THE PROPAGATION, CHARACTERISATION AND OPTIMISATION OF *CANNABIS SATIVA* L AS A PHYTOPHARMACEUTICAL

A thesis submitted by

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Abstract

In response to known pharmacology, and an increasing weight of anecdotal evidence of efficacy, clinical trials have been performed to support the licensing of cannabis-based botanical medicines. The initial applications envisaged were the treatment of cancer pain, neuropathic pain and various symptoms associated with multiple sclerosis. With effective alternatives often unavailable, otherwise law-abiding UK patients have regularly turned to illicit cannabis for medical relief. The main active ingredients in this are the cannabinoids THC and CBD, but other pharmacologically active cannabinoids are also present. One study reported here quantifies these cannabinoids and assesses the likely implications for efficacy. Using light microscopy, studies are performed to expand current knowledge of the form and function of trichomes in *Cannabis sativa* L. Supporting chemical analyses ascertain what secondary metabolites are biosynthesised within these trichomes, and determines where and when this occurs. To comply with the demands of the pharmaceutical industry, and in marked contrast to illicit cannabis, a phytopharmaceutical feedstock must meet high expectations regarding the minimum and maximum content of a range of compounds. Specific studies are performed to ascertain how growing methods affect the secondary metabolite content. They also aim to find out how a tight specification can be met while satisfying commercial and environmental expectations. This involves studying plant development and secondary metabolite biosynthesis in both indoor and outdoor conditions. The first approved cannabis-based botanical medicine supported by this research is Sativex®. This became available in Canada in 2005 for the treatment of central neuropathic pain in multiple sclerosis and in 2007 for intractable cancer pain. The medicine is also available in the UK and many other countries on a 'named patient basis'. This thesis has also supported the production of a range of other cannabinoids which are undergoing in-vitro and in-vivo testing. This could lead to the commercial production of an increasing range of phytopharmaceuticals.

Cannabis sativa L cv Gayle

CBD Chemotype.

Awarded European Plant Variety Rights EU 16301, 10th Oct 2005

To my best mate John Pook

and those who cared for him at

St Leonard's Hospice, York

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List of Publications

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Abbreviations

ACMD	Advisory Council on the Misuse of Drugs
ANOVA	Analysis of Variance
API	Active pharmaceutical ingredient
B_D/B_D	Homozygous CBD chemotype
B_D/B_T	Heterozygous mixed THC/CBD chemotype
BDS	Botanical Drug Substance
BMA	British Medical Association
BRM	Botanical Raw Material
B_T/B_T	Homozygous THC chemotype
cAMP	cyclic Adenosine 5'- monophosphate
CB_1 and CB_2	Cannabinoid Receptors 1 and 2
CBC	Cannabichromene
CBCA	Cannabichromenic acid
CBCV	Cannabichromevarin
CBCVA	Cannabichromevarinic acid
CBD	Cannabindiol

CBDA	Cannabidiolic acid
CBG	Cannabigerol
CBGA	Cannabigerolic Acid
CBN	Cannabinol
CI	Confidence Interval
CNS	Central Nervous System
EMEA	European Medicines Agency
ERK	Extra-cellular regulated kinase
ETP	Enriched trichome preparation
GC	Gas chromatography
GAP	Good Agricultural Practise
GDP	Guanosine 5’-diphosphate
GMP	Good Manufacturing Practice
GTP	Guanosine 5’-triphosphate
GWP	Good Wild crafting Practice
HPLC	High Performance Liquid Chromatography
HPS	High Pressure Sodium

MBFU	Mercury Vapour Lamp
MH	Metal Halide
MHRA	Medicines and Healthcare Products Regulatory Agency
MS	Multiple Sclerosis
NIAB	National Institute of Agricultural Botany
PAR	Photosynthetically Active Ration
SD	Standard Deviation
SE	Standard Error
TEM	Transmission Electron Microscope
THC	Tetrahydrocannabinol
THCA	Tetrahydrocannabinolic acid
THCV	Tetrahydrocannabivarin
THCVA	Tetrahydrocannabivarinic acid
UNICEF	United Nations Childrens's Fund formally United Nations International Emergency Fund
UV-B	Ultra violet (Band B)
W m^{-2}	Watts per square meter
WHO	World Health Organisation

Chapter 1 INTRODUCTION

1.1 Plants as a source of medicines – past and present

Since modern man (*Homo sapiens* L) first walked this earth he has probably always exploited plants as medicines. Evidence from burial sites suggests that the Neanderthals (*Homo neanderthalis*) also shared this practice. Man's present day closest relative the chimpanzee (*Pan troglodyte* L), medicates with many plant species. Observations suggest that when the chimp purposefully swallows unchewed leaves of one of these, *Aspilia mossambicensis,* it is making use of the dense foliar trichomes to ensnare gut parasites (Huffman *et al.* 1996). Conceivably, common ancestors of *Homo* and *Pan* also shared similar practices, since many less developed animal species are frequently observed consuming plants for what appear to be medicinal, rather than nutritional, purposes (Sumner, 2000).

There are written records dating back several millennia BCE, from early civilisations on most continents, which describe man's use of plants as medicines. These include American Indian, early European, Middle Eastern, Ayurvedic (Indian sub-continent), Chinese, Korean and Japanese, and Aboriginal cultures. Cannabis features in many of these, and in oriental and Middle Eastern countries its use can be traced back many thousands of years. Evidence suggests that around 3000 BCE *Cannabis sativa* L was used as an Ayurvedic (Russo, 2004) and Chinese medicine (Mechoulam, 1986). In Egypt, mention of medicinal uses of cannabis was written in the Papyrus Ramesseum III (circa 1700 BCE). More detailed uses were recorded in the Ebers Papyrus (circa 1600 BCE) which describes the use of cannabis as a decoction in enemas, applications to the eye and topically in the form of medicated bandages (Mannische 1989). An archaeological discovery of cannabis in China dating back 2700 years is also supportive evidence of its early medicinal use (Russo *et al*., 2008). In Hebrew, Greek and Roman texts there are references to the sedative hypnotic uses of cannabis. These sources refer, inter alia to its use in obstetrics and gynaecological products. There are also ancient references to the inhalation of smoked cannabis. Most ancient Middle Eastern and Asian civilisations record it being smoked for medical and ritual purposes. An archaeological find in Jerusalem, from the fourth century AD, indicates the use of cannabis vapour in an enclosed environment by women during labour (Zias *et al*., 1993). In museums around the Mediterranean (e.g. Empurius North of Barcelona) there are collections of surgical artefacts, which include pipes for smoking drugs. This is at least a millennium before the introduction of smoking tobacco from the New World.

According to a joint UNICEF and WHO report (UNICEF, 1992) of the 80% of the world's population living in developing countries, only 15% had access to modern scientific medicine; the rest depended on traditional indigenous systems of health care in which herbal medicines played a part. For about three-quarters of the world population there is therefore complete reliance on plant-derived medicines. In 1976, in modern western medicine, plant derived active ingredients were still represented in up to 25% of prescription-drugs (Farnsworth and Morris, 1976). In developed countries plant-derived pharmaceuticals contribute more than $30 billion of sales revenue to the industry. Over 60% of anti-cancer drugs and 50% of cardiovascular and analgesic treatments are derived from plants (Fowler and Law, 2006).

The World Health Organisation (WHO) reported that more than 21000 plant species are regularly harvested for the production of medicines. The vast majority of these are harvested in their natural environment. This is especially the case in areas with low technological and economical development (Europam, 2006). The collection of wild plants – so called wild crafting – has many drawbacks. Over zealous collection of wild plants for medicinal use has actually threatened the existence of some species e.g. *Galanthus woronowi* for the production of galanthamine, and *Coleus forskohlii* for the supply of forskolin (Evans, 2002). Consequently this type of collection has led to some plant species receiving CITES protection to help prevent their extinction. To maximise plant quality and to minimise the impact of wild-crafting on species survival and the environment, the modern pharmaceutical industry stipulates that the harvest should comply with the Good Wild-crafting Practice (GWP) Guidelines (Europam, 2006). To maximise the sustainable yield from some threatened wild species, research has established strategies for regulating and improving harvest techniques. An example is the Chinese tree *Camptotheca acuminata* (Vincent *et al.* 1997), which is the source of the major anticancer drug camptothecin, and had a market value of $5500 million in 2006 (Fowler and Law, 2006). Wild plants are generally highly variable in their secondary metabolite content and they may at times also be in short supply. These concerns, and the threat to species survival, can at least be partly overcome by commercially growing crops for the pharmaceutical industry. Surprisingly perhaps, only about a hundred plant species are specifically cultivated for this purpose (Europam, 2006). Growing plants for medicinal use goes back many millennia. By 660 BCE, Assyrian herbalists were cultivating many plants (Baker, 2002). Pliny the Elder, in his Naturalis Historia of 77AD, described the medicinal qualities of cannabis, and detailed the recommended planting and harvest timings (Pliny, 1951).

A vast number of plant secondary metabolites form the active ingredients of modern drugs. By definition, these are organic compounds which are not directly involved in the normal growth, development or reproduction of organisms. However they add to the plant's survival chances by improving its resistance to predators, parasites and a range of environmental stresses. Some secondary metabolites are highly purified before being formulated as medicines. Others provide a starter material from which semi-synthetic drugs are produced. Examples of both types are shown in Table 1.1.

Plant-derived ingredient/ precursor	Semi-synthetic drug Product	Chemical Type	Medical Use	Plant Source
Codeine, Morphine	_	Opiate alkaloid	Analgesic	*Papaver somniferum*
Taxol	_	Diterpene ester	Antineoplastic	*Taxus Brevifolia*
Vinblastine, Vincristine	_	Bis-indole alkaloid	Antineoplastic	*Catharanthus roseus*
Podophyllotoxin	Etoposide	Lignan	Antineoplastic	*Podophyllum peltatum*
Diosgenin	Progesterone	Aglycone Steroid	Birth control	*Dioscoria sylvatica*
Camptothecin	Topotecan	Indole alkaloid	Antineoplastic	*Camptotheca acuminata*
Physostigmine	Neostigmine	Alkaloid	Cholinergic	*Physostigma venenosum*

Table 1.1 Examples of plant derived drugs and modern semi-synthetic drugs made from the secondary metabolites of outdoor grown plants.

The plant kingdom has also enabled the production of so called phytopharmaceutical or 'botanical drugs'. These are defined as well characterised, multi-component standardised drugs extracted from plant sources. The medicine Veregen™, derived from green tea *Camellia sinensis*, and approved for the topical treatment of warts (Medigene Inc.) is such an example. In 2004 the United States Food and Drug Administration issued the Botanical Drug Guidance which made it possible to bring to market a complex mixture for which evidence of adequate safety and efficacy had been established (FDA, 2004). This could result in the successful company being awarded a period of exclusivity. By 2006 Veregen™ was the only medicine to have been successful (NDA 21-902). Other botanical medicines are prescribed in some European countries but as of 2008 none were dispensed in the UK. GW Pharmaceuticals

commenced clinical trials in the US in 2008, to evaluate the efficacy of the cannabis-based botanical medicine Sativex® for the control of pain in terminal cancer patients. If trials were successful, a New Drug Application (NDA) would be sought.

1.2 Cannabis Botany

Cannabis is a tall upright annual herb. It is generally dioecious i.e. producing separate male and female plants (Figure 1.2a), but fibre hemp varieties have been specifically bred to be monoecious (hermaphrodite) (Small and Cronquist, 1976). The leaves are palmate, and in the iconic image of a cannabis leaf there are seven lobes, the lowest pair showing as backwards-facing spurs. However this number and shape is not fixed. On seedlings the first pair of leaves is typically monophylous (single lobed), the second pair having three lobes and the next pair five. In many plants, especially of central Asian origin, the number does not extend beyond five while in others the number can extend to around thirteen. Leaf size and shape differs markedly according to genetic origin, three contrasting examples being illustrated in Figures 1.1a – c.

Figure 1.1. Contrasting leaf morphology in three clones of *Cannabis sativa* L., (a) CBD chemotype G5 M16 cv Gill, (b) THC chemotype G1 M3 cv Guinevere (c) Afghan landrace clone M146 (Illustrations by Valerie Bolas, commissioned by GW Pharmaceuticals).

Cannabis is a wind pollinated species. The males, which are generally taller than the females (Figure 1.2a), commence flowering first. The specimen in Figure 1.2a was grown in still conditions and leaves appear yellow under the deep covering of pollen. When mature, the sepals on the male flowers open to expose the anthers, which hang freely on fine filaments (Figure 1.2c). The exposed anthers soon dehisce to shed pollen onto any passing air current. Shortly after the cessation of pollen production the male dies, but females from the same population will continue to mature for up to several weeks. Females produce inflorescences containing vast numbers of florets over a

period of several weeks. This period is extended if pollen is not received. An example of a well-developed inflorescence is shown at the front of this thesis (Page ii). Just as leaf shape varies according to provenance, the shape of the inflorescence does also. The inflorescences of the female can become very sticky due to a covering of resinous glandular trichomes. These are the main source of the cannabinoids, a group of terpenoid compounds unique to *Cannabis*. Consequently, the female inflorescence is the most importance plant part to those exploiting it for its recreational or medicinal properties. Part of a fertile female inflorescence is shown in Figure 1.2b, and the fertile white stigmas are clearly visible against the bracts of this purple variety.

As a result of pollination the female develops copious numbers of seeds, or more correctly achenes. These have been collected by man more several millennia for their great nutritional value, the oil produced from crushed seeds also being used for culinary purposes and to light lamps. The stems of some varieties are a rich source of fibre which has also been used by man for several millennia for the production of paper, rope, textiles and more latterly building materials and automotive parts (Wills, 1998). During the reign of Henry VIII and Elizabeth I, farmers in this country were legally required to grow hemp to ensure that the navy had sufficient rope and sail cloth (Hansard (Australia), 1996). So common was *Cannabis sativa* L in the English countryside that in his herbal of 1653 Nicholas Culpeper wrote, “This is so well known in this country that I shall not need to write any description of it”.

(a) (b) (c)

Figure 1.2. (a) Male (left) and female cannabis (right) in later stage of flowering. (b) Female cannabis inflorescence. (c) A cluster of male flowers with sepals split open and reflexed to expose the anthers.

As a consequence of its various uses, growing of *Cannabis sativa* L spread to all continents, apart perhaps from Antarctica. The original source of the species is heavily debated but is commonly thought to have evolved in central Asia in a region approximately 30° - 35°N with the Himalayas to the south, Turkestan to the west,

Pakistan to the East and Southern China as its probable northernmost extreme (Wills, 1998). This is just a few degrees south west of Western China where its closest relative the hop *Humulus lupulus* L is believed to have originated (Neve, 1991a). From these regions the two species spread and, heavily influenced by man, adapted to a range of latitudes, habitats and growing methods.

1.3 *Cannabis* Taxonomy

The binomial *Cannabis sativa* L. carries the suffix L to record that the taxonomist Carl Linneaus adopted this name in his *Species Plantarum* of 1753. However, the binomial had been used much prior to this, by Leonardt Fuchs in his Kreuterbuch of 1543. Just as Linnaeus recognised one species, most modern day taxonomists also regard *Cannabis* as monotypic, with the species as one isolated gene pool (Harlan and de Wet, 1971). Within that species several subspecies are sometimes identified (Small and Cronquist, 1976). The biological/reproductive definition of a species states that all specimens of a population are of a single species if they are naturally able to sexually reproduce, generating fertile offspring. This is the case in the genus *Cannabis,* and by this definition therefore there are no clear biological grounds to separate it into different species (Schultes *et al*., 1974). However modern *Cannabis* taxonomy remains confused, as some scientists and commentators prefer to define species according to typological or morphological characteristics. Chemotaxonomy has also been used to catagorise cannabis populations according to their terpenoid content (Hillig and Mahlberg, 2004).

In 1785 Lamark described the genus as polytypic and introduced the separate name *Cannabis indica* for plants grown in India. Such plants he regarded as being a different species to the European '*Cannabis sativa* type' based upon their different morphology, geographic range, pronounced smell and greater narcotic potency. In the twentieth century Lamark's name *Cannabis indica* came to be widely used to describe the short wide-leaved plants indigenous to Afghanistan, like the example in Figure 1c. However, re-examination of the Lamark's original *Cannabis indica* herbarium samples shows his plants to have been narrow leaved. Those modern day taxonomists who adhere to a belief that *Cannabis sativa* L and *Canabis indica* Lam were truly separate species would more likely have identified Lamark's samples as *Cannabis sativa* L. Many other species of *Cannabis* have been proposed of which just *Cannabis ruderalis* Janisch has met wide use. This name was used to identify weak low-potency ruderal (road side) plants from eastern Europe which produced small marbled achenes with a strongly constricted abscission layer (de Meijer, 1994).

