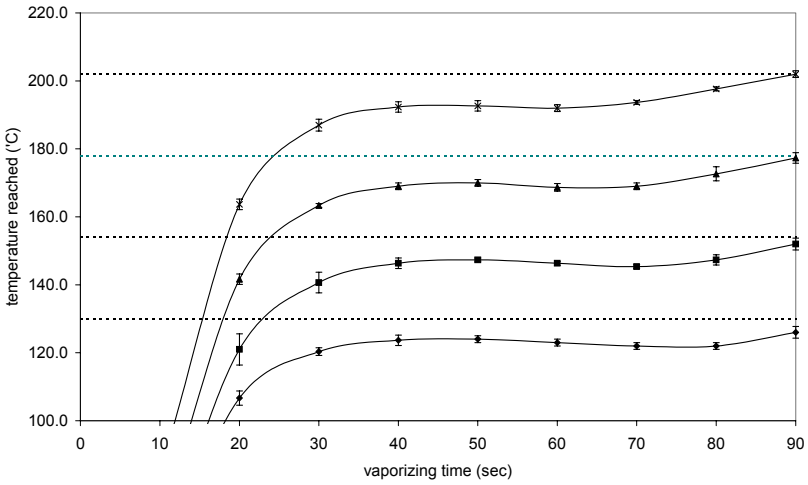
10.3.1 Trapping and recovery of THC for analysis

Since no trace of THC could be found in the ethanol fraction of the wash bottle inserted after the filter, it was concluded that THC was completely trapped onto the used type of filter. Recovery of THC was found to be 99.3 (± 1.1) % from the filter and 83.0 (± 2.5) % from the liquid pad. All measurements were corrected for these values.

10.3.2 Accuracy of the temperature setting

At all tested temperature settings it was found that temperature reached a first plateau after about 30 s. After that, temperatures remained relatively stable for some time, but kept somewhat below accepted limits (target temperature ± 4°C, as claimed by the manufacturer) for all tested settings. Results can be seen in figure 10.2a. However, after about 45-60 seconds, depending on the setting, the heating element was activated again by the temperature sensor, and about 20 s later temperatures increased by a few degrees, bringing the temperature within specified limits. It must be concluded that the liquid pad and the filling chamber need some time to heat up to the target temperature.

a)



b)

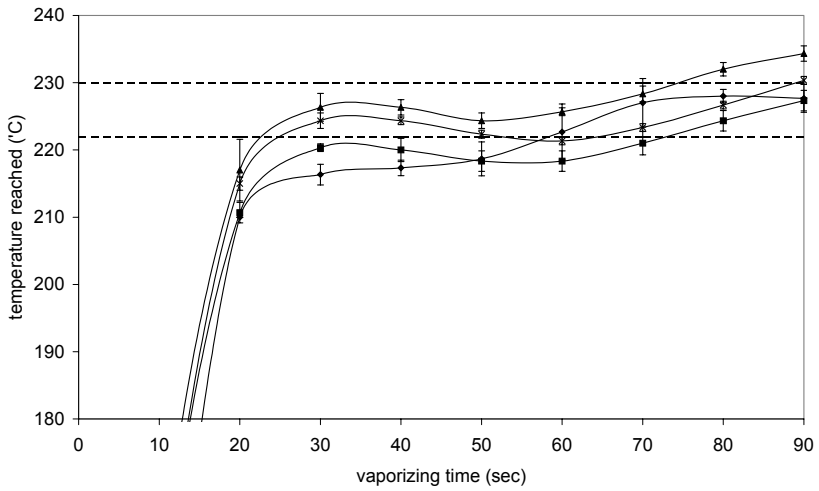


Figure 10.2: accuracy of the temperature setting
a): Temperature profile over time of the Volcano at different settings. Dotted lines indicate target temperatures at settings 1, 3, 5 and 7.
b): Comparison of temperature profile of four different Volcano devices at setting 9. Dotted lines indicate allowed target temperature range (±4°C). Data is shown as mean values of three experiments, and errorbars indicate standard deviation.

10.3.3 Reproducibility of the temperature setting

When four different Volcano devices were evaluated under equal conditions to evaluate inter-device variability (figure 10.2b), some small differences in heating profile were found. Only temperature setting 9 was evaluated here after it was found to be the optimal temperature for THC delivery. Although two devices reached target temperature (accepted variation ± 4°C) already after 30 s, the two others needed 60 s or more to do so. For two devices the temperature increased above the maximum limit of target temperature in the 90 s duration of our experiment. In conclusion, each individual Volcano device shows little variability during sequential uses (intra-device variability), although small differences do exist between different devices (inter-device variability).

10.3.4 Optimizing of vaporizing parameters with different substrates

THCA: Under the influence of heat THCA can be converted into THC by decarboxylation. Indeed, when THCA was used it was observed that this conversion increased with temperature, and maximum delivery of THC was about 33% at the highest temperature setting (figure 10.3). However, conversion was not complete and THCA was present in the vapor extracts at a level of about 5.5 (± 1.3) % relative to THC.

Crude flower tops: The use of plant material (200 mg at 12% THCA) resulted in a maximum THC delivery of only 29% (figure 10.3). In fresh cannabis plant materials, THC is present in the form of its acidic precursor THCA, and the use of plant material resulted in an incomplete decarboxylation with about 3.8% residual THCA present in the vapor. Besides THC, several other cannabinoids as well as a range of other plant components were detected. Therefore, the use of cannabis plant material in the Volcano should not be recommended for the administration and study of THC alone.

Pure THC: Evaporation of THC was shown to increase with temperature, with a maximal delivery of about 53% at setting 9 (figure 10.3) while no degradation products (delta-8-THC (Δ^8-THC), cannabinol (CBN) or other unknown peaks in the HPLC-chromatogram) were observed at any setting (see figure 10.4). Therefore, using the Volcano device, it was concluded that the highest delivery yield was achieved with an ethanolic of pure THC. When liquid pads were extracted after vaporizing it showed a very low amount of residual THC, indicating a very high yield of evaporation at the highest temperature setting. This strongly suggests that nondelivered THC does not remain on the liquid pad, but is probably lost by condensation after initial evaporation.

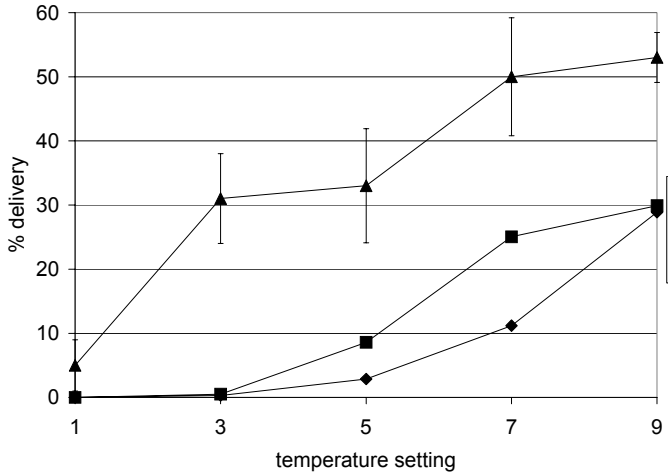


Figure 10.3: Delivery of THC into the balloon after vaporizing THC (▲, 8mg), THCA (■, 9mg) or plant material (♦, 200 mg) at different temperature settings (in % of amount loaded in filling chamber). Data is shown as mean values. Errorbars indicate standard deviation.

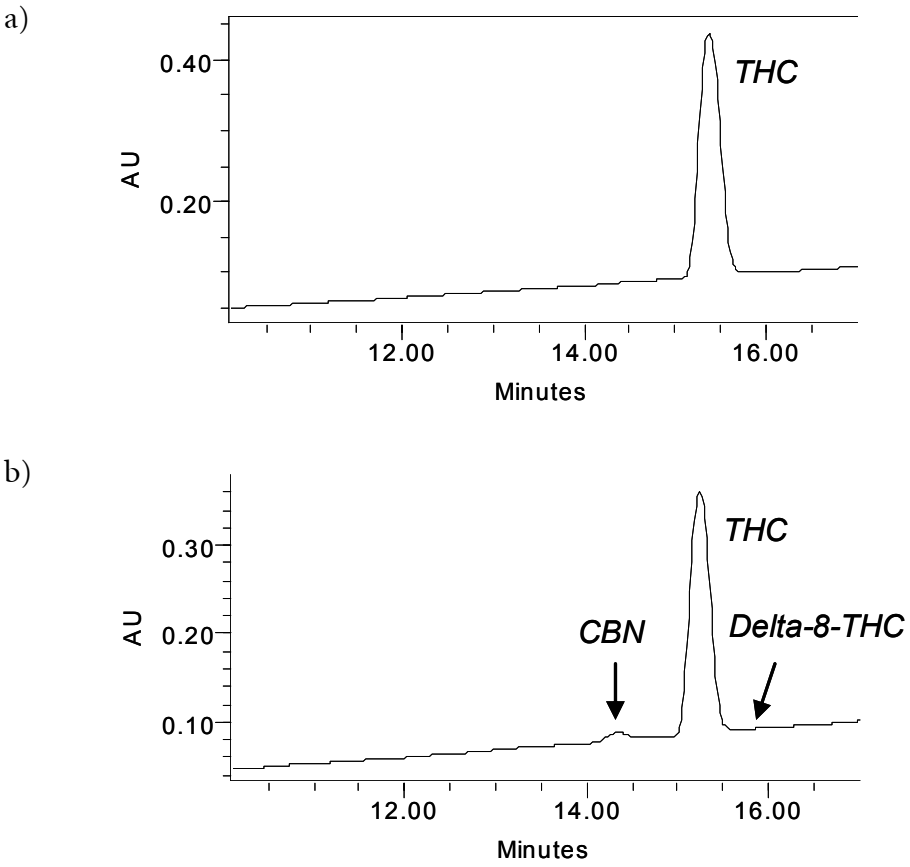


Figure 10.4: HPLC chromatogram (228nm) of THC before vaporizing (a) and recovered from the balloon after vaporizing (b) at setting 9. No decomposition products of THC are observed as a result of vaporizing.

The minimal time needed for the maximal evaporation of THC from the liquid pad was determined by measuring residual THC after vaporizing. Figure 10.5 shows that the amount of residual THC rapidly decreases between 20 and 40 s after starting of the vaporizing. This corresponds with the observation that in the same time-period the (near) target temperature of the Volcano is reached (figures 10.2a and 10.2b). After 45 s most of the THC is evaporated and just a small fraction of THC can be found in the liquid pad extract, indicating that vaporizing time should be at least 45 seconds. Indeed, when using a temperature setting of 9 with a balloon volume of 4 liters (filling time around 30 s), a low THC delivery (only 30% for 8 mg of THC) with a high dose variability (relative s.d. ± 22%) was observed, indicating that the maximum delivery yield was not yet reached.
It was observed that the maximal evaporation of THC is reached after 120 s (figure 10.5), meaning that a longer evaporation time does not release more THC. Since the Volcano is blowing air at a constant rate of about 9 liters per minute, this corresponds to a balloon volume of about 18 liters. However, by empirical testing in our laboratory (data not shown) it was found that a maximum volume of about 8 liters could be inhaled within three minutes when following the protocol of the clinical trial. Therefore a balloon volume of 8 liters (filling time of about 55 s) was selected for further study. Under these conditions, only about 5% THC remained on the liquid pad after evaporation.

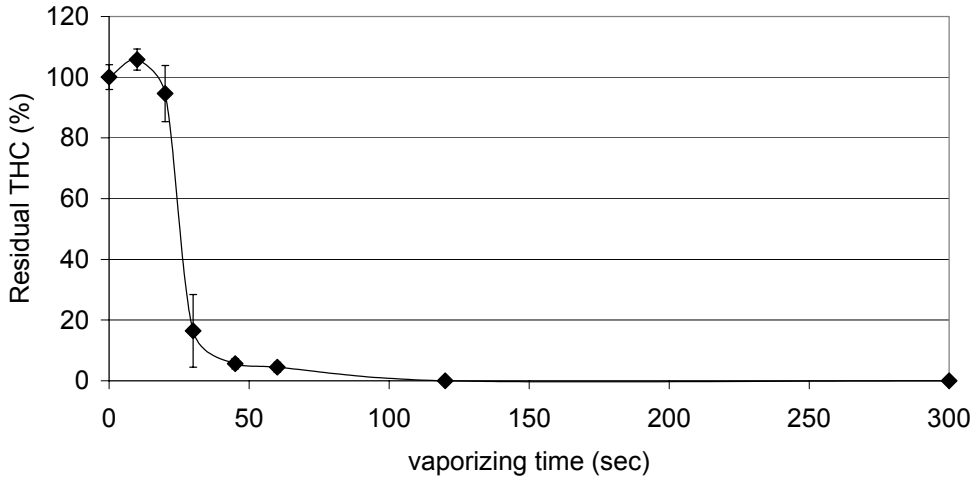


Figure 10.5: Residual THC on liquid pad after varying vaporizing time at setting 9. Data is shown as mean values of three experiments, and error bars indicate standard deviation. Values were corrected for the maximum recovery of 83% for extraction of the liquid pads.

10.3.5 Relationship between loaded dose and delivery under optimal conditions

With a Volcano operating under the aforementioned optimized conditions (temperature setting 9, balloon volume 8 liters) the delivery was determined with an increasing amount of THC ranging from 2 to 8 mg. It is shown in figure 10.6 that the delivery was proportional to

the loaded dose of THC; A linear curve was obtained with a regression coefficient (R^2-value) of 0.99. From the slope of the line, a mean delivery yield (THC loaded / THC recovered from balloon) of 57.8 (±6.9) % could be calculated.
Four available devices were then tested under the optimized conditions using a sample of 4 mg of THC. Differences in delivery between the Volcano devices were relatively small. Average delivery of all four Volcanos was 53.9 (±8.1) %, and this value was taken as the average delivery for further considerations.

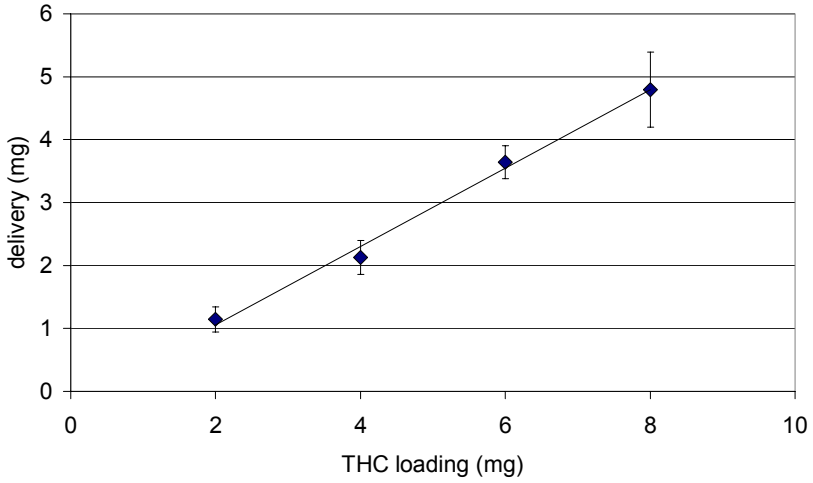


Figure 10.6: Delivery of THC under optimized conditions with THC loading dose ranging from 2 to 8 mg. Data is shown as mean values of three experiments and error bars indicate standard deviation. Linearity (r^2-value) was more than 0.99, as determined by linear regression.

10.3.6 Condensation onto balloon and filling chamber

Loss of THC during experiments could partially be accounted for by incomplete evaporation and condensation onto parts of the Volcano vaporizer. Prolonged storage of the balloon at room temperature after vaporizing led to a steadily increasing loss of THC by condensation, up to the point that after 180 minutes almost no THC could be detected anymore in the vapor extract (figure 10.7). However, if the balloon was extracted within 5 minutes after vaporizing, less than 2% of the total dose was recovered as a precipitate from the inner surface of the balloon. However, condensation of THC onto the other parts of the Volcano setup was found to be of more significant importance. Visual inspection of the filling chamber shows the presence of a condensate, mainly on the inside of the filling chamber just above the liquid pad. Extraction of the filling chamber together with the valve, but excluding the liquid pad, showed that an average of 23.6 (± 14.1) % of the loaded THC had condensated onto these parts of the Volcano, and could therefore account for a large part of the nondelivered THC.

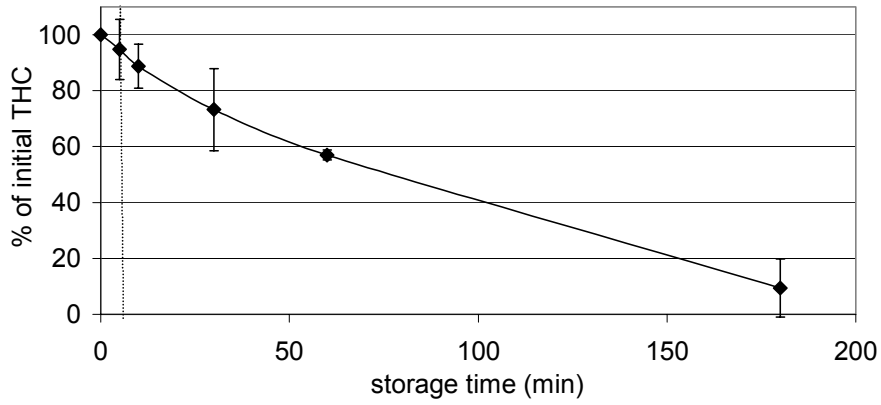


Figure 10.7: Amount of THC recovered from the balloon as result of prolonged storage time after vaporizing. Data are shown as mean values of three experiments, expressed as % of initially recovered THC. Errorbars indicate standard deviation. During this study all balloons were processed within 5 minutes after evaporation, which is indicated by the dotted line.

10.3.7 Clinical study and loss by exhalation

The clinical trial was finished without any serious complaints by the test subjects. Some mild complaints included irritation of the throat and lungs, and coughing. However, these effects were also observed during inhalation of placebo and therefore could be an effect of residual ethanol. The development of significant physiologic changes after inhalation of vaporized THC indicates that THC can be effectively administered by this route.
Interestingly, it was shown that a large proportion of inhaled THC was not absorbed by the lungs. The total amount of THC used for evaporation was 20 mg of THC for each subject (Rising dose of 2, 4, 6 and 8 mg resulting in a total sum of 20 mg). Taking into account the average delivery yield into the balloon of 53.9%, as found in this study, only an average of 10.8 mg of THC was totally available for inhalation from the balloon. The amount of THC recovered from exhaled breath ranged from 2.5 to 4.4 mg, which means that up to 30-40% of inhaled THC was not absorbed by the lungs. The variability of THC in exhaled breath (relative s.d. ± 5.4%) is comparable to the variability in delivery of THC by the Volcano. Taking this into account it could be concluded that absorption of THC by the lungs is probably very similar between different subjects.

10.4 Discussion and conclusion

The Volcano® vaporizer was validated for the efficient and reproducible delivery of delta-9-tetrahydrocannabinol (THC), and was found to be able to deliver an average amount of about 54% of the dose of THC (applied onto the liquid pad) into the balloon for inhalation. In a variety of studies using different types of smoking procedures [Manno, 1970; Fehr, 1972; Davis, 1984], THC recoveries from smoke have been found to range from 34% to 69%.

Because the plant material is not burnt in the Volcano, no significant harmful cancer causing combustion products are expected, and the noxious intake, when compared to smoking, is greatly reduced [Gieringer, 2001, 2004]. Therefore, when using the Volcano device for pulmonary administration of THC, a delivery is reached that is comparable to smoking, but without the presence of degradation products or harmful byproducts in significant amounts.
Loading the Volcano with Cannabis plant material or with pure THCA resulted in a residual amount of THCA in the vapor in the order of 5% relative to THC. Not much is known about biological effects or metabolism of THCA, and therefore the use of THCA as sample for intended clinical administration of pure THC should be avoided. Older studies at least indicate that THCA is not psychoactive in monkeys [Edery, 1972]. Although in our study cannabis plant material was used only for comparative reasons, it was clear that a variety of cannabinoids and other compounds such as terpenoids are present in the vapor.
With pure THC as the loading sample, temperature setting and balloon volume were optimized for a maximal and reproducible delivery of THC, without formation of detectable amounts of degradation products. Using the highest temperature setting together with a balloon volume of 8 L was found to yield optimal results. Balloon volumes over 8 L were not tested because of restraints in the clinical trial protocol.
The target temperature of the Volcano was found to be not completely accurate and stable. Possibly this is a contributing factor to the relative variability in the delivery of THC, which was about 15% at setting 9. However, this is reasonable when compared to the variability that has been previously found in smoking studies of cannabis plant material [Fehr, 1972]. Accuracy of temperature control therefore does not seem to be of crucial importance under these conditions, although a more accurate temperature control may result in an even lower variability in THC delivery.
In the range of 2 to 8 mg THC, the delivery was found to be linear with the amount of THC loaded. Prolonged storage of the balloon before inhalation resulted in an increasing loss of THC by condensation inside the balloon, and after 3 hours almost no THC could be recovered from the vapor in the balloon. However, if the content was extracted within 5 minutes after vaporization, not more than 2% of THC present in the balloon was lost. Vaporized THC was visible inside the balloon as a thin gray mist which was absent in placebo balloons, so during the clinical trial balloons had to be wrapped with a black plastic cover, in order to keep the study blinded.
During the clinical administration, it was found that about 35% of total THC was exhaled directly after inhalation and was therefore not absorbed by the lungs. When the efficiency of delivery during vaporizing and incomplete absorption by the lungs is considered, the final administered dose equaled about 6-8 mg of THC of the total amount of 20 mg loaded. The subjective effect upon the subjects seemed to be in accordance with such a dose as described in other papers [Abood, 1992; Leweke, 2002]. So it seems that a final uptake of 30-40% was reached (relative to loaded amount of THC), which is comparable to the efficiency commonly reached by smoking of cannabis.

It has been shown that the administration of THC by aerosol is capable of producing the full constellation of cannabinoid effects in mice. These effects were CB1-receptor mediated, as shown by the use of selective CB1 antagonists [Wilson, 2002], which confirms that the pulmonary administration of cannabinoids certainly has a clinical potential. Several studies have been performed using an aerosol for the administration of THC [Hartley, 1978; Lichtman, 2000; Wilson, 2002; Naef, 2004]. But because cannabinoids are almost completely insoluble in water, this requires the use of solubilizers that are to be inhaled together with THC, which frequently results in irritation of the lungs and coughing. Moreover, part of an administered aerosol can be swallowed and thereby administered orally, complicating the effect, kinetics and metabolism of the administered compound. This has already been shown for aerosol administration of radio-labeled isoproterenol [Lyons, 1973].
Using the Volcano vaporizer for administration seems to eliminate at least part of the problems associated with the use of an aerosol for the inhaled delivery of THC. It is likely that the Volcano also produces an aerosol, i.e. droplets of various sizes in a gas phase made up of vapor and air. However, in an artificial lung model the majority of vaporized THC could reach the deepest compartment (personal communication with Volcano manufacturer) indicating that the exhaust blown from the Volcano consists for a large part of the very finest droplets and vapor. Nonetheless, the composition of an aerosol is partially dependent on the ambient conditions such as humidity and presence of nuclei for condensation. So although our results were found to be reproducible with a relatively low variability, these factors must be taken into consideration for further development of the Volcano.
What is currently needed for optimal use of medicinal cannabinoids is a feasible, non-smoked, rapid-onset delivery system. With the Volcano a safe and effective cannabinoid delivery system seems to be available to patients. Although our current study has concentrated on the delivery of THC, it should be noted that other cannabinoids may also have a role to play for some indications. In several medical studies, the effect of THC or dronabinol alone could not match the effect of a total cannabis preparation, indicating there might be other active cannabinoids needed for a full range of effects [Williamson, 2000]. As an example, a combination of THC with CBD is now under clinical investigation for the treatment of chronic pain conditions [Notcutt, 2004]. The next step in the evaluation of the Volcano vaporizer should therefore include the study of mixtures of pure cannabinoids.

10.5 Acknowledgements

The authors would like to thank the manufacturer of the Volcano vaporizer, Storz & Bickel GmbH&Co., for providing the department of Pharmacognosy with the Volcano devices for this study. Bedrocan BV (The Netherlands) is acknowledged for providing us with medical grade cannabis plant materials. Farmalyse BV (Zaandam, The Netherlands) was involved in the development of the procedure to produce clinical grade cannabinoid samples of THC and THCA.

Concluding remarks and perspectives

Although a huge number of scientific papers have been published on cannabis over the past decades, many aspects still remain unclear. The world today is full of cannabis myth and mystery. The work described in this PhD thesis is a contribution to solve some of these mysteries. In general, the results of this thesis have played a supporting role in the introduction, and, possibly more important, the acceptance of medicinal use of cannabis in the Netherlands. It has become a consistent source of information on the cannabis plant and its main constituents, the cannabinoids, and the obtained results cover a wide range of aspects that are important for further research on medicinal cannabis. For example, new cannabinoid standards have become available to the analytical and clinical researcher. And the use of the Volcano vaporizer can now be advised to patients that currently could only treat their symptoms by smoking of cannabis. Moreover, it further opens up the possibilities to perform inhaled studies without smoking. Also it has become clearer in what situations cannabis tea, with its relatively low potency, can be useful for medical users. In short, it has been possible to bring science and patients a bit closer together.

A major argument of health authorities against the use of herbal cannabis as a medicine is that it is a highly variable product with respect to composition and microbiological safety. However, the experience of the Dutch Office of Medicinal Cannabis has shown this doesn't have to be the case if a serious effort is made to address these problems. After all, high-grade medicinal cannabis has been available in The Netherlands for several years now. And by sharing knowledge and applying the same analytical methods, a growing group of Dutch academics as well as industrial partners is currently working together in order to make medicinal cannabis a success story. It is obvious that the shared use of reference standards and analytical procedures (as partially developed in this thesis) by different groups facilitates the comparison of analytical results. As a result of the collaborative work, we now have a better understanding of the cannabis plant, its main active components, i.e. the cannabinoids, and its administration forms. Hopefully, in my opinion, the Dutch situation can act as a good example on how to get out of the cannabis controversy that has already lasted much too long.

The main challenges for the near future are standardization of cannabis-based medicines, obtaining clinical proof of its claimed activities, and improving the acceptance among authorities and health-professionals. It is clear that, in time, cannabis-based medicines should be standardized, efficacious and safe preparations, as much as any other approved medicine. And this should be demonstrated in statistically significant randomized clinical trials, acceptable to regulatory bodies in various countries and adhering to the modern scientific method. However, the continuing fear of potential psycho-active effects of cannabis frequently interferes with such studies: the largest clinical trial ever conducted with a cannabis preparation (on multiple sclerosis), with over 600 patients [Zajicek, 2005], apparently failed because of under-dosing the amount of THC. So maybe it is time to stop focusing on the effects of low-dose oral administration of pure THC, when most beneficial effects are claimed by patients based on the smoking of significant amounts of herbal cannabis. There should be renewed attention for different administration forms such as tea, inhalation, and maybe even cookies, even when these administration forms have no direct value for pharmaceutical development. After all, an open mind is an important part of successful research, and the research on cannabis is certainly no exception.

Fortunately, attitudes worldwide seem to be slowly changing in the right direction. To show an important example: until recently, scientists in the U.S. could only turn to a single government agency (National Institute for Drug Abuse, NIDA) to obtain cannabis materials for their studies. NIDA's frequent denial to supply the requested cannabis, and the low quality of the materials led a group of frustrated scientists and lobbyists to file a lawsuit against the authorities [Pearson, 2004]. Their demand for a more scientific approach to cannabis research have so far resulted in a series of court rulings that were supportive of this idea. Simultaneously, in several other Western countries the restrictions that hinder access to medicinal cannabis are slowly becoming less stringent and even recreational cannabis use is occasionally decriminalized. Italy is in the process of changing the law in order to allow the import of Dutch medicinal cannabis. Sometimes a bit more pressure is needed from lobbyists or patients: recent court rulings in Germany have opened the way for patients to demand cannabis-based medicines, if alternative treatments have failed. It seems that for many skeptics it's becoming clear that the evil cannabis plant may have some benefits after all.

So what is necessary now is that scientists simply do their jobs, without the restrictions that are currently holding them back. Exciting modern techniques such as NMR-spectroscopy, Principal Component Analysis, mass-detection and various chromatographic improvements make it possible to isolate, identify and study any desired constituent of the cannabis plant. Lifting the restrictions that are currently present would be like opening a scientific floodgate; it would be possible to conduct research that should have been done a long time ago, if only someone was allowed to do it. After all, cannabinoids have a unique structure that can not be found anywhere else in nature, and many of them are already known to have at least some biologically activity. Initially a focus is needed on quantitative analysis using validated methods, which requires high quality reference standards of a broad range of cannabis constituents, such as those described in this thesis. The results should finally be evaluated by a variety of laboratories in order to develop a generally accepted method for the analysis of cannabis preparations. In fact, we should simply go back to generally accepted quality control assays for cannabis preparations, as they existed in Pharmacopoeia before introduction of the Single Convention on Narcotic Drugs of 1961. With such methods at hand, we should study medicinal cannabis in the forms it is used by real patients, out in the real world, with a broad scope on why some cannabis preparations have certain activities, while others do not. After all, the renewed interest in medicinal cannabis is largely due to the strong and continuous lobby of these patients, especially in countries like the U.S. and U.K. These cannabis pioneers deserve to be heard. Putting synthetic THC in capsules of sesame oil (Marinol®), thereby increasing the price per dose several orders of magnitude, may have more to do with good marketing than with scientific proof.

Now that the significance of the human endocannabinoid system becomes increasingly clear, cannabinoids should have a brighter future. After decades of severe legal restrictions on cannabis research, herbal cannabis and its constituents, the natural cannabinoids, are again in focus for their medicinal properties. A large number of cannabinoid-based medicines are expected to enter the market in the coming years, particularly in the field of cannabinoid receptor-agonists and antagonists such as Rimonabant® and ajulemic acid (CT-3). But even without considering these pharmaceutical developments, research on the medicinal use of cannabis is important simply because cannabis is already used for self-medication by an enormous number of people worldwide, often risking punishments as severe as life imprisonment. Therefore, I think that a future without cannabis-based medicine is very unlikely.

Summary

Cannabis (*Cannabis sativa* L.) has a long history as a recreational drug and as part of traditional medicine in many cultures of the world. But by no means is the medicinal use of cannabis a thing of the past. Nowadays, a large number of people worldwide claim that the use of cannabis ameliorates the symptoms of their medical condition, and cannabis is used medically by patients suffering from diseases varying from cancer and HIV/AIDS to multiple sclerosis and chronic pain. At least since the 19th century, the effect of cannabis on society has been a topic of discussion. However, somewhere in recent history cannabis definitely ended up on the wrong side of the law and as a result, all discussion on the medicinal use of this plant has become extremely complicated. And although some people were willing to challenge the law and end up in jail for continuing the use of their valued medicine, it was unfortunate for them that the medicinal effects of cannabis were not scientifically proven.

But now, after decades of focusing on the negative aspects of cannabis use on health and society, scientists are slowly discovering that the medicinal effects may indeed exist. The significance of the medicinal use of cannabis is becoming increasingly clear, mainly as a result of two relatively recent discoveries: first, the cannabinoid receptors, and second, the existence of endogenous cannabis-like compounds, the endocannabinoids. As a result, we are slowly learning that our own human body is controlling some of its vital functions by using of signaling compounds that have a lot in common with the constituents of the cannabis plant. In recent years, the development of new medicine based on pure constituents of the cannabis plant, or their synthetic analogs and derivatives has become a major target for pharmaceutical companies. It seems the medicinal users were not so wrong, after all.