The argument that *Cannabis* is polytypic gained legal significance from 1972 onwards, when an increasing number of court cases occurred in the USA, with defence lawyers challenging the taxonomy in convictions involving marijuana. United States law attributed the illegal recreational marijuana solely to the species *Cannabis sativa* L. Defence lawyers, claiming that their defendants were involved with *Cannabis indica* or other suggested species, argued that there was no case to answer (Small, 1976). Perhaps partly stemming from this challenge to the law, a large proportion of the commercial suppliers, advisors and commentators currently in the recreational cannabis industry still commonly refer to the 'species' *Cannabis indica* and *Cannabis ruderalis* in addition to *Cannabis sativa* (Snoeijer, 2002). Within this field, many appear to feel some empathy or romantic association with (or a perceived financial dependence upon) the important part that *Cannabis* has held within the anti-establishment movement. For the remainder of this thesis, the species is regarded as monotypic with the name *Cannabis sativa* L.

Within the last hundred years the taxonomy of *Cannabis* above species level has also been cause of much debate. The genus *Cannabis* has most commonly been regarded as belonging to the family Cannabaceae of the order Urticales (Raman, 1998). Taxonomists originally placed Cannabis in Urticaceae (nettle family) but in the early 20th century some moved it to the Moraceae (fig family). However, the number of dissimilarities between *Cannabis* and the nettles or figs led to this genus being allocated, in the 1960s, to the new family – the Cannabaceae – along with just one other genus *Humulus* (the hops). The existence of the family Cannabaceae has gained widespread support. However, its place in the order Urticales (of the superorder Dillenidae) has been challenged in recent years. Some taxonomists now place the family in the order in Rosales along with the Urticaceae and the Moraceae, as shown in Table 1.2.

The various suggested sub-species are described elsewhere (Raman, 1998). As stated earlier, chemotaxonomy has been used to split *Cannabis* populations between putative species and subspecies (Hillig and Mahlberg, 2004). European sources of *Cannabis*, usually grown for fibre or seed, typically contain a much higher cannabidiolic acid (CBDA) to delta-9-tetracannabidiolic acid (THCA) ratio than more tropical plants where the majority of the field grown illicit crops are raised. (Small and Beckstead, 1973a, b). It has been postulated that the biosynthesis of the cannabinoids THCA or CBDA from the common precursor cannabigerolic acid (CBGA) is controlled by the presence of co-dominant alleles at a common locus. These give rise to homozygous chemotypes with a high THCA or CBDA purity and a heterozygous chemotype

containing a THCA/CBDA mixture. All heterogenous cannabis populations will contain a mixture of all three chemotypes (de Meijer *et al*., 2003). Differences in the THCA/CBDA balance of geographically contrasting populations may be due to environmental pressures e.g. prevailing temperature (Boucher *et al*., 1974). However, the greatest effect on chemotype will be the selective pressure exerted man as a result of a local need of *Cannabis* for fibre, seed or drug purposes. The genotypes used in the research for this thesis were bred from varying putative sub-species, which originated from a wide range of provenances. Many of these genetic sources were landrace materials and others specifically-bred agricultural varieties.

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Hamamelidae
Order	Rosales
Family	Cannabaceae – Hemp family
Genus	*Cannabis* L. – hemp
Species	*Cannabis sativa* L.

Table 1.2 A suggested classification of *Cannabis sativa* L (Sytsma *et al*., 2002)

1.4 UK Medicinal Cannabis Use - History and Legal Complications.

The medicinal properties for cannabis were claimed in the UK many centuries ago. Interest expanded greatly in the nineteenth century following the research of British expatriate surgeon O'Shaughnessy, who used ethanolic tinctures of cannabis for the treatment of pain (Robson, 1999a). While working for the East India Company in Calcutta O'Shaughnessy described the methods of use of cannabis by the native population where It had a variety of applications, particularly as a sedative and analgesic. In a series of careful experiments, O'Shaughnessy defined the method of extraction and useful doses of the preparation so produced. In his monograph he described using alcohol to produce tinctures, which were increasingly imported into the UK. In addition to a description of the useful properties of gallenical preparations based on cannabis, O'Shaughnessy described some of the adverse events which

followed its use and really established the place of cannabis tincture and cannabis extract in Victorian medicine.

By the end of the century the medicinal use of cannabis was well enough established for it to be the subject of a reference in Merck's Manual (1899). This described cannabis as a hypnotic sedative and very useful for the treatment of hysteria, delirium, epilepsy, nervous insomnia, migraine, pain and dysmenorrhoea. General practitioners continued to prescribe complex cannabis formulations up until the middle of the twentieth century. Many of the central nervous system indications for which cannabis was used (analgesic, hypnotic, sedative and antiepileptic) were by then met by the benzodiazepine group of drugs and analgesics like paracetamol and codeine. While these guaranteed reliable dose control, it was difficult to obtain a consistent response to cannabis because of the variable cannabinoid content of the plant material available (Notcutt, 2004). There were also growing international concerns of social problems caused by recreational cannabis use. This, and awareness of the adverse effects of cannabis, finally led to its prohibition in the UK when the UK Government ratified the 1925 Geneva Convention on the manufacture, sale and movement of dangerous drugs. It did however remain available through pharmacies, until it was completely outlawed as a medicine by being declared a Schedule 1 substance in the Misuse of Drugs Act 1971. This act also outlawed the production and possession of cannabis for recreational purposes. Amongst three categories of decreasing seriousness described in Schedule 1 (A, B and C) cannabis was initially placed in Class B. This indicated that those caught in possession of a small or moderate quantity for personal use would likely attract a court fine. Repeat offenders and those supplying moderate quantities of cannabis would be more likely to be sentenced to a community (i.e. non-custodial penalty), while those producing cannabis and/or supplying large quantities could be imprisoned for up to fourteen years. In 2004 the UK Government reclassified cannabis as a Class C drug, which meant that possession could attract a much smaller maximum prison sentence. Possession of small quantities would be more likely to attract a police fine, without the offender needing to attend a Magistrates Court. In 2009, contrary to scientific advice from the Advisory Council on Misuse of Drugs, cannabis was reclassified as Class B (ACMD, 2008).

Despite its continuing prohibition, in the last twenty years in the UK, an increasing number of patients with severely debilitating diseases such as multiple sclerosis have used illicit cannabis to obtain symptom relief (Whittle, 2004). Smoking cannabis for recreational or medicinal reasons in the UK was almost unknown until the 1950s, although recreational cannabis smoking had become common a decade later (Robson,

1999a). Most medicinal users of illicit cannabis would have also smoked the material, although some would have ingested it in cooked form. A number of commercially available books described recipes for foodstuffs containing the drug. The organized production of such foodstuffs for alleged medicinal use resulted in two well publicized convictions in 2006. A small survey carried out by the UK newspaper *Disability Now* in 1997 reported that amongst two hundred medicinal users of illicit cannabis, 20% were using the drug to treat symptoms of multiple sclerosis and similar numbers for spinal injury and back pain (HLSCST, 1998). A more extensive UK survey amongst those using illicit cannabis medicinally received 2969 replies. It suggested that 136 diseases were being treated with cannabis, the most predominant being Chronic Pain 25%, Multiple Sclerosis 22%, Depression 22%, Arthritis 21% and Neuropathy 19% (Ware *et al.*, 2005). As these numbers suggest, some respondents were using cannabis for the relief of more than one disease. The use of cannabis for the treatment of symptoms of multiple sclerosis was well known in the UK. The high incidence of usage in treatment of migraine and the pain of rheumatoid arthritis was perhaps less expected. In the UK there was relatively little use for stimulation of appetite in sero-converted patients with HIV and AIDS, although in the USA this was a significant indication for use of both smoked cannabis and the synthetic-cannabinoid product Marinol®.

1.5 The influence of the BMA and the House on Lords Select Committee on Science and Technology on UK Cannabis Research.

In 1997 the British Medical Association published a highly-influential report on the therapeutic uses of cannabis (BMA, 1997). The report acknowledged the weight of evidence for the drug's efficacy in treating spasticity, nocturia and central pain in those with spastic conditions such as multiple sclerosis, and stated that clinical trials in this field merited a high priority. The report acknowledged that cannabinoids were undoubtedly effective as anti-emetic agents in vomiting induced by chemotherapy and anti-cancer drugs. More research was recommended to identify which cannabinoids had the optimal therapeutic profile. The undoubted analgesic effects of some cannabinoids were also acknowledged. The report cited research with cannabinoids in cancer patients which showed that the pain control and cannabimimetic effects were not inseparable (Evans, 1991). The term cannabimimetic in this context is defined as pertaining to the pharmacological properties of Δ^{-9} tetrahydrocannabinol, which was a cannabinoid identified as the main psychotropic ingredient of cannabis in 1964 (Gaoni and Mechoulam, 1966). Cannabinoids were envisaged as useful adjuncts to standard analgesics and hospices, pain control clinics and post-operative wards were suggested

as ideal settings for such research. This option was further supported by the observation that mixtures of the cannabinoid THC and the opiate morphine had exhibited synergistic analgesic activity (Cichewicz, 2004). To facilitate trials it was stated that the World Health Organisation should advise the United Nations Commission on Narcotic Drugs to reschedule certain cannabinoids under the United Nations Convention on Psychotropic Substances, and in response the Home Office should alter the Misuse of Drugs Act accordingly. In the absence of such action from the WHO, the government was advised to consider changing the Misuse of Drugs Act to allow prescription of cannabinoids to patients.

In the light of heightened interest in cannabis, and particularly the report by the BMA and the House of Lords Select Committee on Science and Technology (HLSCST) under the chairmanship of Lord Perry, was requested to examine the scientific and medical evidence to determine if there was a case for relaxing some of the existing restrictions on the medical uses of cannabis (HLSCST, 1998a, b). The Committee considered was that if there is a clinical benefit accruing from the use of cannabis or one of its constituents, then it should be regarded as a medicinal substance. Legally, under the Medicines Act, medicinal substances could only be supplied properly if they have been assessed by the Medicines Control Agency (now the Medicines Health Regulatory Agency – MHRA) and a license issued by the regulatory authority. It very strongly recommended that government should provide an environment in which interested parties could research and develop cannabis-based medicines. If their quality, safety and efficacy were adequately demonstrated, the developers should be allowed to apply for a Medicines Act License and supply the product on prescription.

One stated criticism of an otherwise excellent report was that the BMA had focused on single molecules. As pointed out, herbal cannabis contains a mixture of active compounds, more than one of which possibly contributed to the therapeutic action. Sensible patients who appeared to be benefitting from herbal cannabis did not report an equal benefit when given just one of the active ingredients. Possible synergy between active compounds was a possible explanation. The action of herbal cannabis itself still needed definition (Wall, 1998).

1.6 International Legal Attitudes to Medicinal Cannabis

The United Nations Single Convention on Narcotic Drugs Schedule IV made cannabis the subject of special restrictions 1961. Article 2 stated that individual countries should protect their public using whatever means were regarded appropriate according to conditions prevailing in their country. Protection of the public could necessitate

prohibition of the production, manufacture, export or import, trade in or possession of any such drug - except for amounts which may be necessary for medical and scientific research only (including clinical trails). In some countries it could be felt appropriate to tolerate the possession of small quantities of the drug for recreational use.

In 1998, the UK legal regime regarding medicinal use of cannabis could be described as one of the most restrictive in the world (HLSCST, 1998a). The following review of the international legal status is not intended to be comprehensive, but describes the history and current situation in a number of major countries.

1.6.1 USA

In the early 19th Century physicians in the USA (the world's largest pharmaceutical market) prescribed cannabis freely and several preparations were widely available. In 1894 the Indian Hemp Commission Report talked favourably of cannabis drugs and recommended that cannabis should be controlled through taxation and regulation rather than prohibition. However, as in Europe, newly discovered opiate-based drugs were eclipsing cannabis as a medicine and its use declined.

Through the early 20th Century recreational cannabis use in the States was commonly linked with hard-drug addiction, and it came to be increasingly regarded as an unwelcome practise favoured by undesirable elements in society. (Bonnie and Whitebread, 1970). In the 1930s Harry J Anslinger, the first commissioner of the newly formed Federal Bureau of Narcotics, cultivated society's belief that cannabis threatened to destroy the country's moral fabric. One result of this was the introduction in 1937 of the Marijuana Tax Act, which placed such draconian burdens on those attempting to use cannabis for medicinal purposes that its use almost ceased. During the 1960s opinions in the States were beginning to change. President Nixon appointed The Shafer National Commission on Marijuana and Drug Abuse to review US policy and their subsequent report, entitled *Marijuana, a Signal of Misunderstanding 1972*, recommended a relaxation in the laws controlling cannabis use. Little changed however. President Nixon had declared a 'War on Drugs' and he rejected the report before even reading it (Russo, 2003).

A number of patients with a firm belief in medicinal cannabis remained outspoken and one of these Robert Randall successfully brought a suit against the Federal Government seeking medically supervised access to cannabis. Citing 'Compassionate Use' he and others were granted legal access to 'research grade material' grown under government supervision in Mississippi. In 1983 thirty four States had enacted legislation making cancer and glaucoma patients eligible to apply for medicinal

cannabis through this scheme. However, in 1992 the Secretary for Health and Human Services closed the program for new patients saying that it 'sent the wrong message' (Mead, 2004). Seeking to drive patients away from herbal cannabis as part of its War on Drugs the federal government diverted them towards synthetic THC (dronabinol). First formulated and launched on prescription in 1985 as Marinol the federal government judged that this product had rendered smoked medicinal cannabis unnecessary. However, absorption of this orally administered drug by the gastro-intestinal tract is highly variable. In contrast to smoked cannabis, patients commonly found it difficult to titrate the dose against their symptoms (Ohlsson *et al*., 1980).

Recognising this, in 1996 the states of California and Arizona authorized seriously ill patients to use or cultivate, possess or use cannabis if recommended by a medical doctor. However, the Federal government Controlled Substances Act prohibited cannabis cultivation for any purpose and high-ranking Federal officials threatened that physicians as well as growers could face criminal prosecution. The backlash from doctors to this threat led the Drug Czar Barry McCaffrey to ask for review of the medical evidence supporting cannabis as a medicine. The report of this review *Marijuana and Medicine: Assessing the Science Base* was published in 1999 and included many recommendations in favour of cannabis (Joy *et al*., 1999). By 2008 twelve states, covering approximately 20% of the population, authorised the use of cannabis for medicinal purposes. To assist these patients, a large number Cannabis Growers Clubs were formed. However, despite this, the federal government still pursued the closure of these clubs and similar outlets with vigour. In California in 2008 over two hundred thousand patients had a written recommendation from a medical doctor supporting their medicinal marijuana use. Of these, 40% could be regarded as having a serious illness (Room *et al*., 2008). In this state the Federal Authorities appeared to be losing their battle.

In the USA, the vehicle for regulation of research into cannabinoids is the National Institute for Drug Abuse (NIDA). Most of the research, which is supported in the USA, is directed towards mode of action studies and the cataloguing of adverse effects produced by cannabis. The majority of the research is concerned with preclinical studies and very little clinical work is supported in the USA other than investigations of adverse effects on the psychological profile of recreational users. The net effect of prohibition of cannabis in the USA has been that little or no clinical research under therapeutic benefit has been carried out to date. In 2009, in the last days of the Bush Administration, there appeared to be minimal appetite within the US government for any type of cannabis reform. Against this difficult background, as stated earlier, in 2008

GW Pharmaceuticals commenced clinical trials in the US to evaluate the efficacy of the cannabis-based botanical medicine Sativex® for the control of pain in terminal cancer patients.

1.6.2 Canada

In 2001, Health Canada defined categories of patient eligible to receive access to medicinal cannabis. These included individuals with acute pain, violent nausea or other serious symptoms caused by multiple sclerosis, cancer, spinal injury or disease, AIDS/HIV, severe arthritis and epilepsy. With doctor's approval, these patients could receive cannabis grown under the name CanniMed by the company Prairie Plant Systems. It was also legal for individuals to grow *Cannabis* for personal medical use. In 2005 Sativex® received provisional approval with conditions for the treatment of central neuropathic pain in multiple sclerosis, and in 2007 for intractable cancer pain.