It may be expected that well-conducted research should be able to make the distinction between the good and the bad uses of cannabis. However, more than in any other field of research, the cannabis researcher is restricted by tough international legislation. Studying cannabis is bound to invite trouble on several levels: practical, legal and even political. Consequently, even though the scientific fields of synthetic cannabinoids and the endocannabinoid system are rapidly expanding, the field of herbal cannabis research still remains one of the most tightly restricted, and therefore it is essentially censored in some ways. Cannabis researchers sometimes proudly state that almost no plant has been studied as much as the cannabis plant, as more than 10,000 papers have been published on the subject. But in contrast, it is amazing how much is still not understood about the effects and dangers of cannabis use.

In fact, the question may arise if the research community so far has been able to create a realistic image of the medicinal potential of cannabis. Because what in fact is really known about the cannabis plant? The problem is already apparent with the plant material itself: because of a prohibition on the breeding, possession or transport of the plant, researchers worldwide virtually have no access to fresh plant materials. Consequently, a large part of plant material used for cannabis research comes from customs seizures, or from governmental agencies that lack the skills, knowledge, or the will to produce high-quality plant materials. Important information, such as the type of cannabis (cultivar), breeding and storage conditions, chemical composition and age of the plant materials are often unknown. Over time this has resulted in an extreme simplification of the complex cannabis plant. In general, nowadays, cannabis is simply called cannabis, with the psychoactive tetrahydrocannabinol (THC) referred to as its (only) active constituent. It seems to be virtually forgotten that more than 700

different varieties of cannabis have been described and that at least 66 cannabinoids are known. In fact, the single parameter usually reported in (scientific) publications is the THC content of the plant material, a parameter frequently determined by the supplier but not independently checked by the researchers themselves. As a result, the potency of the cannabis plant is commonly equaled to its THC content only, even in clinical trials. The media further aggravate this situation by the way they report on cannabis as a psychoactive drug.

Even though an increasing number of studies indicate that many activities cannot be explained by THC receptor binding alone, and that there have to be multiple active constituents present in the cannabis plant, cannabis research in general remains stubbornly focused on THC alone, thereby obscuring the possible effects of other cannabinoids present. A steady stream of discussions can be found in medical journals, discussing the need to continue research on medicinal cannabis, while a growing number of THC based medicines are developed. But even decades after the discovery of the (pharmacologically) most important constituents, the cannabinoids, only a handful of them have been made available as standardized reference compounds for scientific research. This means that most of the cannabinoids never have been tested for their biological activity. Clearly, there is a need to look at cannabis again with a fresh perspective, and to fill the gaps that exist in the current knowledge on cannabis as a medicine.

This PhD thesis aims to be a helpful guidebook for basic research on cannabis. Moreover, it contributes to the investigation of cannabis on the whole, and will hopefully spark interest in its neglected constituents. This thesis is written from an analytical and phytochemical point of view, and deals primarily with biochemical aspects of the cannabis plant and its constituents. Since the cannabinoids are widely considered to be the most important (but not the only!) active components of the cannabis plant, the work has concentrated on them. And since THC is the best studied of all the cannabinoids, this compound has been the focus of several chapters in this thesis. However, the main purpose of this thesis is to bring the cannabis plant, as a whole, back into focus.

A thorough overview of the current scientific understanding of cannabis as a medicinal plant has been given in **chapter 1**. Obviously, sound research on cannabis can only be performed if a reliable and continuous source of plant material is made available. Research projects typically take several years to complete, and the object of study should at least be available for such a period of time. Ideally, the composition of the plant material should be stable and be known in great detail. Fortunately, since 2003 such plant material has been available in the Netherlands, where medicinal grade cannabis is provided on prescription through pharmacies. Growing, processing and packaging of the plant material are performed according to pharmaceutical standards and are supervised by the official Office of Medicinal Cannabis (OMC). The quality is guaranteed through regular testing by certified laboratories, and the cannabinoid composition is guaranteed within a narrow range. However, in the Netherlands a tolerated illicit cannabis market exists in the form of so-called 'coffeeshops', which offers a wide variety of cannabis to the general public as well as to medicinal users of cannabis. Although this facilitates studying the medicinal aspects of cannabis, it is also confusing because the distinction between recreational and medicinal use can not always be clearly made. Ever since cannabis became

available in the pharmacies, many patients started to compare the price and quality of OMC- and coffeeshop-cannabis. As a result, the public debate on the success and necessity of the OMC program has been based more on personal experiences than on scientific data. In 2006, the leading opinion of consumers was that OMC cannabis is more expensive, without a clear difference in the quality.

In **chapter 2** the current status with respect to medicinal cannabis in the Netherlands is discussed in detail. It further describes a study that was performed in order to test for differences in quality between the official and the illicit sources of cannabis for medicinal use. Cannabis samples obtained from 11 randomly selected coffeeshops in different areas of the Netherlands were compared to the 2 different types of medicinal grade cannabis obtained from the OMC. The following parameters were tested by validated methods that have been described in the Dutch monograph for medicinal cannabis: THC content and cannabinoid profile, water content, accuracy of obtained weight, microbiological contamination and price. When the cost of the cannabis was expressed in Euro per 100 mg of its main component THC, it was found that the pharmacy was about 1.5 - 2 times more expensive than the average coffeeshop. The THC content of all samples was found to be in the relatively narrow range of 11.7-19.1% (of dry weight plant material). No obvious differences were found in either cannabinoid profile or water content of the samples. Many coffeeshop samples were found to contain significantly less weight than requested during purchase, and all were contaminated with unacceptable high levels of bacteria and/or fungi, according to pharmaceutical standards. In one of the samples at least 3 different types of harmful microbes could be identified. Each batch of pharmacy cannabis is always fully tested on the absence of such contaminations. Although the number of samples tested was limited, the obtained results show that medicinal cannabis offered through the pharmacies is more reliable and safer for the health of medical users of cannabis.

A major obstacle in the acceptance of medicinal cannabis by medical professionals is in the 'proof' of its effectiveness, meaning that its medicinal value has to be established by quantitative analytical and clinical research. This implies that the major components of the cannabis plant must be available to the researcher as reference standards, i.e.: in high purity and in precisely quantified administration forms. However, currently only a few of the major cannabinoids are commercially available. Many legal and practical obstacles exist for ordering these compounds, because of import-export regulations.

Consequently, a major goal of this thesis was to certify a large-scale supply of cannabinoid standards, which could be used as reference standards for in-house as well as for cooperative studies. In **chapter 3** a simple method is presented for the preparative isolation of seven major cannabinoids from *Cannabis sativa* plant material. Separation was performed by centrifugal partition chromatography, a technique that permits large scale preparative isolation. Using only two different solvent systems, it was possible to obtain purified samples of the neutral cannabinoids; THC, cannabidiol (CBD), cannabinol (CBN), cannabigerol (CBG), as well as the acidic cannabinoids tetrahydrocannabinolic acid (THCA), cannabigerolic acid (CBGA) and cannabidiolic acid (CBDA). Two different cannabis varieties were used for the isolation. Because cannabinoids are produced by plant metabolism in the form of carboxylic acids (acidic cannabinoids), the levels of neutral cannabinoids found in the plant are usually low. By carefully controlled heating of the extract an efficient conversion of acids to neutrals could be achieved, resulting in efficient isolation of the corresponding neutral cannabinoids. All isolated cannabinoids were shown to be more than 90-95% pure by gas chromatography. This method makes

acidic cannabinoids available for the first time on a large scale for biological testing. The method described in this report can also be used to isolate additional cannabinoids from other types of cannabis plant material.

High quality reference standards must be pure and quantified. Because of their oily nature, quantification of cannabinoids is not easily achieved by gravimetric method (i.e. by weighing). In **chapter 4** a ^{1}H-NMR method was therefore developed for the quantitative analysis of pure cannabinoids in ethanolic solution. The same method was also found to be suitable for direct quantification of cannabinoids present in *Cannabis sativa* plant material without the need for chromatographic purification. The study was performed by the analysis of singlets in the range of δ 4.0-7.0 ppm in the ^{1}H-NMR spectrum, where distinguishable signals of each cannabinoid are present. Because the signal response in quantitative NMR is directly proportional with the amount of compound present in the sample, the concentration of a cannabinoid can be determined by direct comparison to the known concentration of an internal standard. Quantification was performed by calculating the relative ratio of the peak area of selected proton signals of the target compounds to the known amount of the internal standard, anthracene. For this method no reference compounds are needed. It allows rapid and simple quantification of cannabinoids with a final analysis-time of only 5 minutes without the need for a pre-purification step. The quantification method was validated over a range of concentrations and found to be very reliable.

In general, the major cannabinoids important for the biological effects of cannabis are considered to be THC, CBD, CBN, CBG and CBC, as well as their carboxylic acids. They can be found in cannabis plant material in varying ratios and concentrations, depending on plant variety, age, breeding conditions and storage. Cannabinolic acid (CBNA) is one of these natural constituent of the cannabis plant, particularly of aged plant materials, and it is therefore a possible candidate for some of the biological or medicinal activities of cannabis. Under degradative conditions, CBNA is formed from THCA, a major constituent of the cannabis plant. However, CBNA could not be isolated from our plant materials, because its concentration and amount in selected plant materials were very low. Synthesis of CBNA must therefore be considered as an alternative to isolation from plant material. However, no method for synthesis has been published so far.

In **chapter 5** we present the semi-synthesis of CBNA from THCA by aromatization using selenium dioxide mixed with trimethylsilylphosphate as catalyst in chloroform. Like all acidic cannabinoids, CBNA is relatively unstable because it easily loses its carboxylic acid moiety to form CBN. Therefore careful optimization of the reaction parameters was needed. Final preparative purification on a milligram scale was achieved by using centrifugal partition chromatography and the final product had a purity of more than 96%. Although the overall yield of the procedure was only 10%, the method is easy to scale up and the used chemicals are inexpensive. The developed method enables the production of CBNA on a preparative scale, making it available for quantitative analysis and for further studies of its biological activity. Spectroscopic data of CBNA such as ^{1}H-NMR-, UV- and IR-spectrum, as well as chromatographic data are presented as useful reference for further research on CBNA.

After a variety of highly pure and quantified cannabinoid standards thus became available, we proceeded to determine their chromatographic as well as spectroscopic properties under standardized

conditions. **Chapter 6** provides a synoptic overview of the chromatographic and spectroscopic properties of 16 major cannabinoids present in *Cannabis sativa* plant material, and of 2 human metabolites of THC. Cannabinoid standards were obtained through our own methods as well as from commercial sources. Spectroscopic analyses included UV absorbance, infrared-spectral analysis, (GC-) mass spectrometry and fluorescent properties of the cannabinoids. Most of this data is also available from other literature but scattered over a large amount of scientific papers from the last decades. In our study analyses were carried out under standardized conditions so spectroscopic data can be directly compared. Different methods for the analysis of cannabis preparations were used and are discussed for their usefulness in the identification and determination of separate cannabinoids. HPLC, GC and TLC retention-index values of the cannabinoids are presented.

The availability of cannabinoid reference standards, and of chromatographic and spectroscopic data are important conditions for cannabis research. Simultaneously, it is important to develop quantitative methods for the analysis of cannabis plant materials, as well as other preparations. However, most of the methods described in the scientific literature are not suitable for the analysis of the acidic cannabinoids, such as THCA, the carboxylic acid precursor of THC. Other methods have not been properly validated for their use in pharmaceutical research. In fact, currently no simple and fully validated method exists for the determination of the authentic cannabinoid content of cannabis plant specimens. For this purpose, in **chapter 7** an HPLC method was developed for the analysis of major cannabinoids present in high-potency (drug-type) cannabis plants. The method was fully validated according to pharmaceutical (ICH) guidelines using our pure cannabinoid standards. HPLC analysis was complemented with a secondary analysis by gas chromatography, which made it possible to quantitatively analyze the tested cannabinoids over a wide range of concentrations. Finally, the application of the method was tested for the quantification of cannabinoids present in cannabis plant samples. Currently, the validated method is part of a monograph routinely used for the analysis of the medicinal grade cannabis provided through pharmacies in the Netherlands.

The cannabis plant is one of the oldest known medicinal plants, which is reflected in the large number of administration forms that have been described. However, little is known about most of these administration forms. Although smoking of cannabis is by far the most common way of consumption, a significant number of medicinal users prefer to consume it in the form of a 'tea'. However, not much is known about how the composition of the tea is influenced by the different ways of preparation, handling and storage. Therefore the parameters involved in tea preparation were systematically studied in **chapter 8**. We used the high-grade medicinal cannabis available in Dutch pharmacies to determine the cannabinoid composition of tea under standardized and quantitative conditions. Experimental conditions were systematically varied in order to mimic the possible variations made by medicinal users. During analysis there was a specific focus on the cannabinoids THC and its acidic precursor, THCA. The obtained results provide a clear quantitative understanding of the physicochemical aspects of cannabis tea preparation and they are believed to contribute to a better appreciation of this ill-understood mode of cannabis administration.

In general, the easiest way of administering a medicine is orally, in the form of tablets or liquids. However, for the cannabinoids this route is not easily available because of their very low water-solubility. In particular the low aqueous solubility of THC is a serious obstacle for the development of

efficient administration forms of this widely studied compound. In **chapter 9**, we studied the use of cyclodextrins (CDs) for improving the aqueous solubility and the stability of THC and other cannabinoids. The aqueous solubility of THC was tested in the presence of α-, β- and γ-CD, and randomly methylated β-CD (RAMEB). It was found that only RAMEB was able to increase the aqueous solubility of THC to a significant level. A THC concentration of about 14 mg/ml was reached by using a 24% (187mM) RAMEB solution, which means an increase in solubility of 4 orders of magnitude. The resulting THC/RAMEB complex was investigated through phase-solubility analysis, complemented by ^{1}H-NMR, NOESY- and UV-studies in order to obtain details on the stoichiometry, geometry and thermodynamics of the complexation. The binding ratio of THC to CD was found to be 2:1, with the second THC molecule bound by non-inclusion interactions. Based on the obtained results a model for the complex structure is presented. The complex was found to be stable for at least eight weeks, when stored under laboratory room conditions. Results show that complexation with RAMEB seems to be promising for the development of water-based formulations of THC as well as other cannabinoids.

Smoking is the most popular way to use cannabis, even though inhalation of toxic pyrolytic compounds can pose a serious hazard to health. The reason is because inhaled administration of the bioactive components of cannabis is very efficient and fast-acting. Previous studies have suggested that the vaporizing of cannabis samples presents several advantages over smoking. Therefore we evaluated in **chapter 10** the use of a vaporizer device that can evaporate the active components of the cannabis plant for inhalation. In this study a vaporizer of the brand 'Volcano' was evaluated as a novel method for the clinical administration of THC. By changing parameters such as temperature setting, type and dose of evaporation sample, and balloon volume, the vaporization of THC was systematically improved to its maximum yield, while preventing the formation of degradation products. Factors that resulted in loss of THC were also evaluated. The reliability of the vaporizer was shown by determining the inter-device reproducibility between 4 Volcano devices. Finally, the results of this study were used in a clinical study for the administration of THC by vaporizing. Our results indicate that the Volcano is a reliable and convenient device for the administration of THC by inhalation.

Samenvatting

Medicijnen uit planten

Farmacognosie, het vakgebied waarin dit proefschrift tot stand is gekomen, is de studie van medicijnen afkomstig van natuurlijke bronnen, en dan met name uit planten. Hedendaagse farmacognosie heeft als voornaamste doel om nieuwe medicinale stoffen op te sporen in natuurlijke bronnen (planten, dierlijke producten, mineralen) of om deze te herkennen in traditionele geneeskunst. Door de aktieve bestanddelen te identificeren, isoleren en vervolgens farmacologisch en klinisch te testen, moeten deze stoffen uiteindelijk leiden tot de ontwikkeling van nieuwe medicijnen die voldoen aan de eisen van de moderne tijd.

Planten als bron van nieuwe medicijnen zijn altijd zeer belangrijk geweest. De Wereldgezondheidsorganisatie WHO schat dat 80% van de bevolking van ontwikkelingslanden voor zijn primaire gezondheidszorg afhankelijk is van traditionele geneeskunst, die voornamelijk gebaseerd is op het gebruik van medicinale planten. Wereldwijd komt dat neer op 3.5 tot 4 miljard mensen, wat wel duidelijk maakt hoe relatief de term 'alternatieve geneeskunst' is, wanneer hij wordt gebruikt voor kruidengeneeskunde. Helaas passen planten of hun extracten, vanwege hun aard, niet gemakkelijk thuis is de moderne Westerse geneeskunde. Planten bevatten een grote verscheidenheid aan bestanddelen, waarbij in veel gevallen niet duidelijk is welke daarvan eigenlijk de medicinaal aktieve stoffen zijn. Bovendien is de exacte samenstelling van een plant vaak afhankelijk van bijvoorbeeld zijn groeiomstandigheden, waardoor er verschillen kunnen optreden tussen diverse partijen van dezelfde plant. Dit alles maakt het moeilijk om een gestandaardiseerd en betrouwbaar medicijn te bereiden uit plantenmateriaal. Ook aan het patenteren van planten en planten-stoffen kleven grote bezwaren, waardoor farmaceutische bedrijven moeite kunnen hebben hun enorme investeringen in de speurtocht naar medicijnen terug te verdienen. Alles bij elkaar maakt dit dat planten geen populair onderwerp zijn voor het ontwikkelen van nieuwe medicijnen.

Ondanks deze bezwaren is toch een aanzienlijk deel van onze hedendaagse medicijnen direct of indirect afkomstig uit plantaardige bron. Het meest succesvolle voorbeeld aller tijden is wellicht aspirine. Al eeuwen geleden kauwde men op een stuk wilgenbast (*Salix alba*) tegen hoofdpijn. Momenteel wordt het bestanddeel verantwoordelijk voor dit effect echter synthetisch (door middel van scheikundige processen) geproduceerd onder de naam aspirine. Het is slechts een van de vele belangrijke medicijnen met een plant als basis. Andere voorbeelden zijn kinine (anti-malaria), taxol (anti-tumor), reserpine (hoge bloeddruk) en galanthamine (bij Alzheimer).

Sommige planten worden echter eerst bekend om hun negatieve effecten op de mens, voordat hun medicinale kwaliteiten worden herkend. De opiumplant (*Papaver somniferum*) werd in de 18e eeuw gezien als een dermate gevaarlijk middel voor de samenleving dat de Chinezen er zelfs twee oorlogen om vochten met de Britten, die het spul in grote hoeveelheden verhandelden in China. Maar het was ook duidelijk dat er in opium aktieve bestanddelen voorkwamen die iets deden met het menselijk lichaam. Een interessant onderwerp voor wetenschappelijk onderzoek binnen de farmacognosie dus. Die bestanddelen bleken de opioïden te zijn, waarvan morfine en codeïne het meest bekend zijn. Als pijnstiller en verdovingsmiddel zijn deze stoffen onmisbaar voor de moderne geneeskunde, terwijl

opiumgebruik uiteindelijk verboden is geworden. Door een duidelijk, wetenschappelijk onderbouwd onderscheid te maken tussen recreatief en medicinaal gebruik is het blijkbaar mogelijk om potentieel gevaarlijke stoffen op een nuttige manier te kunnen gebruiken.

Cannabis als probleem

De plant *Cannabis sativa*, ook wel kortweg cannabis, is beroemd en berucht: vrijwel iedereen kent de term THC, wat staat voor tetrahydro-cannabinol, de stof in de cannabisplant waar je 'high' of 'stoned' van wordt. Daarnaast wordt cannabis verantwoordelijk gehouden voor een eindeloze lijst aan (al dan niet bewezen) negatieve effecten zoals hartkloppingen, hallucinaties, paniekaanvallen, psychose en hersenbeschadigingen. Al vanaf de jaren 1960 is er systematisch gewezen op de gevaren van dit duivelse kruid.

Het is dan ook niet verwonderlijk dat het medicinaal gebruik van cannabis doorgaans leidt tot verhitte discussies. In de ergste gevallen vindt die discussie plaats in de rechtbank, waar soms een straf dreigt die op kan lopen tot levenslange gevangenisstraf, zoals in sommige delen van de Verenigde Staten. Want hoewel het een lange geschiedenis heeft als vezelplant (hennep) en als voedselbron (hennepzaad), wordt cannabis tegenwoordig vooral gebruikt als psycho-aktieve drug, en momenteel is het, na caffeïne (koffie) en nicotine (tabak), de meest gebruikte *stimulant* ter wereld. Het is met afstand de meest populaire illegale drug en schattingen geven aan dat wereldwijd enkele honderden miljoenen mensen regelmatig cannabis gebruiken. In de meeste landen wordt cannabisgebruik dan ook gezien als een bedreiging voor de volksgezondheid of de openbare orde, en is het bezit of gebruik ervan streng verboden. Het onderscheid tussen medicinaal en recreatief gebruik van cannabis bestaat in de meeste landen simpelweg niet, en medicinaal gebruik wordt vaak gezien als een excuus om aan cannabis te komen.

Toch is het gebruik van cannabis als medicijn letterlijk zo oud als onze beschaving. Zo staat het bijvoorbeeld al beschreven in duizenden jaren oude Chinese geschriften over medicinale planten. En vrij recent nog, rond 1930, waren er in Europa zeker 28 verschillende medicijnen beschikbaar met cannabis als ingrediënt. In de jaren daarna ging het echter snel bergafwaarts met de populariteit van cannabis, voornamelijk door de heffing van hoge accijnzen, en de opkomst van nieuwere medicijnen die makkelijker in het gebruik zijn en de rol van cannabis konden overnemen.

Hoewel de reactie van hedendaagse politici op cannabis-gebruik vaak op zijn minst overdreven overkomt, heeft dit een lange traditie. Zo was de Amerikaanse president Nixon ervan overtuigd dat cannabis een geheim wapen was van de communisten, verspreid door Joden, en bedoeld om de Westerse samenleving te ontwrichten. Sinds 1961 bestaat er internationale wetgeving (de United Nations Single Convention on Narcotic Drugs) die het gebruik van cannabis wereldwijd onwettig maakt, en het onderzoek ernaar aan zeer strenge eisen bind. Het gevolg is dat in de afgelopen decennia nauwelijks sprake is geweest van vrij en ongebonden onderzoek naar de effecten van cannabis gebruik. Het onderzoek dat wel is uitgevoerd, wordt (vaak uit noodzaak) gekenmerkt door kortzichtigheid en er is een sterke focus op de vermeende negatieve effecten van cannabis. Iedere stap van medisch onderzoek moet apart worden goedgekeurd en worden getoetst aan de strenge regelgeving. Het resultaat is een gefragmenteerd en zeer incompleet beeld van de potentie van medicinale cannabis.

Cannabis als medicijn

In de laatste jaren lijken de kansen voor het medicinaal gebruik van cannabis echter te keren. Onder toenemende druk van patiënten, en door het langzaam vrijkomen van wetenschappelijk bewijs voor de werkzaamheid van cannabis als medicijn, vinden geleidelijk veranderingen plaats in het cannabisbeleid. Deze variëren van het decriminaliseren van (medicinaal) cannabis gebruik in het Verenigd Koninkrijk en Zwitserland, tot serieuze pogingen om patiënten toegang te geven tot betrouwbare medicinale cannabisproducten, zoals in Spanje, Canada en ook in Nederland.

In de afgelopen 10 jaar zijn enkele zeer significante ontdekkingen gedaan op het gebied van de fysiologische effecten van cannabisstoffen. In de hersenen en het afweersysteem zijn namelijk de plekken ontdekt waar die stoffen hun effect uitoefenen (de receptoren). Vervolgens is gebleken dat ons lichaam zelf stoffen maakt die lijken op de belangrijkste stoffen (cannabinoiden) uit de plant. Deze 'endogene cannabinoiden' (endo-cannabinoiden) reguleren allerlei belangrijke lichaamsprocessen. Bij allerlei medische aandoeningen zijn juist deze processen verstoord, waardoor langzaamaan duidelijk begint te worden waarom cannabinoid-achtige stoffen een positief effect kunnen hebben bij die aandoeningen. Het is dan ook onmogelijk om heden ten dage nog te beweren dat je van cannabis slechts 'high' wordt.

Gebaseerd op deze recent verworven kennis zullen de komende jaren diverse nieuwe medicijnen worden geïntroduceerd die gebaseerd zijn op de effecten van cannabis en cannabinoiden. Het meest significante is wellicht Rimonabant, een middel tegen overgewicht, dat gebaseerd is op het feit dat cannabis-consumptie leidt tot een hevig hongergevoel. Rimonabant is ontwikkeld om juist het tegenovergestelde te veroorzaken: het wegnemen van de hongerprikkel. Een ander middel, ajuleminezuur, lijkt heel sterk op THC en heeft een sterke pijnstillende en ontstekingsremmende werking, maar zonder het psychotrope effect van THC. In tegenstelling tot vele andere potente pijnstillers heeft dit middel geen al te gevaarlijke bijwerkingen.

Al met al leiden deze ontwikkelingen langzaam tot een klimaat waarin het medicinaal gebruik van cannabis weer bespreekbaar wordt. Net zoals in het geval van opium en het daaruit verkregen morfine zou cannabis als bron van problemen wel eens kunnen opbloeien tot bron van belangrijke nieuwe geneesmiddelen. Degelijk wetenschappelijk onderzoek zal daarom moeten uitwijzen onder welke omstandigheden het verantwoord is om medicinale cannabis toe te staan, en hoe de aktieve bestanddelen het best kunnen worden benut.

Dit proefschrift

Nederland is het eerste land ter wereld dat cannabis plant materiaal beschikbaar heeft gesteld als een medicijn via de apotheek. Sinds september 2003 is cannabis van farmaceutische kwaliteit op recept verkrijgbaar voor bepaalde patiënten. Het Bureau voor Medicinale Cannabis (BMC, onderdeel van het Ministerie van VWS) zorgt er daarbij voor dat de benodigde cannabis wordt geproduceerd, getest op kwaliteit, en gedistribueerd naar de apotheken. (Huis)artsen kunnen cannabis voorschrijven voor diverse ernstige aandoeningen, waaronder multiple sclerose en chronische pijn, maar in principe wordt dit alleen gedaan nadat andere, meer gangbare medicatie al is voorgeschreven. In feite is cannabis

daarmee een laatste-keus middel indien andere middelen onvoldoende blijken te werken. Toch zijn er naar schatting enkele duizenden potentiële gebruikers van medicinale cannabis in Nederland aanwezig. Het spreekt voor zich dat de introductie van medicinale cannabis ook de verplichting schept om onderzoek ernaar aan te moedigen. En toevallig was het precies in die periode dat ik besloot om aan een promotieonderzoek te beginnen.

Dit proefschrift is geschreven vanuit een analytisch en fyto-chemisch oogpunt: het houdt zich dus bezig met de biochemische aspecten van medicinale cannabis, ofwel met zijn inhoudsstoffen. Wanneer er gesproken wordt over cannabis, zowel voor recreatief als medicinaal gebruik, dan wordt doorgaans verwezen naar de gedroogde bloemen van de vrouwelijke plant, ook wel bekend als 'wiet'. Dit is namelijk het meest potente deel van de cannabisplant, met het hoogste gehalte aan aktieve bestanddelen. De gedroogde hars afkomstig van deze bloemen wordt weer aangeduid met 'hash'. Deze hars is de bron van de belangrijkste bio-aktieve bestanddelen van de cannabis plant, de cannabinoiden. Ze hebben een unieke chemische structuur en worden in geen enkele andere plant aangetroffen. Deze cannabinoiden zijn het middelpunt van dit promotieonderzoek.

Om te beginnen wordt in **hoofdstuk 1** een uitgebreid overzicht gegeven van alles wat te maken heeft met cannabis als medicijn; van geschiedenis tot chemische aspecten en toekomstperspectief. Hieruit wordt duidelijk dat cannabis wellicht een enorme potentie heeft als bron van nieuwe medicijnen, maar dat de manier waarop het negatieve aspect van cannabis overheerst, nog steeds een enorm obstakel is om op een wetenschappelijk verantwoorde manier, onpartijdig onderzoek te verrichten.

Het werk beschreven in dit proefschrift is uitgevoerd in Nederland, dat een zeer bekende traditie heeft in het accepteren van cannabis als recreatief middel (koffieshops!). Dit maakt het bestuderen van de medicinale aspecten van cannabis een stuk makkelijker, maar tegelijkertijd werkt het ook verwarrend, aangezien het onderscheid tussen recreatief en medicinaal gebruik daardoor niet altijd even duidelijk is. In **hoofdstuk 2** wordt door middel van een vergelijkend warenonderzoek getoond hoe een verschil kan worden gemaakt tussen medicinale en recreatieve cannabis op basis van de kwaliteit, en waarom een gereguleerde bron van betrouwbare cannabis een voorwaarde is voor verdere farmaceutische ontwikkeling. Uit de resultaten blijkt dat de strenge eisen waaraan de Nederlandse medicinale cannabis moet voldoen weliswaar leiden tot een hogere prijs (per gram), maar dat daardoor een produkt kan worden gegarandeerd van betrouwbare samenstelling en constante kwaliteit.

Zoals in elke plant zijn ook in cannabis een grote diversiteit aan bestanddelen aanwezig. Daardoor is het een moeilijke klus om te bepalen welke van deze stoffen verantwoordelijk zijn voor de verschillende medicinale effecten die aan cannabis worden toegeschreven. Een eerste voorwaarde bij het bestuderen van iets zo complex als een plant is daarom het begrijpen van de samenstelling. Dit moet gebeuren door middel van betrouwbare, analytische methodes, die niet alleen aangeven *welke* stoffen aanwezig zijn, maar die bovendien ook iets zeggen over de precieze *hoeveelheid*. Met andere woorden, deze methoden zijn kwantitatief. Voor dergelijke methoden zijn de te bestuderen stoffen nodig in zuivere vorm, die bij de analyse dienen als vergelijkingsmateriaal. De belangrijkste stoffen

voor dit onderzoek, de cannabinoiden, zijn echter niet of nauwelijks te koop of op een andere wijze te verkrijgen. In **hoofdstuk 3** wordt daarom een methode beschreven voor de isolatie van cannabinoiden uit cannabis plant materiaal. In **hoofdstuk 4** wordt vervolgens een methode beschreven om op een snelle en betrouwbare wijze het exacte gehalte van de cannabinoid te bepalen. Helaas bleek het niet mogelijk om een van de gewenste cannabinoiden, cannabinol-zuur (CBNA) uit plantenmateriaal te isoleren. In **hoofdstuk 5** is daarom een methode beschreven voor de productie van CBNA uit het eenvoudig te isoleren cannabinoid tetrahydrocannabinol-zuur (THCA).

De geïsoleerde stoffen (ook wel referentiestoffen of standaarden genoemd) spelen in dit promotieonderzoek een centrale rol, en maken onderzoek mogelijk dat anders niet had kunnen worden uitgevoerd. Om te beginnen werd het tijd om eens de verschillende eigenschappen van al die stoffen op een rijtje te zetten (o.a. UV-absorptie- en massa-spectrum en chromatografische data). Weliswaar waren veel van die eigenschappen al eerder onderzocht en gepubliceerd, maar dit was nooit gebeurd onder gestandaardiseerde omstandigheden. Ofwel: iedere onderzoeker gebruikte zijn eigen type apparatuur en verschillende condities, waardoor de gepubliceerde eigenschappen moeilijk met elkaar vergelijkbaar zijn. In **hoofdstuk 6** is daarom voor het eerst een poging gedaan om al die, voor de fyto-chemisch onderzoeker, belangrijke karakteristieken op exact dezelfde wijze te meten en weer te geven.