1.6.3 Mainland Europe

For decades, the Dutch Government has had the most relaxed attitude to cannabis use within Europe. Possession of small quantities of cannabis for personal recreational use has been tolerated. Many people used cannabis for medicinal purposes, often growing their own plants. Much of the cannabis used would have been bought from the large number of regulated 'coffee shops' that offered the sale and consumption of cannabis on their premises. Under the Guidance of the Ministry for Health, Welfare and Sport, in 2003 a new source of 'medical cannabis' became available through pharmacies in the Netherlands. This material simply consisted of unformulated dried cannabis floral material. It contained high levels of THC and minimum amounts of other cannabinoids. The cannabinoid content was stated as being within a tightly specified range and the product was sterilised using γ-radiation. The product was much more expensive than similar, albeit less-well regulated, floral material on sale in coffee shops. Perhaps because of this, consumption was much lower than government predictions and the continued supply of this material was threatened.

In November 2006, the Ministry stated that the German and Italian governments were interested in accessing the Dutch medicinal cannabis. On 9th July 2008, the Austrian Parliament approved *Cannabis* cultivation for scientific purposes under the Health Ministry's control. Possession of cannabis for recreational purposes remained an imprisonable offence. Spain has undergone a progressive decrimilisation with regard to cannabis possession. In 2001 the Catalonia region permitted the possession of cannabis for medicinal purposes and Sativex® became available in that area.

Early in 2009 the results of a Phase III clinical trial showed that Sativex® was significantly efficacious in treating spasticity in multiple sclerosis. This triggered an application for the regulatory approval for Sativex in the UK and mainland Europe.

1.6.4 Ireland

Cannabis is not recognised as having any medical benefits according to Irish law (Misuse of Drugs (Designation) Order (S.I. 69/1998). By European standards, Irish Courts have treated medicinal users of illicit cannabis harshly. In 2003 however, the Irish Medicines Board permitted clinical trials to be performed to evaluate Sativex®.

1.6.5 Australia

In Australia, laws differ between states. Those caught in possession of cannabis of up in New South Wales would only receive a fine if found with less than 15 g. Other states are more lenient, up to 50 g justifying a fine in Queensland, Tasmania and Victoria (Lenton, 2004). Cultivation of cannabis plants similarly attracts differing sentences, and in the Northern Territory is decriminalised. In January 2009, a four year trial commenced to evaluate the medical use of cannabis to treat chronically or terminally ill patients.

1.6.6 Japan

Cannabis possession in Japan is illegal for both recreational and medicinal use, and hefty fines and imprisonment are imposed. However, the Otsuka Pharmaceutical Company Ltd is collaborating with GW Pharmaceuticals Ltd to research a range of the cannabinoids for use in oncology and CNS ailments. The production of some of these cannabinoids is described in this thesis.

1.7 The choice of active pharmaceutical ingredients (APIs)

At the commencement of this thesis in autumn 2003 the sponsoring company (GW Pharmaceuticals Ltd) was propagating two feedstocks to produce the medicine Sativex®, which was undergoing Phase III clinical trials. One of these studies showed that this medicine significantly reduced spasticity symptoms in patients with multiple sclerosis (Wade *et al*., 2004). Following further studies (Barnes *et al*., 2006), the medicine became available in Canada in 2005 for the treatment of central neuropathic pain in multiple sclerosis and in 2007 for intractable cancer pain. These separate feedstocks were the dried floral and foliar material of two very different chemotypes, which contained predominantly Δ^{-9} tetrahydrocannabinolic acid (THCA) or cannabidiolic Acid (CBDA) as the active ingredients. These terpenophenolic compounds are so called *cannabinoids* and are unique to cannabis (Turner *et al*., 1980). A large

proportion of this thesis concerns the propagation, characterisation and optimization of these two chemotypes. At the completion of this thesis in 2009, Sativex® had received conditional regulatory approval for use against neuropathic pain and cancer pain in Canada. The medicine was also available in parts of the European Union as an unlicensed medicine. In addition to Δ^{-9} tetrahydroxycannabinol and cannabidiol, Sativex® also contains other cannabis derived ingredients, including additional cannabinoids and terpenes. The acceptance of Veregen™ as a botanical medicine in the US opened the door to the testing there of cannabis-based Sativex®, and the product was subsequently approved for testing as a treatment for cancer pain, under the US Adopted Name (USAN) Nabiximols.

The decision to formulate a medicine based on these two cannabinoids was due to several factors. Numerous *in vitro* and *in vivo studies* had shown that THC and CBD exhibited high levels of pharmacological activity, although the mode of action of the two differed markedly. (The activities of the two were compared and contrasted in a review by McPartland and Russo (2001)). The aim of mixing the two cannabinoids was not simply to draw additive benefit from the individual properties of the two molecules. CBD was suspected of being able to attenuate the psychoactive effects of THC, which were undesirable in a medicine (BMA, 1997). A growing weight of evidence also showed that mixtures of THC and CBD offered improved efficacy over THC-alone. Phytomedicines could be produced which contained both these cannabinoids, and these would potentially contain other additional active natural ingredients, including the terpenes and flavonoids (Musty, 2004).

Due to the predilections of smokers, drinkers, tea-totallers and coffee lovers the three most commonly used legal drugs are nicotine, alcohol and caffeine (Robson, 1999). In each case the flavour of the plant-derived source is part of the enjoyment of these drugs. Cannabis is perhaps unique amongst illicit drugs in that, for some users, the wide range of tastes produced by the various sources is a similarly important part of the 'cannabis experience' (Rosenthal, 2001). The ingredients having the greatest effects on the cannabis taste would most probably be the fragrant terpenes within the essential oils. Some of these have their own pharmacology and have been cited as likely synergists in mixtures with cannabinoids (McPartland and Russo, 2001). The potential benefit of these ingredients was demonstrated in a test measuring pain relief in mice, in which unknown powerful synergists produced a 330% increase in activity compared to THC alone (Fairbairn and Pickens, 1981). Synergistically improved efficacy of cannabis extracts over THC-alone was also demonstrated in a mouse model which assessed their antispacticity effects (Williamson, 2001). In subsequent research cannabis

extracts also showed a significantly increased antihyperalgesic effects compared to CBD-alone when tested in a rat model of neuropathic pain (Comelli *et al*., 2008). The potential benefits for mankind were supported by the observation that patients taking synthetic derivative nabilone for neurogenic pain actually preferred cannabis herb and reported that it relieved not only pain but the associated depression and anxiety (Williamson and Evans, 2000). Reasons suggested included the more rapid absorption through the lung than the gut; the presence of other ingredients in plant-derived cannabis which might give additive or synergistic effects; and the ability of smokers to self-titrate their dose (Grinspoon and Bakalar, 1995).

The decision to evaluate THC/CBD mixtures as a potential medicine was further supported by the knowledge that the majority of cannabis used in the UK at the time – at least for recreational purposes – was in the form of cannabis resin. It was suspected, but not confirmed, that the majority of anecdotal reports for the efficacy of cannabis in the UK would be based upon this material. Studies in the USA had shown that cannabis resin (hashish) typically contained approximately equal quantities of both cannabinoids, whereas herbal cannabis (marijuana) contained predominantly THC (ElSohly *et al*, 1984).

A series of clinical trials was subsequently planned which would require the propagation chemotypes dominant in cannabinoids other than THC and CBD. Some of these are also discussed in this thesis.

1.8 Cannabinoid and terpene biosynthesis

The cannabinoids of greatest initial interest in this research, Δ^{-9} tetrahydrocannabinol and cannabidiol are referred to as 'pentyl' cannabinoids, due the presence of a five-carbon chain attached to the aromatic moiety (Figure 1.3). Other pentyl cannabinoids frequently discussed in this thesis are cannabichromene (CBC) and cannabigerol (CBG). All four have important propyl analogues. The first step in the synthesis of pentyl cannabinoids is the condensation reaction of geranylpyrophosphate (GPP) with olivetolic acid. The resulting product CBGA is the direct precursor for the three major pentyl cannabinoids tetrahydrocannabinolic acid (THCA) (Fellermeier *et al.*, 2001), cannabidiolic acid (CBDA) (Taura *et al.* 1996) and cannabichromenic acid (CBCA) (Gaoni and Mechoulam, 1966). The enzymes affecting the synthesis of these three cannabinoids are named THC synthase, CBD synthase and CBC synthase respectively. The first step in the synthesis of propyl cannabinoids is the condensation reaction of geranylpyrophosphate (GPP) with diverinic acid. The resultant product

cannabigerovaric acid (CBGVA) is the direct precursor of the propyl analogues of the three aforementioned cannabinoids ie THCVA, CBDVA and CBCVA.

When herbal cannabis is dried stored and heated, these cannabinoid acids decarboxilize gradually or completely to the neutral forms (e.g. THCA → THC) (de Meijer *et al.* 2003). Complete decarboxilation of the acid form of the cannabinoids occurs during the analytical process, when using gas chromatography (Brown, 1998). To varying extents, and according to storage conditions, these cannabinoids undergo oxidative catabolism with the production of a range of additional cannabinoids. Some of these (eg CBN) have their own reported pharmacological activity and in some cases interact with other cannabinoids (Pertwee, 1998, Wilkinson *et al*, 2003). The principle active cannabinoids, their precursors and catabolites are listed in Table 1.3.

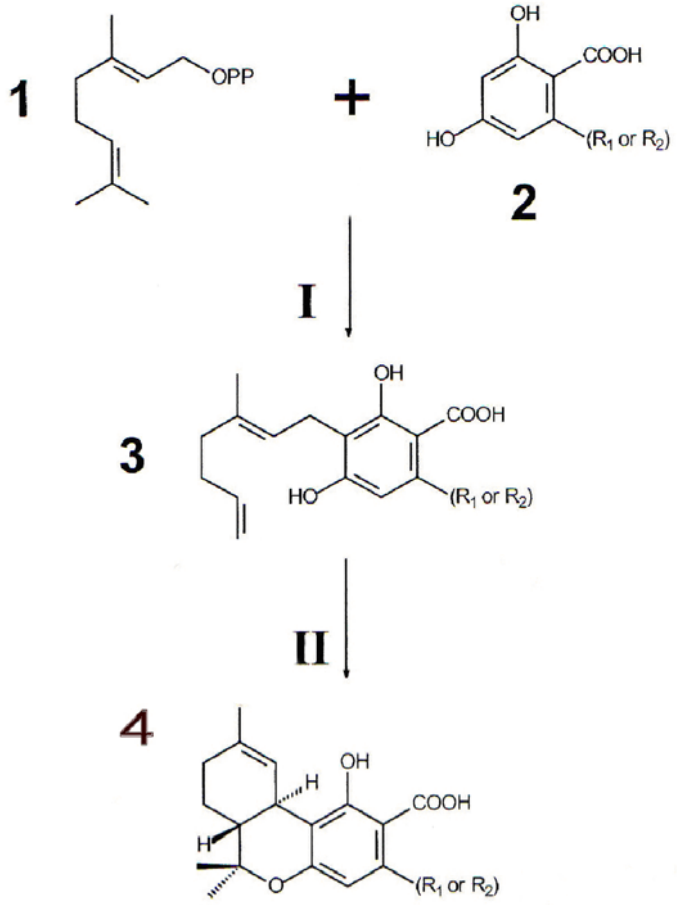


Figure 1.3. Biosynthetic pathway of THC and THCV, via CBG or CBGV. 1, Geranylpyrophosphate; 2, Divarinic Acid (R1) or Olivetolic Acid (R2); 3, Cannabigerovarin (CBGV) (R1) or Cannabigerol (CBG) (R2);. 4, Δ^9- tetrahydrocannabivarin (R1) or Δ^9-tetrahydrocannabinol (R2). R1 (-C3H7) and R2 (-C5H11) indicate the propyl or pentyl forms of the metabolites; Enzyme I: geranylpyrophosphate:olivetolate geranyltransferase(GOT); Enzyme II: THC(V) synthase.

Although the cannabinoids in fresh plant material exist in the acid form (THCA, CBDA etc.,) it is common practice to simply refer to these cannabinoids in their neutral form (THC, CBD etc.). In this thesis, that convention applies unless the acidity of the cannabinoid needs to be highlighted.

Initial Biosynthetic Product	Second Biosynthetic Product	Decarboxylated Product	Principle Initial Oxidative Catabolite
CBGA Cannabigerolic Acid		CBG Cannabigerol	–
	THCA Tetrahydrocannabinolic acid	THC Tetrahydrocannabinol	CBN Cannabinol
	CBDA Cannabidiolic Acid	CBD Cannabidiol	CBS
	CBCA Cannabichromenic Acid	CBC Cannabichromene	CBL Cannabicyclol
CBGVA Cannabigero-varic Acid		CBGV Cannabigerovarin	-
	THCVA Tetrahydrocannabivarinic acid	THCV Tetrahydro-cannabivarin	CBNV Cannabivarin
	CBDVA Cannabidivanic acid	CBDV Cannabidivarin	CBSV
	CBCVA Cannabichromevarinic acid	CBCV Cannabichromevarin	CBLV

Table 1.3 The predominant cannabinoids found in *Cannabis sativa* and their main catabolites.

The ingredients in cannabis suspected of contributing to the synergistic pharmacological effects include the main constituents of the essential oil – the monoterpenes and sesquiterpenes. The precursor of cannabinoid biosynthesis GPP also reacts with a range of other structures to produce the monoterpenes. These are a very diverse chemical group, which includes cyclic and acyclic structures. They are relatively volatile and the main source of essence within the essential oil. In addition to the 'true' monoterpenes ($C_{10}H_{16}$) there are also a series of increasingly more oxidized families within the group. These include the alcohols (e.g. linalool $C_{10}H_{18}O$), ethers (e.g. 1,8-cineole $C_{10}H_{18}O$), esters (e.g. bornyl acetate $C_{12}H_{20}O_2$), aldehydes (e.g. citral $C_{10}H_{16}O$) and ketones (e.g. pulegone $C_{10}H_{16}O$). All of these were amongst fifty eight

monoterpenes identified in cannabis in a detailed study by Turner *et al.* (1980), and these were accompanied by thirty eight sesquiterpenes. The latter are reported to be formed from a dominant precursor farnesyl pyrophosphate (FPP). Both FPP and GPP are derived from isopentenyl pyrophosphate (IPP). The cannabinoids, sesquiterpenes and monoterpenes therefore all share a common precursor in IPP. As with the monoterpenes, the true sesquiterpenes ($C_{15}H_{24}$) are also accompanied by series of more oxidized related structures. Higher molecular weight terpenes are also present, the next group within the 'terpenoid skeleton family' being the diterpenes. These are not volatile and not generally considered active constituents of an essential oil. The only significant example in cannabis is phytol which is a precursor and catabolite of chlorophyll and tocopherol Vitamin E, both of which are abundant in cannabis. Phytol has been shown to exhibit antispasmodic activity (Pongprayoon *et al.*, 1992) and to elevate the neurotransmitter GABA levels in central nervous system (Bang *et al.*, 2002). It too, may contribute to the pharmacological properties of cannabis based medicines.

Two core pathways of IPP biosynthesis have been identified. The first of these, the acetate/mevanolate pathway, operates within the cytosol (the fluid component of cytoplasm) while the second and more recently discovered pyrovate/glyceraldehyde-3-phosphate pathway takes place within the specialized membrane-bound subcellular organelles called plastids (or more specifically leucoplasts) (Hallahan and Gray, 2000). These pathways are outlined in Figure 1.4. Terpene biosynthesis has been reviewed in detail by Croteau and Johnson (1984) and is not repeated here.

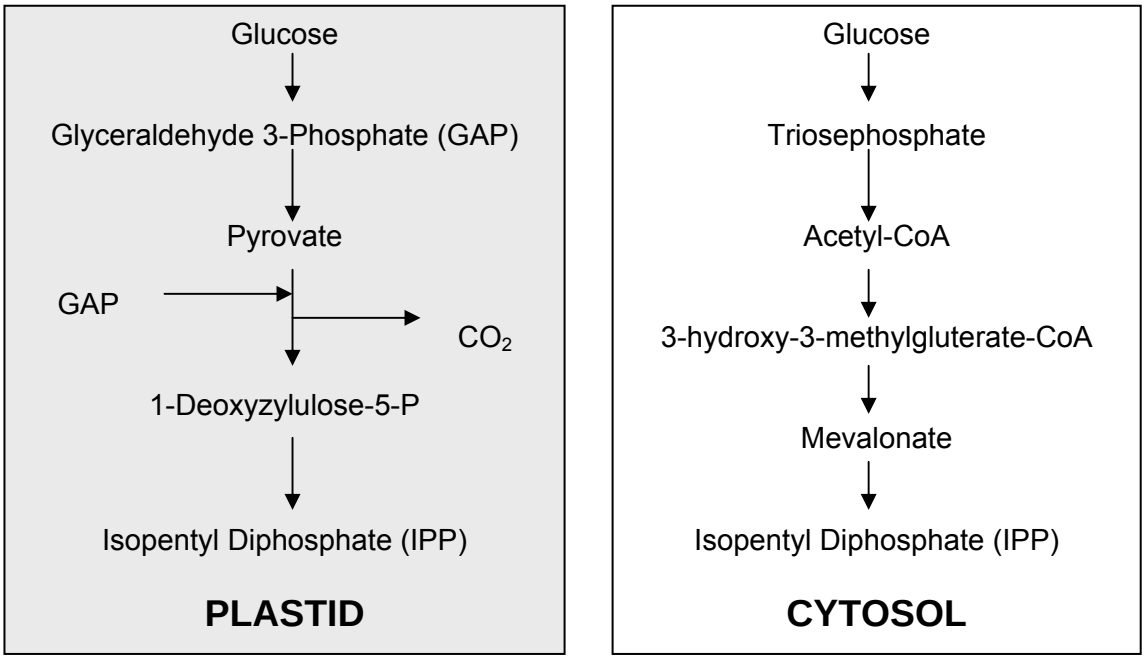


Figure 1.4. The two pathways of isopentyl diphosphate (IPP) biosynthesis in plants, as found in the plastid and cytosol respectively.

1.9 Cannabinoid Receptors and Cannabinoid Pharmacology.

The understanding of the pharmacodynamics of the cannabinoids was greatly enhanced when specific cannabinoid receptors were identified in mammalian brain (Devane *et al*., 1988) and subsequently cloned (Matsuda *et al*., 1990). Named CB_1 receptors (Appendix 3a), these were identified in the central nervous system (CNS) and certain peripheral tissues. These were identified as belonging to a so-called superfamily of G-protein-linked receptors (Matsuda *et al*., 1990). G-protein-linked receptors characteristically exhibit a seven-folded structure which spans the cell's plasma membrane. In some such receptors the ligand attaches to a domain (a section of the polypeptide chain) that is entirely exposed on the outer surface of the cell. Others ligands will have domains which include parts of the chain deep within the cell membrane. The binding of a ligand signals any one of several activities within the cell. Over half of all known drugs work through G-protein receptors (Alberts *et al*., 2002). These receptors include three protein sub-units – α, β and γ. In the unstimulated state of the so-called 'two state model of constitutive activity' no ligand is bound to the receptor 'R' and guanosine diphosphate (GDP) is bound to the α-subunit. This conformation the G protein is termed RG_{GDP}. Upon stimulation, this sub-unit releases its GDP allowing Guanosine 5'- triphosphate (GTP) to bind in its place (Figure 1.5). This conformation of the G protein is termed R^*G_{GTP} (Ross, 2007). As a consequence, the αβγ complex dissociates into an α subunit and a βγ complex. The now-free α subunit is now able to change its shape and interact with target proteins. The α subunits have intrinsic slow hydrolase activity, and as a result the GTP spontaneously hydrolyses to GDP with the release of one phosphate moiety. In doing so, the G-protein resets itself back to the rest position. Within the cell this 'two state' model of constitutive activity suggests that an equilibrium exists: -

$$RG_{GDP} \leftrightarrow R^*G_{GTP}.$$

The balance of these two forms signals a series of separate reactions within the cell. These include alterations to the cyclic adenosine 5'- monophosphate (cAMP) concentration and to the activity of potassium and calcium channels.

Δ^9-THC interacts with the CB_1 receptors as a ligand, causing psychoactive effects (BMA, 1997). A second type, the so called CB_2 receptor (Appendix 3b), was subsequently discovered in spleen macrophages and found not to be present in the CNS (BMA, 1997; Pertwee, 1997). The natural function of the cannabinoid was more easily explained with the discovery of endogenous substances within mammalian tissue which interacted with these receptors. These have been termed as

endocannabinoids to differentiate them from the plant-derived cannabinoids, which are sometimes called phytocannabinoids (Pate, 1994).

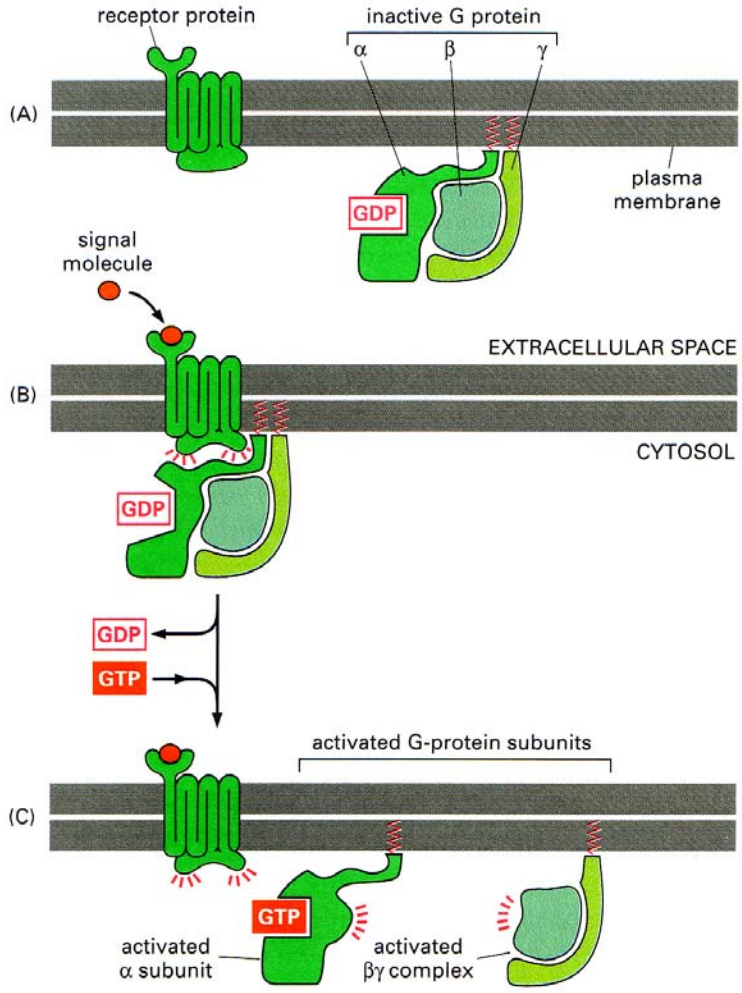


Figure 1.5. The disassembly of an activated G-protein into two signalling components. (Alberts *et al*., 2002)

The first of the endocannabinoids was discovered to be the arachidonic acid derivative arachidonylethanolamide in 1992, and subsequently named anandamide (Devane *et al.* 1992). In addition to the phytocannabinoids and endocannabinoids a number of synthetic cannabinoid ligands have also been manufactured. These include chemicals closely related to the phytocannabinoids such as the Δ^{-9} THC analogue nabilone (Cesamet) and the *nonclassical cannabinoid group* which includes the compound CP 50556 (levonantradol). The pharmacological activity of many of these cannabinoids has been assessed *in vitro* and *in vivo*. In the former case, this has included an assessment of the test cannabinoid's ability to block or reverse the effects of another

standard receptor antagonist (Pertwee, 1997). This method has been used to assess extracts from many of the chemotype studied for this thesis.

The interaction of phytocannabinoids or endocannabininoids with cannabinoid receptors is illustrated schematically in Figure 1.6. In addition to being present in the central nervous system and throughout the brain, CB_1 receptors are also found in the immune cells and the gastrointestinal, reproductive, adrenal, heart, lung and bladder tissues. By altering the cell activity within such a wide range of tissues, several prominent pharmacological responses result from the agonism of CB_1 receptors, including both the control of nociceptive and neuropathic pain. CB_2 receptors are thought to have immunomodulatory effects and to regulate cytokine activity and thereby altering cell-to-cell communication. In combination with CB_1 receptors, they show pain control effects (Pertwee, 2004).

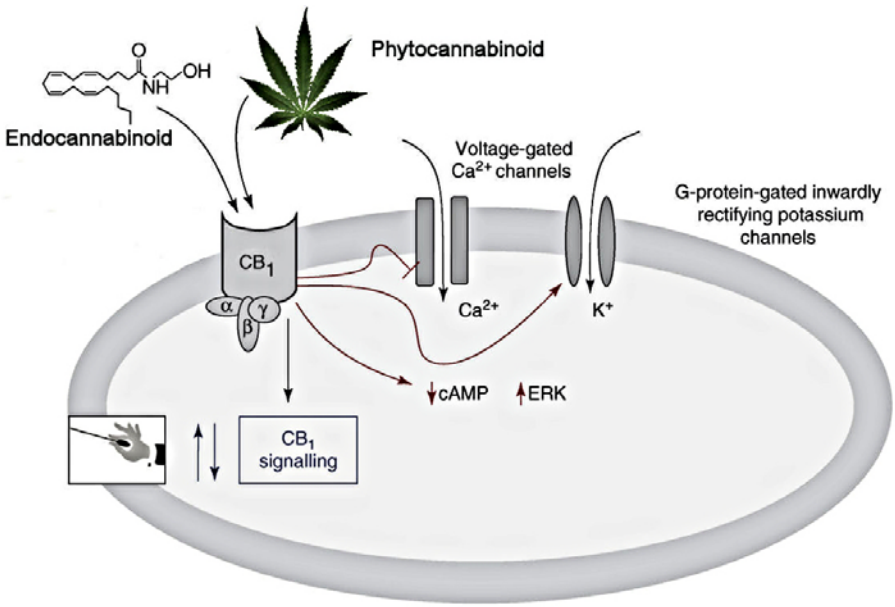


Figure 1.6. The interaction of phytocannabinoids and/or phytocannabinoids with the CB_1 receptor in the eukaryote cell and the consequent effects on cAMP and ERK activity and ion channelling. Modified from Ross (2007).

While many of the pharmacological properties of cannabis can be explained by interactions of its ingredients with the CB_1 and CB_2 receptors, not all of the effects can be explained this way. Cannabidiol has shown significant levels of antipsychotic activity, which are seen as advantageous in medicines containing Δ^{-9} THC, but this cannot be totally explained by interactions with the cannabinoid receptors as CBD is a weak ligand (Iversen, 2008). Both CBD and Δ^{-9} THC have been shown to have equally potent neuroprotective antioxidant properties in rat cortical neuron cultures, where

glutamate otherwise reached toxic levels (Hampson *et al.*, 1998). Indeed, the antioxidant abilities perhaps indicate a natural function of these cannabinoids in plant tissues.

In 1998 an important trial was performed by the Royal Pharmaceutical Society of Great Britain, in collaboration with the UK Medical Research Council. This *CAMS* study (cannabis in MS) involved 630 patients and explored the effects of synthetic THC (Marinol) and a cannabis extract "Cannador" given orally on spasticity and other symptoms related to multiple sclerosis (Zajicek *et al.*, 2003). The results of the study were mixed, and a large placebo effect was noted, but both active treatments demonstrated significant improvements in subjective measures of spasticity, muscle spasms, pain and sleep, and also in an objective measure of mobility. No effect was apparent on irritability, depression, tiredness, tremor or loss of energy. The authors noted an unexpected reduction in hospital admissions for relapse in the two active treatment groups. The known interaction of cannabinoids with the immune system, and the fact that MS was still regarded as an auto-immune condition, led them to comment that this finding was worthy of further investigation.

A highly significant recent finding was the observation that β-caryophyllene, a major sesquiterpene in cannabis, selectively binds to the CB_2 receptor and is a CB_2 receptor agonist with anti-inflammatory activity *in vivo* (Gertsch *et al.*, 2008).

1.10 Aims and Outline of Thesis

Supported by the GW Pharmaceuticals plc, this thesis was performed to improve the reliable production of phytopharmaceutical feedstock for the production of botanical medicines from Cannabis sativa L. As the title of this thesis suggests, the challenging research discussed here was multifaceted and covered a broad range of scientific disciplines. The majority of the work was divided between a pharmaceutical research laboratory and a glasshouse, but it also necessitated much involvement with the agricultural industry and law enforcement agencies. The research had five broad aims, as covered in Chapters Two to Six.

The first botanical medicine from GW Pharmaceuticals to be widely tested in clinical trials contained the two cannabinoids THC and CBD in approximately equal quantities. A significant reason for choosing an equal mixture of these two cannabinoids was the supposition that this balance existed in most of the illicit cannabis in the UK. Chapter Two describes research that investigates what cannabinoid profile actually existed in illicit material. For many patients in the UK, such material was the only form available. Indeed, the anecdotal evidence that many multiple sclerosis patients were finding this

illicit material to be efficacious was a major consideration in allowing GW Pharmaceuticals a license to evaluate cannabis based medicines. Despite this, little was known of its cannabinoid profile. As stated at the 1998 House of Lords Select Committee on Science and Technology enquiry into Cannabis, the action of herbal cannabis needed defining (Wall, 1998). The suspected 1:1 THC:CBD ratio in UK cannabis was based on research into the cannabinoid profile of illicit material in the USA. No such large scale research had been performed here in the UK. Seeking in part to check if a one to one ratio of the two cannabinoids was indeed the typical cannabinoid profile of 'street cannabis', Chapter Two describes work to evaluate the cannabinoid content of this material in what was the first such large scale UK study. In addition to supporting the business aims of GW Pharmaceuticals plc, the research also provided an insight into the apparently escalating potency of UK cannabis, which was being increasingly linked with psychotic disorders amongst Britain's youth. This data was eagerly welcomed by the scientific, medical and law enforcement communities. The co-ordination of the collection of the illicit samples from a number of constabularies necessitated much police time.

The cannabinoids and most terpenes in cannabis are synthesised in small structures called glandular trichomes. A pharmacognosist could reasonably argue that they are the most important part of the plant. Chapter Three looks in depth at the differing structures of Cannabis trichomes, with the aim of improving the knowledge of their form and function. Although not all types formed cannabinoids, the inactive non-glandular types would frequently occur as contaminants when collecting the glandular types for research purposes, and they needed to be routinely identified. The aim of the work in Chapter Four was to compare and contrast the different glandular trichome forms and examine their secondary metabolite profiles. The effect of the state of trichome maturity on the cannabinoid content and profiles was also investigated. Techniques are evaluated in which trichomes are separated so as to exert some control over the cannabinoid profile within a phytopharmaceutical feedstock.

From a purely botanical perspective it was essential to gain a knowledge of how the plant develops. From a horticultural perspective it was imperative to learn how to propagate plants that were healthy and high yielding. This latter aim is shared by growers of illicit cannabis, but the grower for the pharmaceutical industry has the additional aim of learning how to repeatedly propagate crops that were uniform in secondary metabolite content. Chapter Five describes the research to address these aims in an indoor environment. Chapter Six addresses the same aims but in an agricultural setting. Although the research is reported in sequence in Chapters Two to

Six, the differing areas of study were performed in parallel. Growing a crop through to harvest takes time, especially in an outdoor environment. The horticultural and agricultural investigations took several years to complete.

Addressing a seminal cannabis symposium in 1969, organised by the Institute for the Study of Drug Dependence, the internationally renowned expert Dr. R.E. Schultes of Harvard University stated that “A thorough understanding of *Cannabis sativa* L as a plant must be basic to progress in studies of its derivatives and their significance to man and their effects on life and social evolution” (Schultes, 1970). The author of this thesis very much shares that view and this motivated and influenced the studies reported here.

Chapter 2 Characterisation of Illicit Cannabis in the UK

2.1 INTRODUCTION

It is often stated that a drug is a substance with the ability to interact with the metabolism of an animal (usually man). A medicine however is a beneficial drug formulated in such a way as to optimise its absorption and performance. The cannabis-based medicine Sativex® is a medicine formulated as a sub-lingual spray. In ancient civilisations the medicinal benefits of cannabis are achieved by smoking the dried plant material or resin. Much of the 'medicinal cannabis' used around the world is supplied as an unformulated drug. To achieve the desired effect, this material would normally be smoked, but it could sometimes be vaporised or ingested.