Nadat de beschikbaarheid van referentie-standaarden goed was geregeld, was het nodig om een definitieve methode te kiezen voor het analyseren van cannabis-preparaten. Iedere methode heeft namelijk zowel voor- als nadelen. In **hoofdstuk 7** is een methode beschreven die is gevalideerd in overeenstemming met de meest recente eisen voor farmaceutisch onderzoek. Dit betekent dat de betrouwbaarheid van het systeem op diverse punten moest worden bewezen. Met het ontwikkelen van deze analyse methode werd het mogelijk om op betrouwbare en reproduceerbare wijze iets te zeggen over de exacte (complexe) samenstelling van cannabisplantenmateriaal of van medicijnen met cannabis als bestanddeel. De methode is vervolgens in gebruik genomen door verschillende laboratoria, waardoor allen op dezelfde wijze konden communiceren over cannabinoid-gehaltes in allerlei cannabispreparaten. Een van de belangrijkste voorwaarden voor degelijk onderzoek, namelijk standaardisatie (overeenstemming), was daarmee bereikt. Hierdoor konden we in meer detail gaan kijken naar de verschillende vormen waarin medicinale cannabis werd geconsumeerd buiten het laboratorium, door patiënten in de echte wereld.

Cannabis als medicijn kan in allerlei vormen worden gebruikt, maar afgezien van roken (inhaleren) is van de meeste vormen niet erg veel bekend. Zo prefereert een aanzienlijk deel van medicinale gebruikers consumptie in de vorm van thee, maar er is vrijwel niets gepubliceerd over de eigenschappen van cannabis-thee. Om die reden is een systematische studie uitgevoerd die is beschreven in **hoofdstuk 8**. Hierbij zijn alle mogelijke variabelen die een rol spelen bij het bereiden van thee opzettelijk gevarieerd om de invloed op de uiteindelijke samenstelling van de thee te bepalen. Denk hierbij aan bijvoorbeeld de kooktijd, hoeveelheid gebruikte cannabis en volume thee dat bereid wordt. Ook het effect van bewaren na de bereiding is hierbij meegenomen. Uiteindelijk blijkt dat

cannabisthee een redelijk betrouwbare toedieningsvorm kan zijn voor bepaalde groepen patiënten. Daarnaast worden aanwijzingen gegeven die de bewaartijd van de thee sterk kunnen verbeteren.

In het algemeen is de makkelijkste manier van medicatie toedienen de orale weg, ofwel via de mond. Maar helaas is deze route niet eenvoudig toepasbaar voor de cannabinoiden, vanwege het feit dat zij vrijwel niet oplosbaar zijn in water. Naast het feit dat dit moeilijkheden geeft bij het maken van 'cannabis-pillen', leidt het er ook toe dat cannabinoiden moeilijk door het lichaam worden opgenomen vanuit de ingewanden. Om dit obstakel te overkomen zouden we cannabinoiden dus makkelijker in water oplosbaar moeten maken. In **hoofdstuk 9** wordt het gebruik van verschillende typen cyclodextrines (CDs) onderzocht om dit doel te bereiken. CDs worden veelvuldig gebruikt voor het verbeteren van de oplosbaarheid van medicijnen en ze zijn geschikt voor menselijk consumptie. De resultaten tonen aan dat bij het gebruik van een specifiek type CD zowel de wateroplosbaarheid als de stabiliteit van verschillende cannabinoiden sterk verbetert. Mogelijk opent dit de weg voor oraal toedienbare cannabispreparaten.

Helaas is het zo dat de meest efficiënte toedieningsvorm van cannabis, namelijk roken, tegelijkertijd de minst gezonde is. Met een verdamper is het echter mogelijk om cannabis op een milde manier te verhitten en daardoor de aktieve dampen te inhaleren, zonder dat er schadelijke verbrandingsproducten ontstaan. In **hoofdstuk 10** is een van de meest professionele verdampers van dit moment, de Volcano®, uitvoerig getest voor de toediening van de aktieve bestanddelen van de cannabisplant. THC is hierbij gebruikt als model. Gebaseerd op de positieve resultaten is de verdamper vervolgens daadwerkelijk gebruikt voor toediening van THC aan proefpersonen in een klinische test.

Conclusie

Dit proefschrift heeft als doel gehad om wat meer structuur te scheppen in de chaotische wereld van het cannabisonderzoek door cannabis simpelweg te behandelen als een medicinale plant, zonder al het 'gedoe' eromheen. Dankzij de unieke (wettelijke) situatie in Nederland is dat de afgelopen jaren mogelijk geweest. Want zoals bij elke plant die onderzocht wordt, kan ook het mysterie van de cannabisplant ontrafeld worden door degelijk wetenschappelijk onderzoek, en een goede samenwerking tussen verschillende disciplines, zoals biologie, farmacie en geneeskunde. Hiervoor is het echter wel noodzakelijk dat men met elkaar kan communiceren. De resultaten in dit proefschrift hebben hieraan zeker een bijdrage kunnen leveren. Diverse bedrijven en onderzoeksinstellingen hebben de opgedane kennis benut waardoor er nu voor het eerst een 'standaardwijze' is om met elkaar over cannabis te praten. Het is duidelijk dat daardoor niet meer telkens het wiel opnieuw hoeft te worden uitgevonden. De samenwerkingen die zijn gestart in de afgelopen jaren lopen ook door na het afronden van mijn onderzoeken. Deze synergie heeft duidelijk effecten. De Nederlandse medi-wiet is al bekend geworden in de gehele wereld, en in toenemende mate komt het buitenland kijken hoe die Hollanders dat toch allemaal doen. Italië lijkt nu het eerste land dat in grote hoeveelheden het Nederlandse materiaal gaat importeren. Canada zal wellicht binnenkort gaan volgen.

De structuur van dit proefschrift stond aan het begin van de promotietijd overigens nog allerminst vast. De opdracht was eigenlijk om 'iets te gaan doen' met medicinale cannabis. In de afgelopen jaren heb ik echter goed om me heen gekeken en mijn ideeën constant laten beïnvloeden door de ontwikkelingen op cannabisgebied. Daarnaast heb ik niet alleen contact gehad met wetenschappers, maar ook met beleidsmakers, ondernemers, apothekers en patiënten. Hierdoor zijn er vragen beantwoord die niet alleen academisch interessant zijn, maar die ook waarde kunnen hebben voor de werkelijke dagelijkse praktijk van medicinale cannabis, bijvoorbeeld bij het te volgen cannabis-beleid en de voorlichting van nieuwe gebruikers van medicinale cannabis. Ik ben van mening dat het proefschrift hierdoor een hoop aan relevantie heeft gewonnen. Hopelijk wordt het dan ook regelmatig nog eens gelezen.

References

Abood ME, Martin BR (1992) Neurobiology of marijuana abuse. Trends in Pharmacological Sciences 13(5): 201-206

Adams R, Baker BR, Wearn RB (1940) Structure of Cannabinol. III. Synthesis of Cannabinol, 1-Hydroxy-3-n-amyl-6, 6, 9-trimethyl-6-dibenzopyran. JACS 62: 2204–2207

Agilent Technologies (2000) Electronic database: Wiley Registry of Mass Spectral Data, 7th Edition, published by John Wiley & Sons, Inc. and Wiley Mass Spectral Libraries, Rev. D.02.00.

Ahmed K, Chowdhury HM (1992) Dehydrogenation of cyclohexane and cyclohexene over supported nickel and platinum catalysts. Chem. Eng. J. Biochem. Eng. J. 50: 165-168

Anderson LC (1974) A study of systematic wood anatomy in *Cannabis*. Bot. Mus. Leafl. Harv. Univ. 24: 29-36

Backstrom B, Cole MD, Carrott MJ, Jones DC, Davidson G, Coleman K (1997) A preliminary study of the analysis of Cannabis by supercritical fluid chromatography with atmospheric pressure chemical ionisation mass spectroscopic detection. Sci. Justice 37(2): 91-97

Baker D, Pryce G, Giovannoni G, Thompson AJ (2003) The therapeutic potential of cannabis. The Lancet Neurology 2: 291-298

Barni-Comparini I, Ferri S, Centini F (1984) Cannabinoid level in the leaves as a tool for the early discrimination of cannabis chemiovariants. For. Sci. Int. 24: 37-42

Beardsley TM (1990) Cannabis comprehended. The 'assassin of youth' points to a new pharmacology. Sci. Am. 263: 15-16

Ben Amar M (2006) Cannabinoids in medicine: A review of their therapeutic potential. J. Ethnopharmacol. 105(1-2): 1-25

Beutler JA, Der Marderosian AH (1978) Crossbreeding between *Cannabis sativa* and *C. ruderalis*, with analysis of cannabinoid content. Econ. Bot. 32: 378-394

Bidaut-Russell M, Devane WA, Howlett AC (1990) Cannabinoid receptors and modulation of cyclic AMP accumulation in the rat brain. J. Neurochem. 55: 21-26

Bifulco M, DiMarzo V (2003) The endocannabinoid system as a target for the development of new drugs for cancer therapy. Recenti Prog. Med. 94(5): 194-198

Boekhout van Solinge T (1996) Ganja in Jamaica. Amsterdams Drug Tijdschrift 2: 11-14

Brenneisen R (1983) Psychotrope drogen I. *Cannabis sativa* L. Pharm. Acta Helv. 58: 314-320

Brenneisen R, Kessler T (1987) Die variabilität der Cannabinoidführung von Cannabispflanzen aus Schweizer Kulturen in Abhängigkeit von genetischen und ökologischen Faktoren. Pharm. Acto Helv. 62: 134-139

Brenneisen R and ElSohly MA (1988) Chromatographic and spectroscopic profiles of cannabis of different origins: part I. J. For. Sci. 33(6): 1385-1404

Brenneisen R, Egli A, ElSohly MA, Henn V, Spiess Y (1996) The effect of orally and rectally administered delta 9-tetrahydrocannabinol on spasticity: a pilot study with 2 patients. Int. J. Clin. Pharm. Ther. 34: 446-452

Budzikiewicz H, Alpin RT, Lightner DA, Djerassi C, Mechoulam R, Gaoni Y (1965) Mass spectroscopy and its application to structural and stereochemical problems. 68. Mass spectroscopic studies of constituents of hashish. Tetrahedron 21: 1881-1888

Burstein SH, Karst M, Schneider U, Zurier RB (2004) Ajulemic acid: a novel cannabinoid produces analgesia without a "high". Life Sci. 75(12): 1513-1522

Carod Artal FJ (2003) Neurological syndromes associated with the ingestion of plants and fungi with a toxic component (II). Hallucinogenic fungi and plants, mycotoxins and medicinal herbs. Rev. Neurol. 36(10): 951-960

Chan PC, Sills RC, Braun AG, Haseman JK, Bucher JR (1996) Toxicity and carcinogenicity of delta-9-tetrahydrocannabinol in Fischer rats and B6C3F1 mice. Fundamental and applied toxicology 30(1): 109-117

Chankvetadze B, Pintore G, Burjanadze N, Bergenthal D, Strickmann D, Cerri R, Blanschke G (1998) Capillary electrophoresis, nuclear magnetic resonance and mass spectrometry studies of opposite chiral recognition of chlorpheniramine enantiomers with various cyclodextrins. Electrophoresis 19: 2101-2108

Chemic Laboratories (2000) Proof of concept: release of chemical constituents in cannabis sativa at 170-185ºC versus combustion. Unpublished report to California NORML and MAPS, Nov 17th, 2000

Choi YH, Choi HK, Hazekamp A, Bermej P, Schilder Y, Erkelens C, Verpoorte R (2003) Quantitative analysis of bilobalide and ginkgolides from Ginkgo biloba leaves and Ginkgo products using (1)-H-NMR. Chem. Pharm. Bull. 51: 158-161.

Choi YH, Hazekamp A, Peltenburg-Looman AMG, Frédérich M, Erkelens C, Lefeber AWM, Verpoorte R (2004) NMR assignments of the major cannabinoids and cannabiflavonoids isolated from flowers of Cannabis sativa. Phytochem. Anal. 15(6): 345-354.

Cichewicz DL (2004) Synergistic interactions between cannabinoid and opioid analgesics. Life Sci. 74: 1317-1324

Clarke RC (1981) Marijuana Botany. Berkeley: Ronin Publishing

Clifford DB (1983) Tetrahydrocannabinol for tremor in multiple scelrosis. Annals of Neurology 13(6): 669-671

Conert HJ, Jäger EJ, Kadereit JW, Schultze-Motel W, Wagenitz G, Weber HE, Hegi G (1992) Illustrierte Flora von Mitteleuropa. Paul Parey, Berlin/Hamburg, 1964-1992, p.283-295, p.473-474

Connors KA (1997) The stability of cyclodextrin complexes in solution. Chem. Rev. 97: 1325-1357

Corchero J, Manzanares J, Fuentes JA. (2004) Cannabinoid/opioid crosstalk in the central nervous system. Crit. Rev. Neurobiol. 16: 159-172

Correia I, Bezzenine N, Ronzani N, Platzer N, Beloeil JC, Doan BT (2002) Study of inclusion complexes of acridine with beta- and (2,6-di-O-methyl)-beta-cyclodextrin by use of solubility diagrams and NMR spectroscopy. J. Phys. Org. Chem. 15: 647-659

Corrigan D, Lynch JJ (1980) An investigation of potential staining reagents for the glandular trichomes of Cannabis sativa. Planta Medica: 163-169

Crombie L, Crombie WML (1975) Cannabinoid formation in *Cannabis sativa* grafted inter-racially, and with two *Humulus* species. Phytochem. 14: 409-412

Crombie L, Ponsford R, Shani A, Yagnitinsky B, Mechoulam R (1968) Hashish components. Phytochemical production of cannabicyclol from cannabichromene. Tetrahedron Lett. 55: 5771-5772

Cronquist A (1981) An integrated system of classification of flowering plants. CUP, New York, p.193

Dansak DA (1997) As an antiemetic and appetite stimulant in cancer patients, In ML Mathre, ed., Cannabis in medical practice: a legal, historical and pharmacological overview of the therapeutic use of marijuana. NC Jefferson: McFarland & Co., pp. 69-83

Davis KH (1984) Some smoking characteristics of marijuana cigarettes. In Agurell S, Dewey WL and Willette RE (eds), The Cannabinoids: chemical, pharmacologic and therapeutic aspects. Academic Press, New York, NY

De Jong FA, Engels FE, Sparreboom A, Loos WJ, De Bruijn P, Friberg LE, Mathot RA, Verweij J, Mathijssen RH (2005) Influence of medicinal cannabis (MC) on the pharmacokinetics (PK) of docetaxel (DOC) and irinotecan (CPT-11). AACR Meeting Abstracts 2005: 938-c-939

De Petrocellis L, Melck D, Bisogno T, Milone A, Di Marzo V (1999) Finding of the endocannabinoid signalling system in Hydra, a very primitive organism: possible role in feeding response. Neuroscience 92(1): 377-387

Devane WA, Dysarz FA, Johnson MR, Melvin LS, Howlett AC (1988) Determination and characterization of a cannabinoid receptor in rat brain. Mol. Pharmacol. 34: 605-613

Devane WA, Hanuš L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258: 1946-1949

Dewey WL, Martin BR, May EL (1984) Cannabinoid stereoisomers: pharmacological effects. In: DF Snith (Ed.), Handbook of stereoisomers: drugs in psychopharmacology, CPC Press, Boca Raton, FL, pp. 317-326

Dewey WL (1986) Cannabinoid pharmacology. Pharmacol. Rev. 38: 151-178

Di Marzo V, Melck D, Bisogno T, De Petrocellis L (1998) Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. Trends Neurosci. 21: 521-528

Duran M, de Las Heras MJ, Laporte JR, Capella D (2005) [Therapeutic use of cannabis: results of a prospective survey in Catalonia (Spain)]. Med. Clin. (Barc.). 124(2): 76-77

Eddy NB (1965) The question of cannabis. Bibliography United Nations Commision on Narcotic Drugs, E/CN7/49

Edery H, Grunfeld Y, Porath G, Ben-Zvi Z, Shani A, Mechoulam R (1972) Structure-activity relationships in the tetrahydrocannabinol series. Modifications on the aromatic ring and it the side-chain. Arzneimittelforschung 22(11): 1995-2003

ElSohly MA, ElSohly HN, Turner CE (1983) Cannabis: new constituents and their pharmacological action, in: Topics in pharmaceutical sciences (Breimer DD, Speise P, eds.), Elsevier Science Publishers BV, p. 429-439

Elsohly MA, Slade D (2005) Chemical constituents of marijuana: the complex mixture of natural cannabinoids. Life Sci. 78(5): 539-548

Emboden WA (1974) *Cannabis* – a polytypic genus. Econ. Bot. 28: 304-310

Épshtein N (2004) Validation of HPLC techniques for pharmaceutical analysis. Pharmaceutical Chemistry Journal 38: 212-228

Fairbairn JW, Liebmann JA, Rowan MG (1976) The stability of cannabis and its preparations on storage. J. Pharm. Pharmacol. 28: 1-7

Fehr KO, Kalant H (1972) Analysis of cannabis smoke obtained under different combustion conditions. Can. J. Physiol. Pharmacol. 50: 761-767

Fellermeier M, Zenk MH (1998) Prenylation of olivetolate by a hemp transferase yields cannabigerolic acid, the precursor of tetrahydrocannabinol. FEBS Letters 427:283-285

Fellermeier M, Eisenreich W, Bacher A, Zenk MH (2001) Biosynthesis of cannabinoids. Incorporation experiments with 13C-labeled glucoses. Eur. J. Biochem. 268:1596-1604

Fenselau C, Kelly S, Salmon M, Billets S (1976) The absence of tetrahydrocannabinol from hops. Fd. Cosmet. Toxicol. 14:35-39

Ferioli V, Rustichelli C, Paves G, Gamberini G (2000) Analytical characterization of hashish samples. Chromatographia 52: 39-44.