Smoking cannabis for recreational or medicinal reasons in the UK was almost unknown until the 1950s, but recreational cannabis smoking had become common a decade later (Robson, 1999a). Most medicinal users of illicit cannabis would have also smoked the material, although some would have ingested it in cooked form. Organized production of foodstuffs containing cannabis for alleged medicinal use resulted in several criminal convictions in the UK in recent years. Although thought to be widespread, until now the true extent and pattern of medicinal use of illicit cannabis in the UK was not known. A number of surveys have been performed to gain a better knowledge of this activity. Consroe *et al*. (1997) asked people with MS to describe which of their symptoms were relieved by smoking cannabis. Spasticity and muscle pain were reported to show the greatest improvement (97% and 95% of responders). The published results did not report what form of cannabis was typically used.

In what was thought to be the most extensive survey of illicit cannabis use for medicinal use among chronically ill patients, Ware *et al*. (2005) reported the effects of cannabis in 2969 candidates. The patients were not selected randomly, or by any systematic procedure, and the study is therefore skewed towards highly motivated responders. 82% of users smoked the cannabis and 43% reported eating it. The survey invited respondents' to report their preference for cannabis resin or herbal cannabis (including sinsemilla). The replies were not included in the published report. However, a random sub-sample of 500 unnamed reply forms was acquired from the authors. Just 72 of the 500 responders had answered this question. Of these, 50 (69%) expressed a preference for 'herbal cannabis', 16 preferred resin (22%) and 5 (7%) were satisfied with both. One respondent reported a preference for 'cannabis oil'. The term 'herbal cannabis' in this context was used to collectively categorize materials described by

respondents as ‘herbal’, ‘skunk’, ‘bud‘, ‘leaf’, ‘marijuana’ and similar terms. It was plain from these replies that several different forms of cannabis were being used. It can be assumed that these would have varied greatly in cannabinoid content and profile. Without further research it could not be fully explained why some users preferred one form of cannabis over another. The British Medical Association publication *Therapeutic uses of cannabis* (1997) listed the pharmacological properties of the phytocannabinoids and recognised that the illicit cannabis circulating in the UK was a very inconsistent product and its THC content varied widely. This was confirmed by King *et al*. (2004) in the most detailed study of UK cannabis potency to date. However, the content of CBD and other cannabinoids in illicit UK cannabis remained little studied.

In recent years Cannabis has been by far the most commonly used illicit recreational drug in the UK (Szendrei, 1997). In the early 1970s, resin consistently accounted for about 70% of cannabis seizures in England and Wales but from 1997 to 2002 the proportion fell yearly from 72% to 53% (Mwenda *et al*., 2005). By the following year, resin appeared to have been surpassed by locally grown cannabis as the main form of used (Hough *et al*., 2003). This change would be expected to affect the balance of THC, CBD and other cannabinoids in those cannabis materials circulating. In addition to having significant implications for the pharmacological properties of street cannabis, changes in CBD also had the potential to affect its psychoactive potential.

It was highlighted by Smith (2005) that future studies of potency in the UK cannabis should include an assessment of CBD as well as THC levels. This was reiterated by the United Nations Office on Drugs and Crime in the World Drug Report 2006 (UNODC, 2006). Both publications stated that CBD was a cannabinoid with antipsychotic activity, having the potential to alter the potential harm attributable to the recreational use of THC. Neither publication however was concerned how CBD might affect the pharmacological properties of illicit cannabis. The term ‘potency’ in this context refers entirely to the concentration of cannabinoid in a specified sample. This meaning of potency is widely accepted in forensic science (e.g. ElSohly *et al* 1984). However, in pharmacology potency is a measure of drug activity expressed in terms of the amount required to produce an effect of given intensity (Page *et al* 2006). In this chapter potency is defined as a concentration of cannabinoid.

In the absence of a prescribable efficacious cannabis-based medicine in the UK, illicit cannabis continues to be used widely for medicinal purposes. However, the cannabinoid content and pattern of use of this material has not been comprehensively studied.

2.2 AIM and OBJECTIVES

The aim of this part of the study was to gain an understanding of the cannabinoid content and variability of illicit cannabis circulating in England. Within this study there were a number of objectives.

2.2.1. The study would initially assess how the market was split between cannabis resin, herbal cannabis and sinsemilla.

2.2.2. The cannabinoid profile of these different types would be measured and compared, to gain an understanding of the potential efficacy and safety of illicit cannabis – when used for medicinal or recreational purposes.

2.2.3. The potency of samples would be compared to previous data, generated by the Forensic Science Service. The existence of any trends in THC content would thus be identified.

2.3 MATERIALS

2.3.1 Cannabis samples

The samples analysed from this study had been seized by police in Derbyshire, Kent, London Metropolitan (SE1 area), Merseyside and Sussex between 2004 and 2005.

2.3.2 Microscopy, Photography and other Apparatus

The microscopes and associated apparatus for this study are shown in Table 2.1.

Apparatus	Source
Photonic PL2000 - double arm cold light source. MX3 Low Power Light Microscope	Brunel Microscopes Unit 12 Enterprise Centre, Bumpers Industrial Estate Bumpers Way, Chippenham, Wiltshire. SN14 6QA
High Power Stereo Light Microscope with Trinocular Head for camera attachment.	STE UK Ltd., Staplehurst Rd Sittingbourne, ME10 2NH
Colour Charts	RHS Enterprises Ltd, RHS Garden, Wisley, Woking, Surrey, GU23 6QB

Table 2.1. Photographic, microscopy and other miscellaneous items and commercial sources.

2.4 METHODS

2.4.1 Collection of Representative Samples

The collection of samples was coordinated with the assistance of the South East Government Home Office. Authorization was gained from the Chief Constables of Derbyshire, Kent, London Metropolitan, Merseyside and Sussex. This spread of constabularies would capture populations of differing socio-economic conditions and varying influences of local ports. To obtain a pool of samples which most accurately represented the illicit material circulating amongst cannabis users, police were requested to forward materials that had been seized within the last year, during street arrests or while raiding the property of minor drug suppliers. To help prevent any skewing of the data towards imported material, the study excluded large seizures from those arrested for smuggling cannabis into the country. Such materials would have likely entered the cannabis market higher up the supply chain, and not been offered directly to individuals purchasing cannabis for their own use.

2.4.2 Storage of illicit cannabis samples

Upon arrival, samples were individually numbered and stored in darkness at ambient temperature in 30-35% RH. All were analysed within 28 days of arrival.

2.4.3 Categorisation of the form of each sample

The materials were assessed visually, using a Brunel MX3 low power light microscope where necessary, and the form of the cannabis sample was established. Four categories were identified:

2.4.3.1 Cannabis resin

Figure 2.1. Examples of cannabis resin samples (<1g up to 230g) seized by police in 2004/5.

Cannabis resin consists of the glandular trichomes and other fine particles collected from the inflorescences and upper leaves. The material is compressed into hard blocks prior to importation (Raman, 1998). All samples were dark brown in colour (Figure 2.1). These varied in shape and size and, when present in sufficient quantity, generally had a light characteristic odour.

2.4.3.2 Herbal cannabis

As adopted by King *et al*. (2004), the term 'herbal cannabis' was used only to include imported dried plant material collected from outdoor grown plants. The material was light to dark brown in colour. The glandular trichomes were always brown, due to ageing (Mahlberg *et al*., 1984). Seeds were frequently present. The material was sometimes in loose form (Figure 2.2a), but was also frequently encountered in hard blocks (Figure 2.2b) where it had been compressed to reduce volume during importation. The material had a light fragrant odour. Fungal mycelium was occasionally visible, suggesting that decay had occurred at some point during importation or storage.

(a) (b)

Figure 2.2. (a) Loose herbal cannabis material showing separated seeds; (b) Compressed herbal cannabis material with selection of removed seeds.

2.4.3.3 Sinsemilla

The sinsemilla form of cannabis was light green or grey-green in colour. The material consisted of resinous female floral material only. Close examination often revealed where bracts and leaves had been physically removed. Large intact sections of inflorescence, up to several grams in weight, were sometimes present. More commonly, the material had been broken into smaller pieces and packaged into small packets for marketing (Figure 2.3). Seeds were always absent as a result of the all-female crops being grown without exposure to pollen. The glandular trichome colour varied between crystal clear, white and light brown. The odour was clearly stronger

than that of resin and herbal cannabis. There was no visible sign of fungal deterioration. This pungent, light material was generally regarded as having been grown in the UK, but some could have entered the UK from mainland Europe.

Figure 2.3. A typical confiscated sample of illicit sinsemilla cannabis consisting of three separate packets, each containing approximately one gram.

2.4.3.4 Cannabis powder

Some herbal cannabis and sinsemilla samples were recovered from portable cannabis grinders. These are used to break herbal cannabis and sinsemilla into a suitably fine texture for smoking. More complex grinders included a fine metal mesh within the construction, as shown in Figure 2.4b.

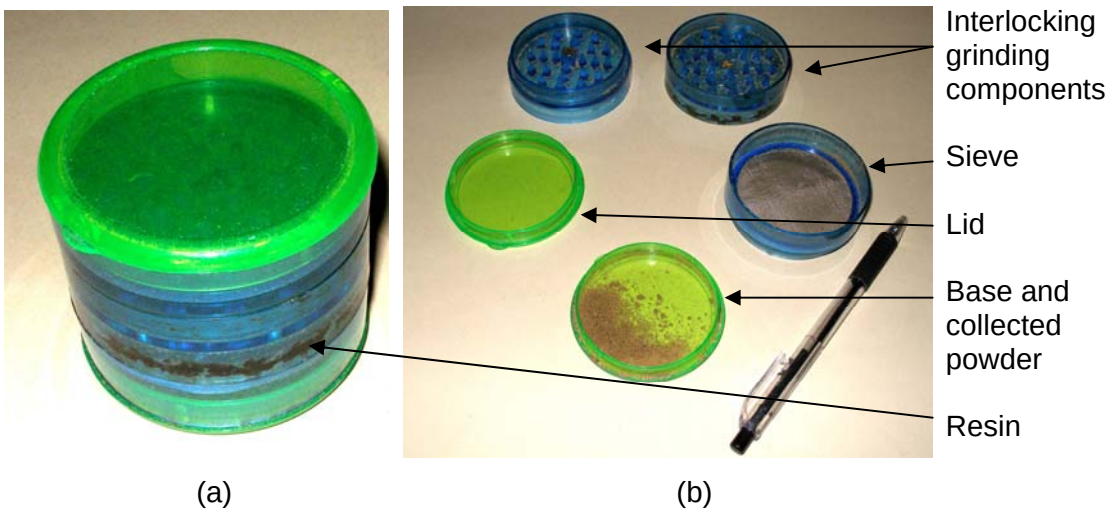


Figure 2.4. (a) A herb grinder in closed position; (b) An open herb grinder revealing the component parts.

In this example dry cannabis would be placed between the top two interlocking sections. As these were contra-rotated by hand, sharp projections on the face of these sections would abrade the cannabis and break it into small portions. These would fall

through 4 mm diameter holes in the top right hand section onto the sieve section below. Any dislodged glandular resin heads would fall through this mesh into the base. Glandular trichomes dislodged from the plant during grinding could fall through this sieve and be collected in a separate chamber within the device. One grinder was found with approximately 1 cm^{-3} of separated yellow powder. This consisted almost entirely of glandular trichomes. Some of these have become trapped and crushed between walls of the interlocking sections forming a ring of black resin.

2.4.3.5 Other categories not included

Previous studies on cannabis in the UK described a hash oil. This preparation is made by dissolving, and subsequently concentrating, cannabis extracts in an organic solvent (Barber *et al*., 1996, Hough *et al*., 2003). No such samples were identified during this study. Many samples were seized which consisted of a mixture of cannabis and tobacco; all were excluded. Three seized suspected-cannabis samples were also analysed and found to be plant material other than cannabis or tobacco.

2.4.4 Measurement of cannabinoid potency and profile

Where detectable the THC, CBD, CBC, CBN, CBG and THCV content of each sample was measured, using gas chromatography, as described in Appendix 1.

2.4.5 Statistical Analysis

Analyses of variance (ANOVA), regression and F-tests were used as appropriate, utilising Microsoft Excel 2003 related software. Kolmogorov-Smirnov and Wilcoxon Rank Sum tests and Hodges-Lehman Estimates were performed using SAS software, with the assistance of colleagues in the Statistical Analysis Department, GW Pharmaceuticals Ltd.

2.5 RESULTS and DISCUSSION

2.5.1 Categorisation of cannabis type between regions

Four hundred and sixty samples were analysed in this study. The proportion of sinsemilla, herbal cannabis or resin in each area is shown in Table 2.1. Sinsemilla was the most common form found overall, accounting for 55% of the samples seized. However, differences were found between regions. In Kent, resin accounted for 85 of the 146 samples (59%), possibly due to importation through the major ports in this region. Herbal cannabis is presently the most common form of cannabis in the USA (ElSohly *et al*., 2000). In contrast, this study revealed this type to be the least common in England. Little or no herbal cannabis was identified in four of the regions, but it did account for 30 of the 159 samples (19%) seized in the South East London Metropolitan area.

Constabulary	Sinsemilla	Herbal	Resin
Derbyshire	15	1	28
Kent	61	0	85
London Metropolitan	97	30	32
Merseyside	49	1	9*
Sussex	34	3	15
Total	256	35	169

Table 2.2. Number of each type of sample received from each constabulary. (In addition, one sample of cannabis powder was received from Kent). *The low number of resin samples from Merseyside was due to the late inclusion of such samples from this constabulary.

2.5.2 The range of cannabinoids in each cannabis category

The range of cannabinoids (the cannabinoid profile) in each type of cannabis is shown in Table 2.2. The distribution of potencies of sinsemilla, herbal cannabis and resin samples were all non-parametric and the median is therefore shown as a more appropriate average than the arithmetic mean. The potency of resin samples varied widely from almost 0% THC up to nearly 11% (Table 2.2). The majority of the samples were at the weaker end of this range. 40% had a THC content of <2% THC, and more than 80% had <6% THC. The range of potencies in herbal cannabis was similar. The maximum THC content found was nearly 12% but approximately 90% had a content of <6%.

Sinsemilla potency ranged from about 1% to 23%, the majority being toward the high end of this range. The cannabis powder, retrieved from the herb grinder, was the most potent of the samples analyzed (40.6% THC, Table 2.2). This material had been prepared using a very simple piece of equipment, and this illustrates that extremely potent cannabis preparations are readily available.

Type		THC	CBD	CBC	THCV	CBG	CBN
Sinsemilla (n = 256)	Median	**13.98**	**<0.10**	**0.20**	**<0.03**	**0.41**	**0.16**
	Minimum	1.15	<0.10	<0.10	<0.10	<0.10	<0.10
	Maximum	23.17	0.56	1.41	2.74	2.16	2.98
Herbal (n = 35)	Median	**2.14**	**<0.10**	**0.22**	**0.17**	**0.21**	**0.55**
	Minimum	0.28	<0.10	<0.10	<0.10	<0.10	<0.10
	Maximum	11.81	1.97	0.42	0.43	0.76	3.62
Resin (n = 169)	Median	**3.54**	**4.17**	**0.34**	**0.10**	**0.29**	**1.55**
	Minimum	0.44	0.36	<0.10	<0.10	<0.10	0.38
	Maximum	10.76	6.97	0.66	0.29	1.05	4.30
Powder (n = 1)		**40.63**	**0.18**	**0.41**	**0.29**	**1.59**	**0.57**

Table 2.3. The median and the range of potencies of five cannabinoids (% w/w) in resin, herbal cannabis, sinsemilla and cannabis powder, seized in five constabularies in England in 2004/5.

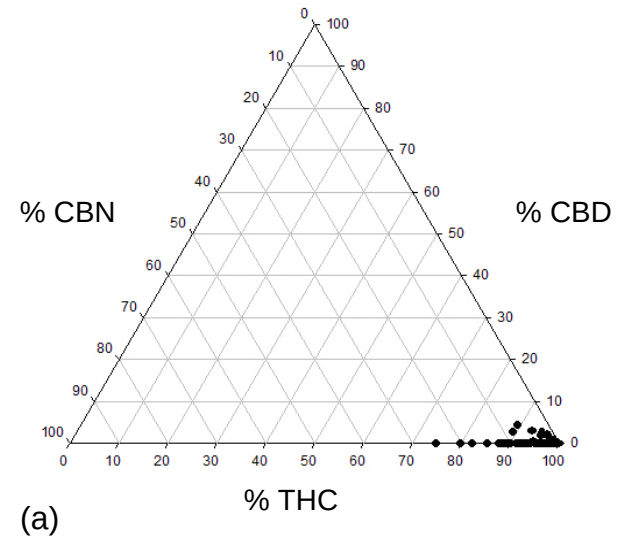
In sinsemilla samples, THC typically accounted for approximately 94% of the total detectable cannabinoid content. The THCV, CBG and CBN content of individual samples occasionally exceeded 2% w/w, but this was approximately one tenth of the maximum potency recorded for THC. The CBD content of sinsemilla was typically very low and fell below detectable levels (0.1%) in the majority of samples. The lack of the THC catabolite CBN, suggested that samples were comparatively fresh when seized, and had remained in good condition in police stores.

THC was also the dominant cannabinoid in herbal cannabis and CBD levels were similarly mostly below the detectable threshold (0.1%). CBN levels were much higher in herbal cannabis than in sinsemilla. The ratio of THC and CBN in these samples varied greatly. This was at least partly due to the varying lengths of time that herbal cannabis encounters on its route to the UK, the majority coming overseas from South Africa (UNODC, 2006). A long transport period would favour the breakdown of THC to the catabolite CBN (Ross *et al*., 1997). As a result of THC catabolism, and CBD, CBG,

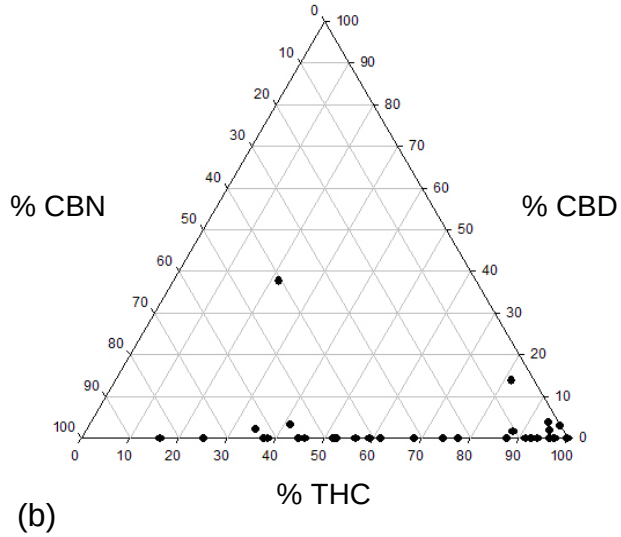
CBC being more stable, THC only accounted the 65% of the median total cannabinoid content of herbal cannabis.

The balance of THC and CBD in the different forms of cannabis was clearly affected by their contrasting genetics. Research suggests that the production of THC or CBD, from the common precursor CBG, is closely controlled by two co-dominant alleles at a single locus (de Meijer *et al*., 2003). As a result, cannabis plants can be identified as belonging to any one of three chemotypes; i.e. THC dominant, CBD dominant or an approximately equal mixture of the two (de Meijer *et al*., 2003, Small *et al*., 1973a). Sinsemilla appeared to be entirely derived from the THC dominant chemotype. Two of the herbal cannabis samples had a substantial CBD content due to the presence of the Bd gene. The resultant effect of these contrasting genetics is illustrated in Figures 2.4a to 2.4c.

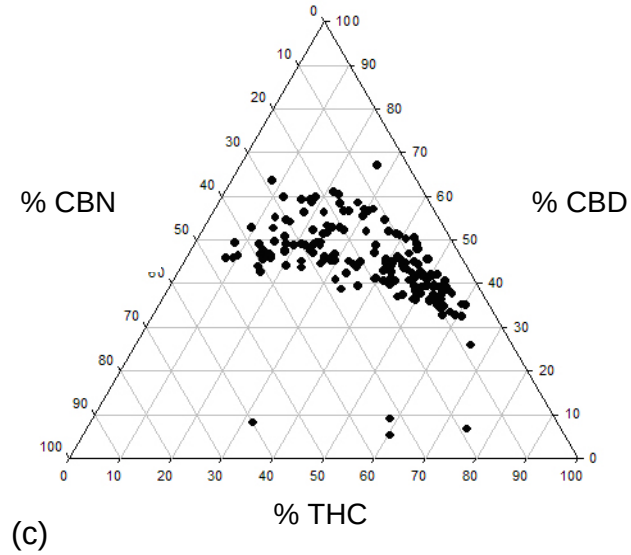
Cannabis resin had a very different cannabinoid profile to that of herbal cannabis and sinsemilla. The majority of the cannabis resin would appear to be prepared from landrace populations of plants which contain all three chemotypes. CBD appeared slightly more dominant (mean content 4.3%) than THC (mean content 3.5%) in this material. CBN was present in much higher quantities than in herbal cannabis or sinsemilla. Cannabis resin samples showed very variable contents of THC (0.4 – 10.8 %) and CBD (0.4 – 7.0%). Compared to sinsemilla, the THC content of resin was significantly much more variable (two-sided f-test, $p < 0.001$). The ratio of these cannabinoids within individual samples also varied widely, as shown in Figure 2.4c.



(a)



(b)



(c)

Figure 2.5. (a) The balance of THC, CBD and CBN in sinsemilla (n = 256); (b) The balance of THC, CBD and CBN in herbal cannabis (n = 35); (c) The balance of THC, CBD and CBN resin (n = 169).

It was hypothesized that much of the variation in THC:CBD ratios within the resin samples was due to THC being a less stable product than CBD. Indeed, in Figure 2.4c resin samples exhibited a wide range of ratios between THC and its main catabolite CBN. To test the hypothesis, an estimate was made of the original THC content of each sample before catabolism to CBN had commenced. A simple arithmetic calculation formula was not available because the breakdown of THC to CBN is not quantitative (Phillips, 1998). Studies with herbal cannabis (Ross and ElSohly, 1999) and resin (Martone and Della Casa, 1990) suggested that a concentration of one mole of CBN implied the original presence of four to six moles of THC. To estimate the likely correlation between the CBN content and original THC content in these samples, the relative contents of the two cannabinoids were plotted (Figure 2.5).

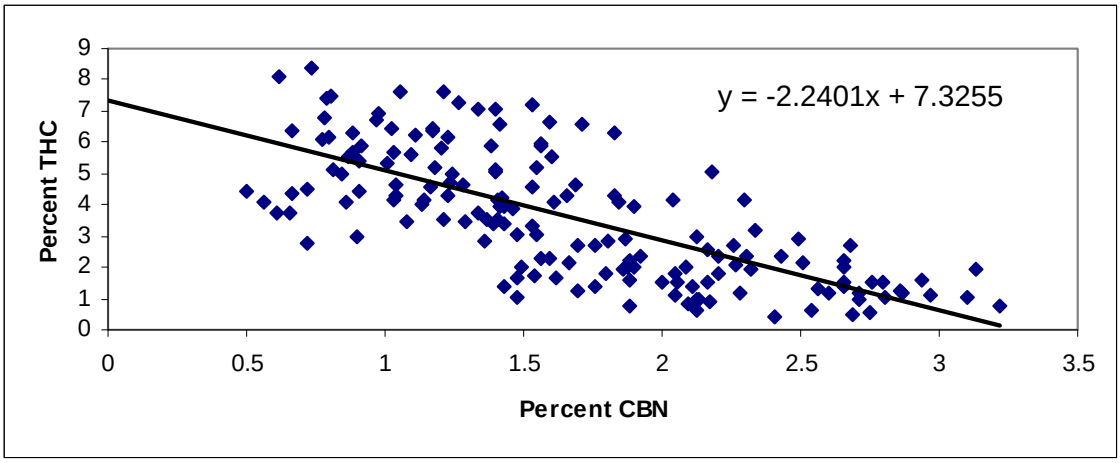


Figure 2.6 The correlation between THC and CBN content in resin samples seized in five constabularies in 2004/5 (n = 169).

The coefficient value of -2.24 suggested that the extrapolated original median THC content of these samples was 7.3% THC, compared to the actual median THC content of 3.5% at the time of analysis. Previous studies have suggested that the half-life of CBD in resin is approximately three times that of THC (Martone and Della Casa, 1990). As a result, in the plants used to produce this resin, the original CBD content was estimated to have been approximately 5.5% compared to 4.2% at seizure. Figure 2.4c also shows that of the one hundred and sixty nine resin samples, four were almost devoid of CBD. These were therefore made from the THC dominant chemovar and probably from a very different source.

2.5.3 Comparison of cannabis potency and profile between regions

Sinsemilla potency ranged from about 1% to 23%, the majority being toward the high end of this range. There were small but statistically significant differences in mean THC

content of sinsemilla seized in different areas. The highest mean THC content was found in the Derbyshire region (16.3%). This material was significantly more potent ($p < 0.05$) than that seized in London (mean 12.9%). The remaining counties returned mean sinsemilla potency values between these two extremes, the differences in potency not being statistically significant ($p > 0.05$). Due to the small number or absence of herbal cannabis samples in most regions, a meaningful comparison of herbal cannabis potency levels between regions was not possible.

There were proportionally larger differences in the mean potencies of resin between regions. Resin seized in Sussex (6.6%) and Derbyshire (5.4%) had significantly higher mean THC contents ($p < 0.05$, ANOVA) than those seized in Kent (4.2%), London (3.6%) or Merseyside (2.8%). Conversely, Sussex resin was notable for having a significantly lower mean CBN content than that from the other counties ($p < 0.05$). The mean CBN content of the Derbyshire resin was also significantly lower than that from London and Merseyside. These data suggest that the Sussex and Derbyshire samples were less aged.

2.5.4 Trends in Cannabis Potency

The mean THC content of imported herbal cannabis in this study (3.1%) was lower than the 4.1% observed by the UK Forensic Service in 1998 (King *et al.*, 2004). Little inference can be drawn regarding potency trends from this because of the small number of samples in both studies, the lack of a reported median in 1998 and the highly non-parametric data (Figure 2.7). However, the potency ranges and distribution patterns are broadly similar, and no major trend is apparent.

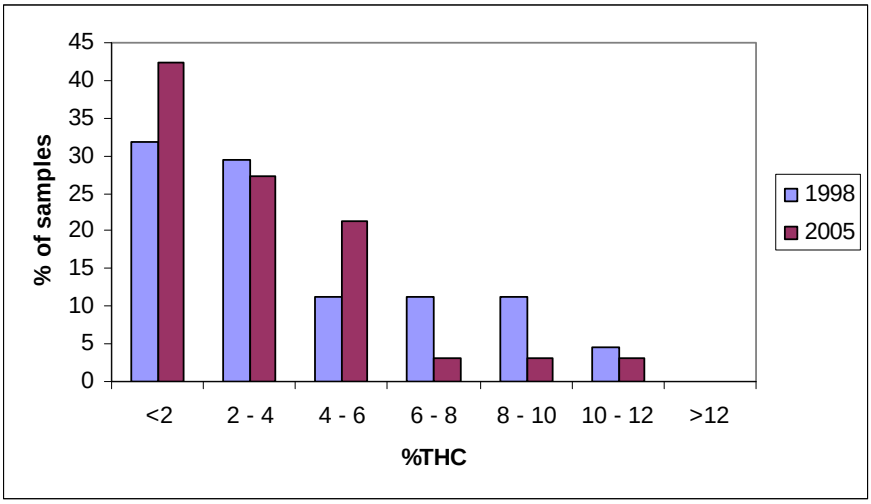


Figure 2.7. A comparison of the range and distribution patterns of THC content of seized imported herbal cannabis samples in 1998, King *et al.* (2004) (n = 44) and 2005 Potter *et al.* (2008) (n = 33).

The mean THC content of resin (3.7%) was typical of that found previously, where potency levels varied from approximately 3 to 6% THC between 1998 and 2002 (King *et al*., 2004). The mean sinsemilla potency value of 13.3% (sd 4.21%) is higher than that reported over the period 1995 to 2002 when the Forensic Science Service reported that THC content of seized cannabis rose annually from approximately 6.0 to 12.5% (King *et al*., 2004). This supports the belief that sinsemilla potency in the UK is potentially increasing but, due to the large standard deviation in the 2005 data, and the lack of reported detail in earlier data, the 2002-2005 rise cannot claimed to be significant.

In both this study and in those analyses performed by the Forensic Science Service from 1996-98, the THC content of samples ranged from <2% THC to >20%. The range of sinsemilla potency levels observed in each study is compared directly in Figure 2.8. To assess the significance of this change in distribution of sinsemilla potencies, the data was analyzed using the Kolmogorov-Smirnov test for different distributions. This showed that the two distribution curves were significantly different ($p < 0.001$). A Wilcoxon Rank Sum test showed that the potency levels in 2005 were significantly higher than those in 1996/8 ($p < 0.001$). A Hodges-Lehman Estimate of median difference suggests an increase of 4.86% THC in the median potency between the 1996/8 data and the 2005 data (C.I. = 3.77, 5.54) (Potter *et al*., 2008).

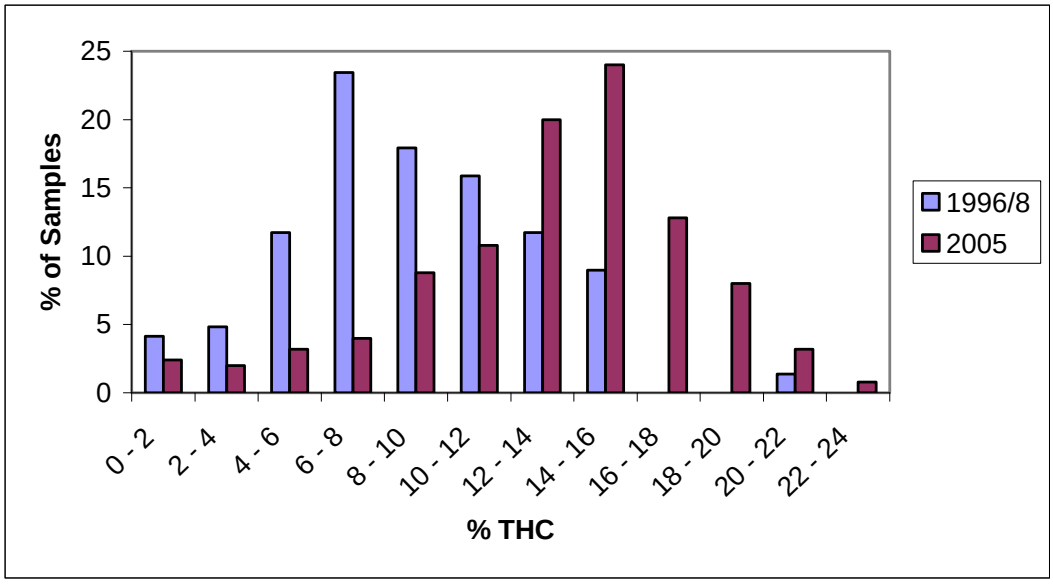


Figure 2.8. A comparison of the ranges of THC contents of Sinsemilla seized in the UK and analysed by the Forensic Science Service in 1996-8 (n = 145) and samples seized by police in Derbyshire (n=15), Kent (n=58), London Metropolitan (n = 96), Merseyside (n = 44) and Sussex (n = 34) in 2004/5 (total n = 247).

Large increases in cannabis potency achieved in the 1970s were largely attributed to the achievements of cannabis breeders (Clarke, 2001). Many named cultivars produced in the 1970s and 1980s are still widely marketed. Seeds of new cannabis cultivars are continually produced in large numbers (Snoijer, 2002) but these do not appear to produce plants of significantly higher THC content (confidential GW Pharmaceutical data). However, cannabis seed production is unregulated and there is no guarantee that seeds currently marketed under established variety names are truly identical to those circulating thirty years previously. The rise in reported potency is more likely due to increasing expertise amongst the illicit UK growers in recent years, more of whom are able to push THC levels closer to the possible maximum. In the UK during this period there has been a large increase in the number of retail outlets selling cannabis seeds and sophisticated growing equipment. Many books, videos and DVDs have been produced, advising growers how to maximise cannabis potency. The internet has facilitated on-line purchasing of these items and has also provided easier access to advice on cannabis growing and processing through expert web pages and focused 'chat-rooms'. During this period the UK Government and Police express the opinion that the production and dealing of cannabis have not always been targeted sufficiently vigorously (Clarke, 2006).