Fetterman PS, Doorenbos NJ, Keith ES, Quimby MW (1971a) Simple gas-liquid chromatographic procedure for determination of cannabinoidic acids in Cannabis sativa L. Experientia 27: 988.

Fetterman PS, Keith ES, Waller, CW, Guerrero O, Doorenbos NJ, Quimby MW (1971b) Mississippi grown *Cannabis sativa* L.: preliminary observation on chemical definition of phenotype and variations in tetrahydrocannabinol content versus age, sex, and plan part. J. Pharm. Sci. 60: 1246-1249

Field BI, Arndt RR (1980) Cannabinoid compounds in South African *Cannabis sativa* L. J. Pharm. Pharmacol. 32: 21-24

Foucault AP (1994) Centrifugal Partition Chromatography; Marcel Dekker, Inc: New York.

Frohne D, Jensen U (1973) Systematic des Pflanzenreichs. Verlag G. Fischer, Stuttgart, p.100

Furler MD, Einarson TR, Millson M, Walmsley S, Bendayan R (2004) Medicinal and recreational marijuana use by patients infected with HIV. AIDS Patient Care STDS 18(4): 215-28

Gaal LF van, Rissanen AM, Scheen AJ, Ziegler O, Rossner S (2005) Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factors in overweight patients: 1-year experience from the RIO-Europe study. Lancet 365: 1389-1397

Gaoni Y, Mechoulam R (1964a) Isolation, structure, and partial synthesis of an active constituent of hashish. JACS. 86: 646-647

Gaoni Y, Mechoulam R (1964b) The structure and synthesis of cannabigerol, a new hashish constituent. Proc. Chem. Soc. 82

Gaoni Y, Mechoulam R (1971) The isolation and structure of delta-1-tetrahydrocannabinol and other neutral cannabinoids from hashish. JACS 93(1): 217-224

Garrett ER, Hunt CA (1974) Physiochemical properties, solubility, and protein binding of delta9-tetrahydrocannabinol. J. Pharm. Sci. 63: 1056-1064

Georggiett OC, Muino JC, Montrull H, Brizuela N, Avalos S, Gomez RM (2000) Relationship between lung cancer and aflatoxin B1. Rev. Fac. Cien. Med. Univ. Nac. Cordoba 57(1): 95-107

Gieringer D (1996) Marijuana research: waterpipe study. MAPS (Multidisciplinary Association for Psychedelic Studies) Bull 6(3): 59-66

Gieringer D (2001) Cannabis vaporization: a promising strategy for smoke harm reduction. Journal of Cannabis Therapeutics 1(3-4): 153-170

Gieringer D, StLaurent J, Goodrich S (2004) Cannabis vaporizer combines efficient delivery of THC with effective suppression of pyrolytic compounds. Journal of Cannabis Therapeutics 4(1): 7-27

Giroud C, Augsburger M, Rivier L, Mangin P (1997) Hemp tea versus hemp milk: subjective effects and elimination studies of THC and its main metabolite. In: Proc. 35th TIAFT meeting, Padova, Italy, pp.112-121

Giroud C, Augsburger M, Favrat B, Menetrey A, Pin MA, Rothuizen LE, Appenzeller M, Buclin T, Mathieu S, Castella V, Hazekamp A, Mangin P (2006) Effects of oral cannabis and dronabinol on driving capacity. Ann. Pharm. Fr. 64(3): 161-172

Grinspoon L (1997) Marijuana, the forbidden medicine. Revised edition. Yale University Press, New Haven, CT

Grlic L (1968) A combined spectrophotometric differentiation of samples of cannabis. Bull. Narcot. 20: 25-29

Grotenhermen F, Russo E (2002) Cannabis and cannabinoids. Haworth Press, New York

GW pharmaceuticals (2003) Cannabis-based medicines. Drugs R. D. 4(5): 306-309

Hall W, Degenhardt L (2000) Cannabis use and psychosis: a review of clinical and epidemiological evidence. Austr. N.Z. J. Psychiatry 34: 6-34

Hamadeh R, Ardehali A, Locksley RM, York MK (1988) Fatal aspergillosis associated with smoking contaminated marijuana, in a marrow transplant recipient. Chest 94(2): 432-433

Hanus L, Tesarik K, Krejci Z (1985) Capillary gas chromatography of natural substances from Cannabis sativa L. I. Cannabinol and cannabinolic acid--artefacts. Acta. Univ. Palacki. Olomuc. Fac. Med. 108: 29-38

Hartley JP, Nogrady SG, Seaton A (1978) Bronchodilator effect of delta1-tetrahydrocannabinol. Br. J. Clin. Pharmacol. 5(6): 523-525

Harvey DJ (1977) Cyclic alkylboronates as derivatives for the characterization of cannabinolic acids by combined gas chromatography and mass spectrometry. Biomed. Mass Spectrom. 4: 88-93

Harvey DJ (1992) Cannabinoids. In: Mass spectrometry: clinical and biomedical applications, volume 1; Desiderio, D.M., Ed.; Plenum press: New York, 1992; 207-257

Hazekamp A, Simons R, Peltenburg-Looman A, Sengers M, Van Zweden R, Verpoorte R (2004a) Preparative isolation of cannabinoids from Cannabis sativa by centrifugal partition chromatography. J. Liq. Chrom. Rel. Technol. 27: 2421-2439

Hazekamp A, Choi YH, Verpoorte R (2004b) Quantitative analysis of cannabinoids from Cannabis sative using ^{1}H-NMR. Chem. Pharm. Bull. 52(6): 718-721

Hazekamp A, Giroud C, Peltenburg A, Verpoorte R (2005) Spectroscopic and chromatographic data of cannabinoids from Cannabis sativa. J. Liq. Chrom. Rel. Technol. 28(15): 2361-2382

Hazekamp A (2006a) An evaluation of medicinal grade cannabis in The Netherlands. Cannabinoids 1(1): 1-9

Hazekamp A, Verpoorte R (2006b) Structure elucidation of the tetrahydrocannabinol complex with randomly methylated beta-cyclodextrin. Eur. J. Pharm. Sci. 29(5): 340-347

Hemphill JK, Turner JC, Mahlberg PG (1980) Cannabinoid content of individual plant organs from different geographical strains of *Cannabis sativa* L. J. Nat. Prod. 43: 112-122

Hendriks H, Malingré TM, Batterman S, Bos R (1978) The essential oil of *Cannabis sativa* L. Pharmac. Weekbl. 113: 413-424

Hepler RS, Frank IR (1971) Marihuana smoking and intraocular pressure. JAMA. 217(10): 1392

Herring AC, Faubert Kaplan BL, Kaminski NE (2001) Modulation of CREB and NF-kappaB signal transduction by cannabinol in activated thymocytes. Cell. Signal. 13: 241-250

Higuchi T, Conners KA (1965) Phase solubility techniques. Adv. Anal. Chem. Instr. 4: 117-212

Hiller FC, Wilson FJJ, Mazumder MK, Wilson JD, Bone RC (1984) Concentration and particle size distribution in smoke from marijuana cigarettes with different Δ9-tetrahydrocannabinol content. Fundam. Appl. Toxicol. 4: 451-454

Holdcroft A, Maze M, Dore C, Tebbs S, Thompson S (2006) A multicenter dose-escalation study of the analgesic and adverse effects of an oral cannabis extract (Cannador) for postoperative pain management. Anesthesiology 104(5): 1040-1046

Hollister LE (1974) Structure-activity relationships in man of cannabis constituents, and homologues and metabolites of Δ^9-tetrahydrocannabinol. Pharmacol. 11: 3-11

Hollister LE (1986) Health aspects of cannabis. Pharmacol. Rev. 38: 1-20

Hood LVS, Dames ME, Barry GT (1973) Headspace volatiles of marijuana. Nature 242: 402-403

Howlett AC (1985) Cannabinoid inhibition of adenylat-cyclase. Biochemistry of the response in neuroblastoma cell membranes. Mol. Pharmacol. 27:429-436

Howlett AC, Qualy JM, Khachatrian LL (1986) Involvement of G_i in the inhibition of adenylate cyclase by cannabimimetic drugs. Mol. Pharmacol. 29:307-313

Howlett AC (1987) Cannabinoid inhibition of adenylat cyclase, relative activity of constituents and metabolites of marihuana. Neuropharmacol. 26:507-512

ICH (2006) The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Official website: www.ich.org. Website visited 15 July 2006

Imamoto T, Yokoyama H, Yokoyama M (1981) Tetrahedron letters 22: 1803-1804

Ingkaninan K, Hazekamp A, Hoek AC, Balconi S, Verpoorte R (2000) Application of centrifugal partition chromatography in a general separation and dereplication procedure for plant extracts. J. Liq. Chrom. Rel. Technol. 23(14): 2195-2208

Institute of Medicine (1999) Marijuana and medicine: assessing the scientific base. National Academy Press, Washington DC, MD

Irvine G (2006) Rural doctors' attitudes to and knowledge of medicinal cannabis. J. Law Med. 14(1): 135-142

Jan TR, Rao GK, Kaminski NE (2002) Cannabinol enhancement of interleukin-2 (IL-2) expression by T cells is associated with an increase in IL-2 distal nuclear factor of activated T cell activity. Mol. Pharmacol. 61:446-454

Janse AFC, Breekveldt-Postma NS, Erkens JA, Herings RMC (2004) Pharmo rapport: medicinal gebruik van cannabis. Pharmo institute for drug outcome research, Utrecht, the Netherlands

Jarho P, Pate DW, Brenneisen R, Järvinen T (1998) Hydroxypropyl-β-cyclodextrin and its combination with hydroxypropyl-methylcellulose increases aqueous solubility of Δ9-tetrahydrocannabinol. Life Sci. 63(26): 381-384

Job P (1928) Formation and stability of inorganic complexes in solution. Ann. Chim. (Paris) 9: 113-203

Johnson TE, Casiano RR, Kronish JW, Tse DT, Meldrum M, Chang W (1999) Sino-orbital aspergillosis in acquired immunodeficiency syndrome. Arch. Ophthalmol. 117 (1): 57-64

Jover A, Budal RM, Al-Soufi W, Meijide F, Vázquez Tato J, Yunes RA (2003) Spectra and structure of complexes formed by sodium fusidate and potassium helvolate with β- and γ-cyclodextrin. Steroids 68: 55-64

Joy JE, Watson SJ, Benson JA, eds. (1999) Marijuana and medicine: assessing the science base. Washington DC: Institute of Medicine, National Academy Press, p. 126

Joyce CRB, Curry SH (1970) In: The botany and chemistry of Cannabis; J. & A. Churchill: London.

Kagen SL, Kurup VP, Sohnle PG, Fink JN (1983) Marijuana smoking and fungal sensitization. J. Allergy Clin. Immunol. 71(4): 389-393

Kanter SL, Musumeci MR, Hollister LE (1979) Quantitative determination of delta 9-tetrahydrocannabinol and delta 9-tetrahydro-cannabinolic acid in marihuana by high-pressure liquid chromatography. J. Chromatogr. 171: 504-508

Kim ES, Mahlberg PG (2003) Secretory vesicle formation in the secretory cavity of glandular trichomes of *Cannabis sativa* L. (Cannabaceae). Mol. Cells. 15(3): 387-395

Korte F, Haag M, Claussen U (1965) Tetrahydrocannabinolcarboxylic acid, a component of hashish. 1. Angew. Chem. Int. Ed. Eng. 4: 872

Lachenmeier DW, Kroener L, Musshoff F (2004) Determination of cannabinoids in hemp food products by use of headspace solid-phase microextraction and gas chromatography-mass spectrometry. Anal. Bioanal. Chem. 378: 183-189

Lawi-Berger C, Miège MN, Kapétanidis I, Miège J (1982a) Systématique végétable – Contribution à l'étude chimiotaxonomique de *Cannabis sativa* L. C.R. Acad. Sci. Paris 295:397-402

Lawi-Berger C (1982b) Contribution à l'étude chimiotaxonomique du genre *Cannabis* (Cannabaceae). Dissertation, Univ. Geneva

Lee JG, Kim KC (1992) Aromatization of cyclohexenes and cyclohexadienes with selenium dioxide-trimethyl polyphosphate. Tetrahedron letters 33: 6363-6366

Lehmann T, Brenneisen R (1992) A new chromatographic method for the isolation of (-)-Δ^9-(trans)tetrahydrocannabinolic acid A. Phytochem. Anal. 3: 88-90

Lehmann T, Brenneisen R (1995) High performance liquid-chromatographic profiling of cannabis products. J. Liq. Chrom. 18: 689-700

Leweke FM, Giuffrida A, Wurster U, Emrich HM, Piomelli D (1999) Elevated endogenous cannabinoids in schizophrenia. Neuroreport 10:1665-1669

Leweke FM (2002) Acute effects of cannabis and the cannabinoids. In Grotenhermen F, Russo E (eds), Cannabis and cannabinoids. Haworth Press, New York, NY. pp. 249-256

Lichtman AH, Peart J, Poklis JL, Bridgen DT, Razdan RK, Wilson DM, Poklis A, Meng Y, Byron PR, Martin BR (2000) Pharmacological evaluation of aerosolized cannabinoids in mice. Eur. J. Pharmacol. 399: 141-149

Llewellyn GC, O'Rear CE (1977) Examination of fungal growth and aflatoxin production on marihuana. Mycopathologia 62(2):109-112

Loftsson T, Magnúsdóttir A, Másson M, Sigurjónsdóttir JF (2002) Self-association and cyclodextrin solubilization of drugs. J. Pharm. Sci. 91: 2307-2316