A recognised weakness in this study is that all the samples tested were seized by police from users or dealers on the street. It is not known how representative this was of the cannabis being consumed across the whole population. In ten years personal experience as a magistrate, serving both Adult and Youth Courts, a vast majority of offenders charged with using cannabis were seen to be from Social Grades C2 to F, as defined by the NRS Survey (Appendix 5), and a large proportion were young. A skewing in the average age of charged offenders arose because police were instructed to charge offenders for cannabis use if they were less than eighteen. In contrast, they were allowed some discretion and could issue a warning to adults. Of three million estimated cannabis users in England and Wales, one million were over thirty years old but much less likely to be charged (May *et al*., 2002). Research suggests that the decision to charge an offender, rather than issue a warning, depended partly upon the attitude of the offender (May *et al*., 2007). The more mature, circumspect and sophisticated user is less likely to attract police attention. The cannabis used by this type of user may differ in provenance, potency and price. During informal conversations at Multiple Sclerosis Support Group meetings, attendees frequently revealed that they grew their own cannabis to ensure supply, to remove contact with drug suppliers and to reduce costs. Hough *et al*. (2003) reported the same observation.

This avoidance tactic might have significantly reduced the chances a 'medicinal' cannabis sample being seized.

2.5.5 The efficacy of illicit cannabis

The three main forms of illicit cannabis circulating in England in 2005 were very variable in both their potency and their cannabinoid profile. Herbal cannabis showed the greatest variability in THC content, this being significantly more variable than resin (two-sided F-test $p < 0.05$). Both were significantly more variable in THC content than sinsemilla (two-sided F-test, resin v sinsemilla, $p < 0.001$). Sinsemilla generally contained high concentrations of THC and relatively few other cannabinoids. Herbal cannabis was dominated by THC and CBN, the ratio of these two varying greatly between samples. Resin contained substantial quantities of three cannabinoids – THC, CBD and CBN – and the ratio of these varied greatly between samples. THC and CBD have been demonstrated to act together synergistically in mammalian systems and small differences in relative content of each could have proportionally greater effects on the user (Williamson, 2001).

From its appearance, sinsemilla gave the greatest hint of its cannabinoid content. Although the range of THC levels in sinsemilla ranged from < 2% up to > 22%, 85% of samples had a THC content of between 8% and 24% THC (Figure 2.7), with the majority being within a closer margin of 12% to 18%. It is possible that the lowest potency material was also poor in appearance. Experienced users may thus have had an increased likelihood of being able to judge when a sample's THC content fell within this 12–18% band. This would have enabled a more accurate judgment to be made of the likely potency of the material. Being more variable in their cannabinoid profile as well as their potency, herbal cannabis and cannabis resin would have potentially demonstrated greater variations in pharmacological effect. The appearance of these products gave little indication of their cannabinoid content.

Experienced users of this cannabis, who self-titrated their dose by smoking or vapourising, may have had the ability to adjust their intake according to the potency of the material. Potentially suitable blood plasma cannabinoid level could have been reached within a few minutes (Huestis *et al*., 1992). Users could thus respond quickly to a perceived under-dosing of cannabinoid. The variability in THC content would have presented greater problems for those who relied on eating cannabis-based foodstuff for medicinal purposes. Cannabis that is ingested would experience first-pass metabolism. As a result, a large proportion of the THC would be metabolised to 11-OH-THC. A number of other lesser metabolites would also form and plasma levels of delta^9-THC would not peak until one to six hours after ingestion Consequently, those users

ingesting cannabis would have less ability to accurately self-titrate to achieve their optimum dose. The final bioavailability of ingested THC is estimated to be just 6% compared to 10-27% during smoking (Hawksworth *et al*., 2004). Obviously, those who were supplied with low-potency cannabis may indeed find that they were unable to achieve sufficient THC plasma-levels, especially if ingesting the material.

From 1994 to 1998 resin accounted for 69-72% of all cannabis seizures in England and Wales. From 1997 onwards the proportion of resin seizures fell annually, reaching 53% in 2002 (Mwenda *et al*., 2003). However, although resin still appeared to be the dominant form of illicit cannabis in the recreational market at the time, the study by Hough *et al*. (2003) and an unpublished subset of data from the patient survey by Ware *et al*. (2005), suggested that only a minority of medicinal cannabis users were choosing resin despite the fact that this was the way they could gain access to substantial doses of CBD. It has often been suggested that medicinal users appreciate the 'high' that can be achieved from cannabis, perhaps welcoming it as a pleasant distraction from their symptoms. In a published personal testimony, presented on behalf of the Alliance for Cannabis Therapeutics (HLSCST, 1998b), Clare Hodges stated that when treating her MS symptoms, she did not have to get ''high'' for cannabis to lift her mood and make her feel calm and positive. In many informal conversations with those having multiple sclerosis, psychoactive effects were always described as undesirable. In clinical trials with Sativex®, any psychoactive effects are regarded as undesirable events. There is therefore a paradox. Only resin contained substantial quantities of CBD, a cannabinoid that was efficacious in its own right and able to reduce the undesirable psychoactive properties of THC. In-vitro studies and limited clinical trials also suggested that THC/CBD mixtures were more efficacious than THC alone, when treating cancer pain (Johnson and Potts, 2005) and other medical conditions (confidential GW Pharmaceuticals data). Yet illicit users appear to have preferred to use sinsemilla or herbal cannabis, which evidently lacked CBD.

The lack of preference for resin may have been due to adverse experiences encountered with such a variable product. It also generally contained much less THC than sinsemilla, and may have often been too weak to deliver sufficient active ingredient. For those wishing to smoke cannabis resin, the product would have to be mixed with tobacco or some other herbal material to support combustion. Preparation of such mixtures is more difficult than those incorporating herbal cannabis or sinsemilla. This requires a high level of dexterity which may present difficulties for some patients. In herbal cannabis, and more so in sinsemilla, the natural plant structure is still clearly visible and additives would be relatively easy to detect. In resin

this is not possible. Large quantities of soil are reported to contaminate the material at harvest and adulterants added to increase yield or bind together poor quality resin powder (Clarke, 1998) although forensic analysis has not found clear evidence of the adulteration (King *et al*., 2004). However, suspicion remains.

As stated earlier, many MS patients confided that they were medicating with cannabis that they had cultivated themselves. This finding was also reported in the small-scale exploratory study performed by Hough *et al*. (2003). Cannabis seeds of high-THC cannabis varieties are readily available for purchase. Screening cannabis seed sources, for the research described in forthcoming chapters, tests were performed to ascertain the chemotype of twenty four commercially available varieties. Of these, twenty two produced negligible amounts of CBD. Of the other two varieties, most seeds produced plants with a high-THC chemotype but three seedlings were of a heterozygous mixed THC/CBD chemotype. This approximated to 2% of all the seedlings tested. Patients wishing to grow their own cannabis would therefore find that, although seeds of a vast number of varieties are commercially available, those that produce CBD are rare. This suggests that, as with those buying herbal cannabis or sinsemilla through the illicit market, those growing their own plants are likely to be raising material almost devoid of CBD.

Any medicinal value obtained from sinsemilla would be mostly attributable to the cannabinoid THC. However, several researchers have found that preparations made from the cannabis plant are more efficacious than THC alone (McPartland and Russo, 2001, Costa *et al*., 2007, Ryan *et al*., 2007, Williamson, 2001) due to synergies between THC and other poorly defined components in the plant. Those active ingredients contributing to this synergy (such as volatile monoterpenes) may be more abundant in sinsemilla than in resin, thereby increasing its pharmacological activity. This study did not examine the content of these other agents.

Imported herbal cannabis was typically of low potency, and large quantities of material would possibly be required to provide a useful medicinal effect. This may be a reason why some medicinal users in the UK survey reported using as much as 10g per day. A similar dosage of 7-9 grams a day of marijuana was reported to be used by those with chronic medicinal conditions in the USA (Conrad, 2004), where THC contents of that material are typically low (ElSohly, 2000).

Research for this thesis showed that sinsemilla potency increased significantly between 1996 and 2005 and the efficacy may have altered accordingly. Herbal cannabis and resin potency appeared to have changed little. However, in recent years

the potency of cannabis products in the Netherlands has increased greatly. In 2004 tests showed the mean THC content of resin manufactured and sold in Netherlands *coffee shops* to be 39% and CBD content just 1% (Pijlman *et al*., 2005). This compares to 4% THC and 4% CBD in resin circulating in England. Cannabis resin may experience similar potency trends in the UK with marked effects on medicinal efficacy. For those recreational users who use cannabis for the enjoyment of the psychoactive effects, especially the young with a disposition to psychoses, this increasingly potent material introduces an increased threat to safety.

2.6 CONCLUSIONS

As reported reported by Potter *et al*. (2008), cannabis circulating in England in 2004/5 was a very unpredictable product and the THC content varied greatly. The research performed here recorded for the first time the variability in the CBD content and the THC:CBD ratios of illicit cannabis in England. This variability would affect the pharmacological properties of the material. A decreasing proportion of the material circulating was in the form of resin, which was the only significant source of the anti-psychotic cannabinoid CBD. The market was becoming increasingly dominated by sinsemilla. This was shown to have significantly increased in THC content since 1998.

Increasing concern has been expressed in recent years, regarding the link between cannabis potency and psychosis – especially in teenage users (Smith, 2005). The implications of increasing THC content and diminishing CBD content on users of recreational cannabis has been well documented. For medicinal users, the variability in THC content also implies an unpredictability in the potential efficacy. However, to a certain extent the variability in THC content of sinsemilla and herbal cannabis could be overcome by self-titrating the dose appropriately. In resin however, the ratio of THC, CBD and CBN varies greatly and this variability cannot be overcome by self-titration. In sinsemilla and herbal cannabis, CBD is lacking and the rapid decline in availability of resin indicates that this cannabinoid is becoming less available to medicinal users of illicit cannabis. CBD is similarly scarce in commercially available cannabis varieties sold by seed companies, and not easily accessible to those growing their own cannabis.

A powder found within a ‘herb grinder’ was shown to be dislodged glandular trichomes. This had a THC content of over 40% w/w and was thus ten times more potent than the average resin. This illustrated the part that glandular trichomes played in cannabinoid biosynthesis. It also showed how high cannabinoid purities could be achieved by separating these from the aerial plant material. With the ultimate aim of exploiting

cannabis trichomes, the following chapter reports research performed to gain a greater understanding of their form and function.

Chapter 3 Cannabis trichome form, function, and distribution

3.1 INTRODUCTION

In *Cannabis sativa* L, it is widely accepted that the cannabinoids are predominantly, if not entirely, synthesised and sequestered in small structures called glandular trichomes (Mahlberg *et al.,* 1984). Most of the monoterpenes and sesquiterpenes found in *Cannabis* were also located in these structures (Malingré *et al.,* 1975; Turner *et al.,* 1980.) Trichomes are arguably therefore the part of the *Cannabis* plant of greatest interest to the pharmacognocist. It is reasonable to suspect that the most productive botanical raw material would contain these glandular trichomes in substantial quantities, and at their optimum stage of development. Indeed, the reliable production of optimum feedstocks would be more likely to be achieved if the grower gained a greater understanding of their form, function and distribution. This would guide the grower how to judge trichome maturity. Similarly, improved feedstocks might be possible achieved by selecting plant parts with the optimum array of trichomes. As seen in the previous chapter, one recreational cannabis product predominantly consisted of trichome material. Similar enriched trichome preparations (ETPs) could possibly warrant evaluation as potent sources of secondary metabolites for pharmaceutical use. When characterising any phytopharmaceutical feedstock, a systematic and illustrated report of the microscopical details is generally important (Evans, 2002). This chapter of the thesis constitutes such a report. The following chapter then examines how to exploit these to full potential.

Cannabis trichomes have been studied in depth for many years, a notable example being the detailed descriptive and illustrated work of Briosi and Tognini (1894), which is still regularly quoted. The 1970s was perhaps the period of most intensive study, much of the research being performed with electron microscopes and meeting the growing requirement for forensic identification of illicit cannabis products (Fairbairn, 1972; Ledbetter, 1975; Dayanandan and Kaufman, 1976; Hammond and Mahlberg, 1973, 1977; Turner *et al.,* 1977).

The general term ‘trichome’*,* when applied to plants, refers to a type of epidermal appendage. According to one definition, ‘trichomes’ constitute an intermediate group of appendages between ‘papillae’ and ‘emergences’ (Werker, 2000). ‘Papillae’ (protrusions of the periclinal outer cell wall) and ‘trichomes’ develop from epidermal cells only (Uphof, 1962), whereas ‘emergences’ develop from both epidermal and

subepidermal tissues (Werker, 2000). The distinction between trichomes and emergences is often far from distinct, and both types are commonly referred to collectively as trichomes (Dietmar Behnke, 1984). Plant trichomes derive their name from the Greek word *thrix,* meaning hair. They exist throughout the Plant Kingdom in an extremely diverse number of forms, of which over three hundred have been described (Payne, 1978). Their diversity has attracted much attention since the earliest microscopists studied them and recorded their detail (Hooke, 1665). Workers have chosen to catagorise trichomes in many ways according to their morphological and/or anatomical features. For example, Theobald *et al.* (1980) placed the various trichome morphologies under seven general headings. Dickison (1974) adopted three general headings: - simple, complex and glandular which were each subdivided into various forms. However, the major distinction was between non-glandular and glandular trichomes the former being differentiated by their morphology, and the latter by their secretory materials.

Non-glandular trichomes, in the form of simple plant hairs, occur in the majority of species within the Tracheobionta (vascular plants), but glandular trichomes are found in just 20-30% (Dell *et al.,* 1978). Taxonomists commonly regard the Trancheobionta as being divided into the Spore Forming Division, or ferns (Pteridophyta) and the Flowering Division (Magnoliophyta). The latter is divided into the gymnosperms (conifers) and the much larger angiosperms (broad leaved flowering plants). Secretory glandular trichomes are mostly found within the Magnoliophyta (Dell *et al.,* 1978; Fahn, 1988) but rarer examples are found in existing pteridophyte species, including the ferns *Pityrogramma sp* and *Polypodium virginianum* (Peterson and Vermeer, 1984). Fossilised remains of the fern *Blanzeopteris praedentata* reveal that glandular trichomes existed as long as 300 million years ago, in the Late Carboniferous period (Krings *et al.,* 2003). The functions of trichomes are either guessed at or totally unknown and many of the hypotheses have not been experimentally tested (Werker, 2000). These hypotheses include the deterrence of predators and protection against environmental stresses (Rodriguez *et al.,* 1984; Hallahan *et al.,* 2000; Theis and Lerdau, 2003; Acamovic and Brooker, 2005). Reviews by Werker (2000) and Wagner *et al.* (2004) described seventeen different trichome functions, many of which could be applicable in *Cannabis*.

As a result of funding from the tobacco industry, the species most studied for its multicellular trichomes is *Nicotiana tabacum* (Glover *et al.,* 2000). The plant family receiving most trichome studies is the Labiatae, due to the importance of the terpenoids to the food, cosmetic and pharmaceutical industry. This family incorporates

peppermint (*Mentha piperita*), sage (*Salvia officinalis*), thyme (*Thymus vulgaris*), lavender (*Lavendula officinalis*) and over four thousand other species. Despite legal restrictions to plant access, cannabis trichome structures are also well studied (Fairbairn, 1972; Ledbetter *et al.,* 1975; Dayanandan *et al.,* 1976; Hammond *et al.,* 1977; Mahlberg *et al.,*. 1984; Kim and Mahlberg 2003). There has been a great overlap in the knowledge gained by those studying *Cannabis* and other species. Cannabis trichome researchers have commonly described two types of non-glandular trichome, which have not been associated with terpenoid development. Three types of glandular trichome have been described on female cannabis, viz bulbous, sessile and capitate stalked. Males have been found to exhibit a fourth type – the antherial glandular trichome, which has only been found on anthers (Fairbairn, 1972).