Loftsson T, Másson M, Brewster ME (2004) Minireview; self-association of cyclodextrins and cyclodextrin complexes. J. Pharm. Sci. 93: 1091-1099

Lurie IS, Meyers RP, Conver TS (1998) Capillary electrochromatography of cannabinoids. Anal. Chem. 70(15): 3255-3260

Lyng SMO, Passos M, Fontana JD (2004) Bixin and α-cyclodextrin inclusion complex and stability tests. Process Biochemistry 39: 100-113

Lyons HA, Ayres SM, Dworetzky M, Failliers CS, Harris MC, Dollery CT, Gandevia B (1973) Symposium on isoproterenol therapy in asthma. Ann. Allergy 31: 1-44

Mackie K, Hille B (1992) Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proc. Natl. Acad. Sci. USA 89: 3825-3829

Makriyannis A (1995) The role of cell membranes in cannabinoid activity in cannabinoid receptors. In: RG Pertwee (Ed.), Cannabinoid receptors, Academic press, London, pp. 87-115

Malingré T, Hendriks H, Batterman S, Bos R, Visser J (1975) The essential oil of *Cannabis sativa*. Planta Med. 28: 56-61

Maniara G, Rajamoorthi K, Rajan S and Stockton GW (1998) Method performance and validation for quantitative analysis by H-1 and P-31 NMR spectroscopy. Applications to analytical standards and agricultural chemicals. Anal. Chem. 70: 4921-4928

Mannila J, Jarvinen T, Jarvinen K, Tarvainen M, Jarho P (2005) Effects of RM-beta-CD on sublingual bioavailability of Delta(9)-tetrahydrocannabinol in rabbits. Eur. J. Pharm. Sci. 26(1): 71-77

Manno JE, Kiplinger GF, Haine SE, Bennett IF, Forney RB (1970) Comparative effects of smoking marihuana or placebo on human motor performance. Clin. Pharmacol. Ther. 11: 808-815

Marks WH, Florence L, Lieberman J, Chapman P, Howard D, Roberts P, Perkinson D (1996) Successfully treated invasive pulmonary aspergillosis associated with smoking marijuana in a renal transplant recipient. Transplantation 61 (12): 771-1774

Martin Del Valle EM (2004) Cyclodextrins and their uses: A review. Process Biochem. 39: 1033-1046

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346: 651-654

Matthias P, Tashkin DP, Marques-Magallanes JA, Wilkins JN, Simmons MS (1997) Effects of varying marijuana potency on deposition of tar and delta9-THC in the lung during smoking. Pharmacol. Biochem. Behav. 58: 1145-1150

Maunder MJ (1970) A comparative evaluation of the delta-9-tetrahydrocannabinol content of cannabis plants. J. Ass. Publ. Analysts 8: 42-47

Mc Partland JM, Pruitt PL (1997) Medical marijuana and its use by the immunocompromised. Altern. Ther. Health Med. 3(3): 39-45

McPartland JM, Russo EB (2001) Cannabis and cannabis extracts: greater than the sum of their parts. Journal of Cannabis Therapeutics 1: 103-132

McPartland JM, Pruitt P (2002) Sourcing the code: searching for the evolutionary origins of cannabinoid receptors, vanilloid receptors, and anandamide. Journal of Cannabis Therapeutics 2(1):73-104
McPartland JM, Pruitt PL (1997) Medical marijuana and its use by the immunocompromised. Altern. Ther. Health Med. 3(3): 39-45

Mechoulam R, Gaoni Y (1965) Hashish. IV. The isolation and structure of cannabinolic cannabidiolic and cannabigerolic acids. Tetrahedron 21: 1223-1229

Mechoulam R, Gaoni Y (1967) Recent advances in the chemistry of hashish. Fortsch. Chem. Org. Naturst. 25:175-213

Mechoulam R, Ben-Zvi Z (1969a) Carboxylation of resorcinols with magnesium carbonate. Synthesis of cannabinoid acids. Chem. Commun.: 343-344

Mechoulam R, Ben-Zvi Z, Yagnitinsky B, Shani A (1969b) A new tetrahydrocannabinolic acid. Tetrahedron. Lett. 28: 2339-2341

Mechoulam R (1970) Marihuana chemistry. Recent advances in cannabinoid chemistry open the area to more sophisticated research. Science 168: 1159-1166

Mechoulam R (1973) Marihuana. Academic press, New York

Mechoulam R, Devane WA, Glaser R (1992) Cannabinoid geometry and biological activity. In: L Murphy, A Bartke (Eds.), Marijuana/cannabinoids: neurobiology and neurophysiology, CRC Press, Boca Raton, FL, pp. 1-33

Mechoulam R, Ben-Shabat S, Hanuš L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog A, Martin BR, Compton DR, Pertwee RG, Griffin G, Bayewitch M, Barg J, Vogel Z (1995) Identification of an endogenous 2-mono-glyceride, present in canine gut, that binds to cannabinoid receptors. Biochem. Pharmacol. 50: 83-90

Mechoulam R, Fride E, Di Marzo V (1998) Endocannabinoids (review). Eur. J. Pharmacol. 359: 1-18

Mechoulam R (2005) Plant cannabinoids: a neglected pharmacological treasure trove. Br. J. Pharmacol. 146(7): 913-915

Menetrey A, Augsburger M, Favrat B, Pin MA, Rothuizen LE, Appenzeller M, Buclin T, Mangin P, Giroud C (2005) Assessment of driving capability through the use of clinical and psychomotor tests in relation to blood cannabinoids levels following oral administration of 20 mg dronabinol or of a cannabis decoction made with 20 or 60 mg Delta9-THC. J. Anal. Toxicol. 29(5): 327-338

Molina-Holgado F, Pinteaux E, Moore JD, Molina-Holgado E, Guaza C, Gibson RM, Rothwell NJ (2003) Endogenous interleukin-1 receptor antagonist mediates anti-inflammatory and neuroprotective actions of cannabinoids in neurons and glia. J. Neurosci. 23(16): 6470-6474

Monda AM, Ilias LS (2001) Dehydrogenation of cyclohexane in a palladium-ceramic membrane reactor by equilibrium shift. Separation science and technology 36: 1101-1116

Morimoto S, Komatsu K, Taura F, Shoyama Y (1998) Purification and characterization of cannabichromenic acid synthase from *Cannabis sativa*. Phytochem. 49(6): 1525-1529

Muller-Vahl KR, Kolbe H, Schneider U, Emrich HM (1999) Cannabis in movement disorders. Forsch. Komplementarmed. 6 Suppl 3: 23-27

Munro S, Thomas KL, Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature 365(6441): 12-13.

Murayama W, Kobayashi T, Kosuge Y, Yano H, Nunogaki Y, Nunogaki K (1982) A new centrifugal counter-current chromatograph and its application. J. Chromatogr. 239: 643-649

Nadulski T, Pragst F, Weinberg G, Roser P, Schnelle M, Fronk EM, Stadelmann AM (2005) Randomized, double-blind, placebo-controlled study about the effects of cannabidiol (CBD) on the pharmacokinetics of Delta9-tetrahydrocannabinol (THC) after oral application of THC verses standardized cannabis extract. Ther. Drug Monit. 27(6): 799-810

Naef M, Russmann S, Petersen-Felix S, Brenneisen R (2004) Development and pharmacokinetic characterization of pulmonal and intravenous delta-9-tetrahydrocannabinol (THC) in humans. J. Pharm. Sci. 93(5): 1176-1184

Nahas GG (1973) In: Marihuana-deceptive weed; Raven Press N.Y.; North-Holland Publishing Company: Amsterdam.

Notcutt W, Price M, Miller R, Newport S, Phillips C, Simmons S, Sansom C (2004) Initial experiences with medicinal extracts of cannabis for chronic pain: Results from 34 'N of 1' studies. Anaesthesia 59(5): 440-452

Noye R, Baram DA (1974) Cannabis analgesia. Comprehensive Psychiatry 15(6): 531-535

Ohlsson A, Lindgren JE, Wahlen A, Agurell S, Hollister LE, Gillespie HK (1980) Plasma delta-9 tetrahydrocannabinol concentrations and clinical effects after oral and intravenous administration and smoking. Clin. Pharmacol. Ther. 28(3): 409-416

OMC: Office of Medicinal Cannabis, The Netherlands (2003) Guidelines for cultivating cannabis for medicinal purposes; Annex to the regulation of the Minister of Health, Welfare and Sport of 9 January 2003. GMT/BMC 2340685 (website visited 08 July 2006): http://www.cannabisbureau.nl/pdf/GAP_EN_2003-01-07.pdf

OMC: Office of Medicinal Cannabis, the Netherlands (2006) Official website: www.cannabisbureau.nl. Website visited 20 August 2006

Oomah BD, Busson M, Godfrey DV, Drover JCG (2002) Characteristics of hemp (Cannabis sativa L.-) seed oil. Food Chemistry 76: 33-43.

Page SA, Verhoef MJ, Stebbins RA, Metz LM, Levy JC (2003) Cannabis use as described by people with multiple sclerosis. Can. J. Neurol. Sci. 30(3): 201-205

Panikashvili D, Simeonidou C, Ben-Shabat S, Hanuš L, Breuer A, Mechoulam R, Shohami E (2001) An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. Nature 413: 527-531

Pauli GF (2001) Q-NMR – a versatile concept for the validation of natural product reference compounds. Phytochem. Anal. 12: 28-42

Pearson H (2004) Joint suits aim to weed out agencies' red tape. Nature 430(6999): 492

Perras C (2005) Sativex for the management of multiple sclerosis symptoms. Issues Emerg. Health Technol. 72: 1-4

Pertwee RG (1988) The central neuropharmacology of psychotropic cannabinoids. Pharmacol. Ther. 36: 189-261

Pertwee RG (2005) Pharmacological actions of cannabinoids. Handb. Exp. Pharmacol. (168): 1-51

Pertwee RG (2006a) Cannabinoid pharmacology: the first 66 years. Br. J. Pharmacol. 147 Suppl 1: S163-171

Pertwee RG (2006b) The pharmacology of cannabinoid receptors and their ligands: an overview. Int. J. Obes. (Lond.). 30 Suppl 1: S13-18

Pfleger K, Maurer HH, Weber A (2000) Electronic database: Mass spectral and GC data of drugs, poisons, pesticides, pollutants and their metabolites. Parts 1-4. Second, revised and enlarged edition. Wiley-VCH, Weinheim, Germany; Mass Spectral Libraries, Rev. D.02.00. Agilent Technologies.

Pijlman FT, Rigter SM, Hoek J, Goldschmidt HM, Niesink RJ (2005) Strong increase in total delta-9-THC in cannabis preparations sold in Dutch coffee shops. Addict Biol. 10(2):171-180

Plasse TF, Gorter RW, Krasnow SH, Lane M, Shepard KV, Wadleigh RG (1991) Recent clinical experience with dronabinol. Pharmacology, Biochemistry and Behaviour 40(3): 695-700

Poortman-van der Meer AJ, Huizer H (1998) A contribution to the improvement of accuracy in the quantitation of THC. For. Sci. Int. 101: 1-8

Pragst F, Herzler M, Herre S, Erxleben BT, Rothe M (2001) UV spectra of toxic compounds. Database of photodiode array UV spectra of illegal and therapeutic drugs, pesticides, ecotoxic substances and other poisons. Verlag Dr. Dieter Helm, Heppenheim, Germany.

Raharjo TJ, Verpoorte R (2004) Methods for cannabinoids analysis in biological materials: a review. Phytochem. Anal. 15(2): 79-94

Ramos Cabrer P, Alvarez-Parrilla E, Mejide F, Seijas JA, Rodríguez Núñez E, Vázquez Tato J (1999) Complexation of sodium cholate and sodium deoxycholate by β-cyclodextrin and derivatives. Langmuir 15: 5489-5495

Ramos Cabrer P, Alvarez-Parrilla E, Al-Soufi W, Mejide F, Rodríguez Núñez E, Vázquez Tato J (2003) Complexation of bile salts by natural cyclodextrins. Supramol. Chem. 15(1): 33-43

Ravichandran R, Divakar S (1998) Inclusion of Ring A of cholesterol inside the β-cyclodextrin cavity: evidence from oxydation reactions and structural studies. Journal of Inclusion Phenomena and Molecular Recognition in Chemistry 30: 253-270

Razdan RK (1973) Recent advances in the chemistry of cannabinoids. Progr. Org. Chem. 8: 78-101

Reinarman C, Cohen PDA, Kaal HL (2004) The Limited Relevance of Drug Policy: Cannabis in Amsterdam and in San Francisco. American Journal of Public Health 94(5): 836-842

Ricordy R, Gensabella G, Cacci E, Augusti-Toccol G (2002) Impairment of cell cycle progression by aflatoxin B1 in human cell lines. Mutagenesis 17(3): 241-249

Ross S, ElSohly MA (1995) Constituents of *Cannabis sativa* L. XXVIII A review of natural constituents: 1980-1994. Zagazig journal of pharmaceutical sciences 4: 1-10

Ross SA, Mehmedic Z, Murphy TP, Elsohly MA (2000) GC-MS analysis of the total delta9-THC content of both drug- and fiber-type cannabis seeds. J. Anal. Toxicol. 24(8): 715-717

Rowan MG, Fairbairn JW (1977) Cannabinoid patterns in seedlings of Cannabis sativa L. and their use in the determination of chemical race. J. Pharm. Pharmac. 29: 491-494

Rozou S, Michalaes S, Antoniadou-Vyza E (2005) Study of structural features and thermodynamic parameters, determining the chromatographic behaviour of drug-cyclodextrin complexes. J. Chrom. A 1087(1-2): 86-94.

Rubin V, Comitas L (1975) Ganja in Jamaica, a medical anthropological study of chronic marihuana use. The Hague, Mouton

Russo EB, McPartland JM (2003) Cannabis is more than simply delta(9)-tetrahydrocannabinol. Psychopharmacology (Berl). 165(4): 431-432

Russo E, Guy GW (2006) A tale of two cannabinoids: the therapeutic rationale for combining tetrahydrocannabinol and cannabidiol. Med. Hypotheses 66(2): 234-246

Schultes RE, Klein WM, Plowman T, Lockwood TE (1974) *Cannabis*, an example of taxonomic neglect. Bot. Mus. Leafl. Harv. Univ. 23: 337-367

Schultes RE, Hofmann A (1980) Pflanzen der Götter. Hallwag-Verlag, Bern/Stuttgart, p.93

Shoyama Y, Yamauchi T, Nishioka I (1970) Cannabis V. Cannabigerolic acid monomethyl ether and cannabinolic acid. Chem. Pharm. Bull. 18: 1327-1332

Shoyama Y, Yagi M, Nishioka I, Yamauchi T (1975) Biosynthesis of cannabinoid acids. Phytochem. 14: 2189-2192

Shoyama Y, Morimoto S, Nishioka I (1983) Cannabis. XV. Preparation and stability of delta-9-tetrahydrocannabinol-beta-cyclodextrin inclusion complex. J. Nat. Prod. 46: 633-637

Sibbald B (2005) Conditional okay for cannabis prescription drug. C.M.A.J. 172(13): 1672

Sidney S, Beck JE, Tekawa IS, Quesenberry CP, Friedman GD (1997) Marijuana use and mortality. American Journal of Public Health 87(4): 585-590

Sirikantaramas S, Taura F, Tanaka Y, Ishikawa Y, Morimoto S, Shoyama Y (2005) Tetrahydrocannabinolic acid synthase, the enzyme controlling marijuana psychoactivity, is secreted into the storage cavity of the glandular trichomes. Plant Cell Physiol. 46(9): 1578-1582

Small E, Beckstead HD (1973) Common cannabinoid phenotypes in 350 stocks of *Cannabis*. Lloydia 36: 144-165

Small E, Jui PY, Lefkovitch LP (1976a) A numerical taxonomic analysis of *Cannabis* with special reference to species delimination. System. Bot. 1: 67-84

Small E, Cronquist A (1976b) A practical and natural taxonomy for *Cannabis*. Taxon. 25: 405-435

Smith RN (1975) High pressure liquid chromatography on cannabis. Identification of separated constituents. J. Chromatogr. 115: 101-106

Smith RN, Vaughan CG (1977) The decomposition of acidic and neutral cannabinoids in organic solvents. J. Pharm. Pharmacol. 29: 286-290

Smith RM, Burford MD (1992) Supercritical fluid extraction and gas chromatographic determination of the sesquiterpene lactone parthenolide in the medicinal herb Feverfew (Tanacetum parthenium). J. Chrom. 627: 255-261

Snippe, J, Bieleman B, Naayer H, Ogier C (2004) Preventieve doorlichting cannabisbranche c.a. St. Intraval, Groningen-Rotterdam

Snoeijer W (2001) A checklist of some Cannabaceae cultivars. Part 1: *Cannabis*. Div. Pharmacognosy, Leiden/Amsterdam Centre for Drug Research, The Netherlands

Stahl E (1967) Duennschichtchromatographie, ein laboratoriumshandbuch. Springer Verlag, Berlin: p236

Stahl E, Kunde R (1973) Die Leitzsubstanzen der Haschisch-Suchhunde. Kriminalstatistik, Zeitschrift für die gesamte kriminalistische Wissenschaft und Praxis 9: 385-388

Steinagle GC, Upfal M (1999) Concentration of marijuana metabolites in the urine after ingestion of hemp seed tea. Journal of Occupational & Environmental Medicine 41(6): 510-513

Stolker AA, van Schoonhoven J, de Vries AJ, Bobeldijk-Pastorova I, Vaes WH, van den Berg R (2004) Determination of cannabinoids in cannabis products using liquid chromatography-ion trap mass spectrometry. J. Chromatogr. A 1058(1-2): 143-151

Storz & Bickel website. http://www.storz-bickel.de, website visited January 10, 2005

Strömberg L (1974) Minor components of cannabis resin. IV. Mass spectrometric data and gas chromatographic retention times of terpenic components with retention times shorter than that of cannabidiol. J. Chromatogr. 96: 99-114

Struempler RE, Nelson G, Urry FM (1997) A positive cannabinoids workplace drug test following the ingestion of commercially available hemp seed oil. J. Anal. Toxicol. 21(4): 283-285

Tam J, Ofek O, Fride E, Ledent C, Gabet Y, Muller R, Zimmer A, Mackie K, Mechoulam R, Shohami E, Bab I. (2006) Involvement of neuronal cannabinoid receptor CB1 in regulation of bone mass and bone remodeling. Mol. Pharmacol. 70(3): 786-792

Tampier L, Linetzky R, Mardones J (1973) Effect of cannabinols from marihuana on smooth muscle. Arch. Biol. Med. Exper. 9: 16-19

Taura F, Morimoto S, Shoyama Y (1995a) Cannabinerolic acid, a cannabinoid from Cannabis sativa. Phytochem. 39(2): 457-458

Taura F, Morimoto S, Shoyama Y, Mechoulam R (1995b) First direct evidence for the mechanism of Δ1-tetrahydrocannabinolic acid biosynthesis. JACS 117(38): 9766-9767

Taura F, Morimoto S, Shoyama Y (1996) Purification and characterization of cannabidiolic acid synthase from *Cannabis sativa* L. Journal of Biological Chemistry 271(29): 17411-17416

Thomas A, Stevenson LA, Wease KN, Price MR, Baillie G, Ross RA, Pertwee RG (2005) Evidence that the plant cannabinoid Delta9-tetrahydrocannabivarin is a cannabinoid CB1 and CB2 receptor antagonist. Br. J. Pharmacol. 146(7): 917-926

Turner CE, Hadley K (1973) Constituents of *Cannabis sativa* L. II: Absence of cannabidiol in an African variant. J. Pharm. Sci. 62:251-258

Turner CE, Hadley KW, Henry J, Mole ML (1974) Constituents of *Cannabis sativa* L. VII. Use of silyl derivatives in routine analysis. J. Pharm. Sci. 63: 1872-1876

Turner CE, ElSohly MA, Cheng PC, Lewis G (1979) Constituents of *Cannabis sativa* L., XIV: intrinsic problem in classifying *Cannabis* based on a single cannabinoid analysis. J. Nat. Prod. 42, 317-319

Turner CE, Elsohly MA, Boeren EG (1980a) Constituents of *Cannabis sativa* L. XVII. A review of the natural constituents. J. Nat. Prod. 43(2): 169-234

Turner CE (1980b) Marijuana research and problems: an overview. Pharm. Int. 1: 93-96

Turner JC, Mahlberg PG (1982) Simple high-per- formance liquid chromatography method for separating acidic and neutral cannabinoids in *Cannabis sativa* L. J. Chromatogr. 253: 295-303

Turner CE (1985) Marijuana and cannabis: research. Why the conflict. In: Marihuana'84. proceedings of the Oxford symposium on Cannabis. Harvey, D.J., ed.; IRL press: Oxford: pp 31-36

Veress T, Szanto JI, Leisztner L (1990) Determination of cannabinoid acids by high-performance liquid chromatography of their neutral derivatives formed by thermal decarboxylation in an open reactor. J. Chromatogr. 520: 339-347

Verhoeckx KC, Korthout HA, van Meeteren-Kreikamp AP, Ehlert KA, Wang M, van der Greef J, Rodenburg RJ, Witkamp RF (2006) Unheated Cannabis sativa extracts and its major compound THC-acid have potential immuno-modulating properties not mediated by CB1 and CB2 receptor coupled pathways. Int. Immunopharmacol. 6(4): 656-665

Vigano D, Rubino T, Parolaro D (2005) Molecular and cellular basis of cannabinoid and opioid interactions. Pharmacol. Biochem. Behav. 81(2): 360-368

Wallace JM, Lim R, Browdy BL, Hopewell PC, Glassroth J, Rosen MJ, Reichman LB, Kvale PA (1998) Risk factors and outcomes associated with identification of Aspergillus in respiratory specimens from persons with HIV disease. Pulmonary Complications of HIV Infection Study Group. Chest 114: 131-137

Ware MA, Tawfik VL (2005) Safety issues concerning the medical use of cannabis and cannabinoids. Pain Res. Manage. 10(Suppl A): 31A-37A

Watts G (2004) High hopes for cannabinoid analgesia. BMJ 329: 257-258

Williams IJ, Edwards S, Rubo A, Haller VL, Stevens DL, Welch SP (2006) Time course of the enhancement and restoration of the analgesic efficacy of codeine and morphine by delta9-tetrahydrocannabinol. Eur. J. Pharmacol. 539(1-2): 57-63

Williamson EM,Evans FJ (2000) Cannabinoids in clinical practice. Drugs 60(6): 1303-1314

Wilson DM, Peart J, Martin BR, Bridgen DT, Byron PR, Lichtman AH (2002) Physiochemical and pharmacological characterization of a Δ^9-THC aerosol generated by a metered dose inhaler. Drug and Alcohol Dependence 67: 259-267

World Drug Monitor (2006) United Nations Office on Drugs and Crime. Chapter 2

Yamauchi T, Shomaya Y, Aramaki H, Azuma T, Nishioka I (1967) Tetrahydrocannabinolic acid, a genuine substance of tetrahydrocannabinol. Chem. Pharm. Bull. 15: 1075-1076

Zajicek JP, Sanders HP, Wright DE, Vickery PJ, Ingram WM, Reilly SM, Nunn AJ, Teare LJ, Fox PJ, Thompson AJ (2005) Cannabinoids in multiple sclerosis (CAMS) study: safety and efficacy data for 12 months follow up. J. Neurol. Neurosurg. Psychiatry 76(12): 1664-1669

Zhang X, Gramlich G, Wang X, Nau WM (2002) A joint structural, Kinetic, and thermodynamic investigation of substituent effects on host-guest complexation of bicyclic azoalkanes by β-cyclodextrin. JACS 124(2): 254-263

Zia V, Rajewski RA, Stella VJ (2001) Effect of cyclodextrin charge on complexation of neutral and charged substrates: comparison of (SBE)7M-beta-CD to HP-beta-CD. Pharm. Res. 18: 667-673

Zoller O, Rhyn P, Zimmerli B (2000) High-performance liquid chromatographic determination of delta9-tetrahydrocannabinol and the corresponding acid in hemp containing foods with special regard to the fluorescent properties of delta9-tetrahydrocannabinol. J. Chromatogr. A 872: 101-110

Zuurman L, Roy C, Hazekamp A, Schoemaker R, den Hartigh J, Bender JCME, Pinquier JL, Cohen AF, van Gerven JMA (2004) Effect of THC administration in humans: methodology study for further pharmacodynamic studies with cannabinoid agonist or antagonist. Br. J. Clin. Pharmacol. (59(5): 625

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sørgård M, DiMarzo V, Julius D, Högestätt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature 400(6743): 452-457

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At the start of my project, after studying the literature for several months, I couldn't wait to start researching 'something'. But where should I start? Cannabis is one of the most studied plants, which has resulted in well over 10.000 publications. The first step therefore, was to separate the sense from the nonsense. The publications of Professor Brenneisen, University of Bern, on the chromatographic analysis of cannabis were the first ones that helped me on my way.

Of course, my project could have been a purely academic exercise, performing phytochemical experiments inside the lab without caring too much about the needs of the outside world. However, that certainly didn't happen, and I have to thank Bedrocan BV and Farmalyse BV for that. They helped me to study exactly those aspects of medicinal cannabis that made my research relevant and interesting for myself, but also for a large group of patients, politicians, and other people outside the academic world.

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If I have forgotten to thank anyone, I sincerely apologize. This work would not have been possible without all of you! Thanks.

Curriculum vitae

Arno Hazekamp was born on 15 March 1976 in Bilthoven, the Netherlands. He attended high-school (VWO) at 'Het Nieuwe Lyceum" in Bilthoven, where he graduated in 1994 with the best average grades of his year. Because of his interest in genetics and laboratory science, he then selected Leiden University, the Netherlands, to study Molecular Biology. In his third year, he performed his first research project at TNO, Leiden, The Netherlands, on the isolation of specific enzymes involved in angiogenesis, by means of recombinant microorganisms. Shortly after that he had his first research experience abroad, when he was selected for the annual exchange program between Leiden University, and Kent State University, Ohio, USA. His short project focussed on the molecular mechanisms involved in cancer. Although his study went succesful up to that point, he increasingly felt that molecular biology missed a certain social component that he needed to enjoy the research.

Therefore, in 1998, he contacted the department of Pharmacognosy, Leiden University, to discuss the options to perform research in the field of medicinal plants and phytochemistry. In this way it was possible to combine the previously obtained laboratory experiences, with the social aspects of fieldwork and traditional medicine. In order to learn the basic skills needed in this field, he started a project in the Pharmacognosy department on the use of centrifugal partition chromatography for the isolation of bioactive compounds from plant extracts. After this, in 1999, he visited the Department of Pharmacology, Faculty of Medicine, Chiang Mai University, Thailand to work on a project entitled "Isolation of a bronchodilator flavonoid from the Thai medicinal plant *Clerodendrum petasites*". He graduated in 2000 *with honours* (cum laude) as a general biologist. After that, he was employed in 2000/2001 as a technician at the Pharmacognosy department. In this period he supervised several students, and was strongly involved in the internal moving of the entire department within the Gorlaeus Laboratories.

In November 2001, Arno started as a PhD student in the department of Pharmacognosy, under the supervision of prof. Rob Verpoorte. His research project was focused on the medicinal properties of medicinal cannabis, and on the practical obstacles that stand between this plant and its development into a modern medicine. He spent a lot of time and energy on informing the general public about the potential of medicinal cannabis, and had many fruitful discussions with a variety of professionals in healthcare, pharmacy, politics and science. During his PhD, he spent several periods at the Institut Universitaire de Médecine Légale (IUML) in Lausanne, Switzerland.

Currently, Arno is setting up his own phytochemical contract laboratory. He is working together with a consortium of other companies under the name PRISNA (Product Isolation from Nature). Cannabis continues to have his special interest.

List of publications

Published as first author

Hazekamp A, Simons R, Peltenburg-Looman A, Sengers M, van Zweden R, Verpoorte R (2004) Preparative isolation of cannabinoids from *Cannabis sativa* by centrifugal partition chromatography. J. Liq. Chrom. Rel. Technol. 27(15): 2421-2439

Hazekamp A, Choi YH, Verpoorte R (2004) Quantitative analysis of cannabinoids from *Cannabis sativa* using ^{1}H-NMR. Chem. Pharm. Bull. 52(6): 718-721

Hazekamp A, Giroud C, Peltenburg A, Verpoorte R (2005) Chromatographic and spectroscopic data of cannabinoids from *Cannabis sativa* L. J. Liq. Chrom. Rel. Technol. 28(15): 2361-2382

Hazekamp A, Ruhaak R, Zuurman L, van Gerven J, Verpoorte R (2006) Evaluation of a vaporizing device (Volcano®) for the pulmonary delivery of tetrahydrocannabinol. J. Pharm. Sci. 95(6): 1308-1317

Hazekamp A, Verpoorte R (2006) Structure elucidation of the tetrahydrocannabinol complex with randomly methylated-beta-cyclodextrin. Eur. J. Pharm. Sci. 29(5): 340-347

Hazekamp A, Sijrier P, Verpoorte R, Bender J, van Bakel N (2005) Cannabis uit de apotheek is beter. Pharmaceutisch weekblad 12: 402-404

Hazekamp A (2006) An evaluation of medicinal grade cannabis in The Netherlands. Cannabinoids 1(1): 1-9

Hazekamp A, Bastola K, Rashidi H, Bender J, Verpoorte R (2007) Cannabis tea revisited: A systematic evaluation of the cannabinoid composition of cannabis tea. J. Ethnopharmacol. 113(1): 85-90

Published as co-author

Zuurman L, Roy C, Hazekamp A, Schoemaker R, den Hartigh J, Bender JCME, Pinquier JL, Cohen AF, van Gerven JMA (2004) Effect of THC administration in humans: methodology study for further pharmacodynamic studies with cannabinoid agonist or antagonist. Br. J. Clin. Pharmacol. (59(5): 625

Choi YH, Hazekamp A, Peltenburg-Looman AMG, Frédérich M, Erkelens C, Lefeber AWM, Verpoorte R (2004) NMR assignments of the major cannabinoids and cannabiflavonoids isolated from flowers of *Cannabis sativa.* Phytochem. Anal. 15: 345-354

Choi YH, Kim HK, Hazekamp A, Erkelens C, Lefeber AWM, Verpoorte R (2004) Metabolomic differentiation of *Cannabis sativa* cultivars using 1H-NMR spectroscopy and principal component analysis. J. Nat. Prod. 67: 953-957

Giroud C, Augsburger M, Favrat B, Menetrey A, Pin MA, Rothuizen LE, Appenzeller M, Buclin T, Mathieu S, Castella V, Hazekamp A, Mangin P. (2006) [Effects of oral cannabis and dronabinol on driving capacity] *French.* Ann. Pharm. Fr. 64(3): 161-172

Bastola K, Hazekamp A, Verpoorte R (2007) Synthesis and spectroscopic characterization of cannabinolic acid. Planta Medica 73: 273-275

Zuurman L, Roy C, Schoemaker RC, Hazekamp A, den Hartigh J, Bender JCME, Verpoorte R, Pinquier JL, Cohen AF, van Gerven JMA (2007) Effect of intrapulmonary THC administration in humans. Submitted to Journal of Psychopharmacology

Monnet-Tschudi F, Hazekamp A, Perret N, Zurich MG, Mangin P, Giroud C, Honegger P (2007) Delta-9-tetrahydrocannabinol accumulation, metabolism and cell-type specific adverse effects in aggregating brain cell cultures. Submitted to Toxicol. Applied Pharmacol.