Photosynthesis would be the original source of the carbon utilised in the biosynthesis of the secondary metabolites in glandular trichomes. In some species however, e.g. *Nicotiana tabacum*, photosynthesis would actually take place in chloroplasts within the trichome (Akers *et al.,* 1978). Dayanandan and Kaufman (1976) described finding chloroplasts within the stalks of bulbous trichomes in *Cannabis* (the smallest of the cannabis trichomes) and reported their absence in the other trichome forms. With no chloroplasts present, precursors of secondary metabolite biosynthesis would therefore have to be translocated from elsewhere. Crombie (1977) reported that cannabinoid biosynthesis had been observed to continue in 'sport' tissue lacking chlorophyll, (although data was limited and statistical analysis not possible). It would appear therefore that these precursors can be translocated from tissues well away from the trichome.

As stated in a detailed research paper on cannabis trichomes by Dayanandan and Kaufman (1976), their study is essential to understand the biogenesis, distribution and function of the different cannabinoids. Mahlberg *et al.* (1984) showed that sessile and capitate stalked trichomes differed in their distribution, as well as their cannabinoid content and profile, and this was linked to the differing cannabinoid distribution in the plant. Recent research has greatly increased the knowledge regarding the biogenesis of the cannabinoids within the glandular trichomes, but further studies are required (Sirikantaramas *et al.,* 2005; Taura *et al.,* 2007). These studies have added to the incomplete debate regarding the function of the cannabinoids in the host plant.

3.2 AIM AND OBJECTIVES

In view of the central role of the trichomes in the production of cannabinoid-rich material the work described here investigated trichome development, structure,

function and catabolism in *Cannabis sativa* L. The knowledge gained would hopefully be used to make recommendations as to how cannabis should be grown, harvested and processed to make maximum potential benefit of the secondary metabolites synthesised in the cannabis trichomes. Maintaining a realistic view that fellow growers would be unlikely to have ready access to electron microscopes, this work was unashamedly performed using microscopes costing no more than a few hundred pounds sterling. The research involved a program of studies with a range of linked objectives, these being: -

3.2.1 To study and photograph the structure and apparent function of trichomes in *Cannabis sativa* L.

3.2.2 To assess how the differing sessile and capitate stalked trichome populations affected the secondary metabolite content of cannabis tissues.

3.2.3 To assess the effect of capitate stalked trichome density and colour on cannabinoid content and profile.

3.2.4 To measure the effect of photosynthetic ability, or lack of ability, on the cannabinoid content of green and yellow tissue in variegated Cannabis sativa.

3.3 MATERIALS

3.3.1 Germplasm

Clone	Characteristics	Variety Name	Supplier
G1-M3 G2-M6 G2-M7 G5-M16 G60-M55 M280	High-THC High-THC High-THC High-CBD Variegated High-CBG	Guinevere Galina Gina Gill Unnamed Unnamed	GW Pharmaceuticals
M186	High-THC Pigmented	Sweet Purple	Paradise Seeds P.O. Box 377, 1000 AJ Amsterdam, Holland

Table 3.1 The names and suppliers of the cultivars used.

3.3.2 Microscopy, Tissue Stains, Photography and other Apparatus

Apparatus	Source
Olympus OM10 35mm SLR camera	Olympus UK Ltd, 2-8 Honduras St, London EC1Y 0TX
Olympus SP350 8 megapixel camera Stereo insert 30mm lens tube, SP-350 Unilink (universal adaptor)	Brunel Microscopes Unit 12 Enterprise Centre, Bumpers Industrial Estate Bumpers Way, Chippenham, WILTS. SN14 6QA
Photonic PL2000 - double arm cold light source.	
MX3 Low Power Light Microscope	
High Power Stereo Light Microscope with Trinocular Head for camera attachment.	STE UK Ltd., Staplehurst Rd Sittingbourne, ME10 2NH
Eye Piece Graticule for Specimen Size Measurement	
Hemp Oil (Culinary grade) for mounting of microscope specimens	Motherhemp, Springdale Farm, Rudston, East Yorkshire YO25 4DS
RS 732-139 Hole Punch Kit	RS Components Ltd., Birchington Road, Corby, Northants, NN17 9RS,
Fast Blue Tissue Stain 2,3,5-Tetrazolium Chloride Stain. Glycerol	Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset. BH12 4QH

Table 3.2 Photographic, microscopy and other miscellaneous items and commercial sources.

3.4 METHODS

3.4.1 Photomicrograph Studies

3.4.1.1 Choice of Microscopes

Two levels of light microscopy were used in this study. For more general observations of plant tissue a low power microscope was employed. Using a Brunel MX3 binocular microscope, fitted with WF10X eyepieces and 1X or 3X objectives, samples of plant

tissue up to 15 mm in diameter were observed. For more detailed observations, of small numbers or individual trichomes, a higher level of magnification was required. For this, a high power stereo light microscope fitted with x10 eyepieces and x4, x10 and x40 objectives was utilized. This gave fields of view of up to 4.5mm in diameter.

3.4.1.2 Staining

Two stains were utilised to aid visibility of trichome internal cell strictures and to gain further clarification of the sites of secondary metabolite biosynthesis and storage. The first stain *fast blue*, which is commonly used when analysing cannabinoids by thin layer chromatography, has also been used during histochemical studies on cannabis trichomes (André and Vercruysse, 1976). A 0.3% w/v aqueous solution stains cannabinoids orange or pink, but other phenolic compounds are also stained.

The second stain used was the 'vital stain' 2,3,5-Triphenyl tetrazolium chloride, also known as TTC or *tetrazolium red.* This is an almost colourless water-soluble stain. In the presence of viable tissue this is reduced (probably by dehydrogenase enzyme activity) to insoluble red triphenyl formazan (Smith, 1951). A 1% w/w solution was used for rapid staining (< 1 hour) and a weaker 0.1% w/w used when slowly staining samples overnight.

3.4.1.3 Unmounted Sample Preparation

The majority of the low power microscope observations were made on unmounted specimens. For these, small pieces of plant tissue were cut from the plant and placed directly onto the low-power microscope plate. As a result, areas of pubescence containing over one hundred trichomes could be observed in a single view. The same minimalist method of sample preparation was sometimes utilised when viewing plant tissue on the high power microscope. However, to achieve views where large proportions of the material were simultaneously in focus, flat samples were likely to produce success. For this, areas of tissue up to a few millimetres in diameter would be laid flat on a microscope glass slide. Trichome filtrates were typically observed by smearing these onto a glass slide.

3.4.1.4 Mounted sample preparation

All the mounted samples prepared in this study were designed to be temporary, and disposed of immediately after use. To mount intact pieces of plant tissue, small pieces up to 1cm diameter were placed on a glass slide and a few drops of mounting fluid placed on the specimen. A cover slip was placed on an angle above the specimen,

with one edge of the slip touching the slide. The cover slip was then lowered onto the specimen. As a result, excess fluid and trapped air bubbles would be expelled from the underside of the slip, and removed with paper tissue.

Water (refractive index 1.33) was sometimes used as a mounting medium. However, transparency of biological samples is best achieved by selecting a medium with a refractive index closest to that of the subject (Delly, 1988). Conversely, definition of colourless objects is increased by choice of a mountant with a refractive index different from that of the object, the ideal ratio for clarity being 1.06 to 1.00 (Evans, 2002). The refractive index of glandular trichome contents was not known. However, initially aiming for transparency and knowing the refractive index of two of the major constituents of glandular resin head - myrcene and trans-caryophyllene - to be 1.48 and 1.50, hempseed oil and glycerol (refractive index 1.47) were selected.

3.4.1.5 Illumination

The Brunel MX3 low power microscope incorporated two light sources. These could be directed vertically upwards and/or downwards onto the subject. The material was sometimes illuminated, by incident light, using a Photonic PL2000 - double arm 'cold light source'. By the inclusion of optical fibres, this enabled white light from a halogen lamp to be directed at the subject without any accompanying radiant heat. Alteration of the flexible illuminating arms enabled the angle of incidence of light to be adjusted.

Some samples, when placed on the STE high power microscope, were also illuminated using the cold light source while others were illuminated from below. When viewing samples mounted beneath a cover slip, the microscope was set up using the Köhler illumination method (Delly, 1988), which first ensures that the light from the condenser lens is correctly focused on an empty microscope slide. When viewing unmounted specimens the condenser height and aperture were adjusted while viewing the subject until optimum resolution was achieved.

3.4.1.6 Photography

To enable photographs to be taken through the low power microscope, one eyepiece was replaced with a compatible 30 mm lens tube to which an Olympus OM10 35 mm single lens reflex camera or Olympus SP350 8 megapixel digital cameras would be attached. As in ordinary photography, the depth of field is considered to be the distance from the nearest object plain to the farthest object plain that is in focus. When objects are a long distance from the camera lens the depth of field is large. However, depth

decreases as the image comes closer to the lens. When taking photomicrographs, depth of field is measured in micrometers (Delly, 1988). To maximize the chance of finding substantial areas of tissue simultaneously in focus within this narrow depth of field, multiple samples were laid as flat as possible on glass slides. Aided by surface tension, samples mounted beneath a cover-slip were more likely to be retained within a narrow area of focus.

The earlier studies in this research used single lens reflex photography and colour print film. For high magnification situations, the required exposure times occasionally exceeded ten seconds. Following brief tests (data not shown) high-speed ASA 400 film was selected. This reduced exposure times while maintaining sufficiently high resolving power. Later studies were performed using digital photography. In all cases, photomicrographs were taken on a solid bench and the shutter activated remotely to reduce manually-induced camera-shake.

3.4.1.7 Isolation and Observation of Detached Glandular Resin Heads

Adapting a method developed for studying trichome resin heads of thyme *Thymus* spp, (Yamaura, 1992) individual resin heads from capitate stalked trichomes were simultaneously removed and fixed on adhesive tape. One layer of sellotape® was tightly wrapped around the handles of a domestic clothes peg with the adhesive surface facing outwards. The taught adhesive surface was then allowed to touch the surface of cannabis bracts where glandular stalked trichomes were present. The adhesive surface was promptly fixed to a clean microscope slide. Detached glandular heads were occasionally trapped in the adhesive and readily viewed.

3.4.2 Effect of glandular trichome array on the secondary metabolite content of plant tissues

For this study, bracts of nearly-mature cannabis inflorescences were selected where the pubescence of capitate stalked trichomes was only visible on approximately half of the area (Figure 3.1). In such cases, it was always the proximal tissue that displayed this pubescence. Twenty bracts of each of three high-THC clones were selected. These were cut with a scalpel to separate each bract into portions where stalked trichomes were judged to be present or absent. Using a low power dissection microscope, the density of stalked and sessile trichomes in the separate tissues was assessed by counting the number, within a single randomly-selected field of view of 16.6 mm^{-2}, on both the upper and lower surface of each of these bract sections.

Samples were then bulked to produce one sample of proximal bract tissue, and one sample of distal tissue, for each of the three clones. The samples of proximal and distal bract material of were then separately dried in an oven at 40ºC for twenty four hours. The cannabinoid content was assessed by GC (Appendix 1).

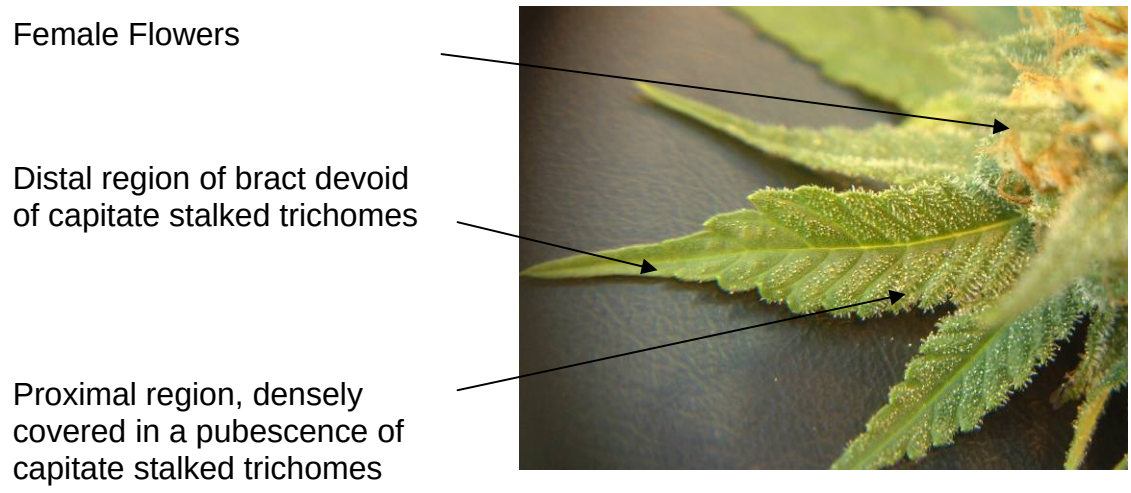


Figure 3.1. Upper surface of a bract within a cannabis inflorescence showing glandular stalked trichomes to be present only within the proximal region (Potter, D..J.).

3.4.3 Organoleptic assessment of the effect of trichome colour and pubescence density on cannabis potency.

This study utilised over two hundred and fifty illicit samples of sinsemilla cannabis inflorescence seized by police from five constabularies during 2004/2005 (Potter *et al.,* 2008). These were collected from police storage and relocated to a central dehumidified store (30% +/- 5% RH) and kept in darkness at ambient temperatures prior to assessment.

All samples were visually examined using a Brunel MX3 low power microscope. Trichome density and trichome colour were awarded single overall scores on a subjective 1-9 scale (Table 3.3). This was based upon those commonly used by the National Institute of Agricultural Botany for the subjective assessment of plant characteristics (NIAB, 2007). For trichome density a score of 1 was awarded to the maximum trichome density and progressively higher scores denoted a thinner pubescence with a score 9 denoting that no intact glandular stalked trichomes were visible. For trichome colour a score of 1 denoted a sample within which the vast majority of glandular trichome heads were completely clear. With maturation these resin heads can typically become turbid and then brown. The score would be awarded

for the colour of the majority within the sample. The samples were subsequently analysed for cannabinoid content by GC (Appendix 1).

Score	Density	Colour
1	Maximum Density	Clear
2	Extremely Dense	Misty White
3	Very Dense	Translucent
4	Moderately Dense	Opaque White
5	Intermediate Density	Slightly Brown
6	Scarce	Light Brown
7	Very Scarce	Mid Brown
8	Extremely Scarce	Dark Brown
9	Absent	Very Dark

Table 3.3 The 1-9 scales for overall capitate stalked trichome density and resin head colour. Within each sample some variation would occur.

3.4.4 Effect of photosynthetic ability, or lack of ability, on cannabinoid biosynthesis in sessile trichomes.

This study utilised a very rare variegated cultivar of *Cannabis sativa* L., G60 M55. In a first of three tests, three plants were selected and leaves collected where pure yellow and dark green could be viewed on either side of the leaf's midrib. Using dissection scissors, areas of approximately equal size were sampled from symmetrically opposite areas on each side of the leaf's midrib. These varied in size but were typically each no more than 100 mm^2. For each colour tissue, one bulked sample was produced per plant - each containing twenty portions of leaf. The emphasis on symmetry was designed to ensure that the green and yellow samples were at identical stages of cell expansion. Samples were oven-dried at 40ºC for 24 hours and analysed for cannabinoid content by GC (Appendix 1). In a second test the above procedure was repeated with just two plants.

A third test was performed where the cannabinoid quantification was based upon leaf area rather than leaf weight. For this test a disk cutter was used to cut equal sized disks from totally green or yellow areas. Leaves of even shape were selected where disks could be cut from symmetrically-located pure yellow and green areas (Figure 3.2). Twenty disks (1 cm^2 diameter) of each colour were cut from each of two plants.

Samples were dried as in the above test. From each plant, the twenty disks of each colour were bulked, thereby providing two replicates per colour. These were analysed by GC.

Figure 3.2. A variegated leaf of clone M60, with 1cm diameter disks cut from symmetrically opposite sides of the midrib (Potter, D..J.).

In both tests the samples were viewed through a microscope and an assessment of sessile trichome density was attempted.

3.4.5 Statistical Methods

Basic calculations of SD (standard deviation), SE (standard error) and ANOVA (analyses of variance) and regression were performed using Microsoft Excel software.

3.5 RESULTS AND DISCUSSION

3.5.1 Photomicrograph studies

All six forms of trichome described by Fairbairn (1972) were examined. These are described in turn.

3.5.1.1 Simple unicellular trichomes

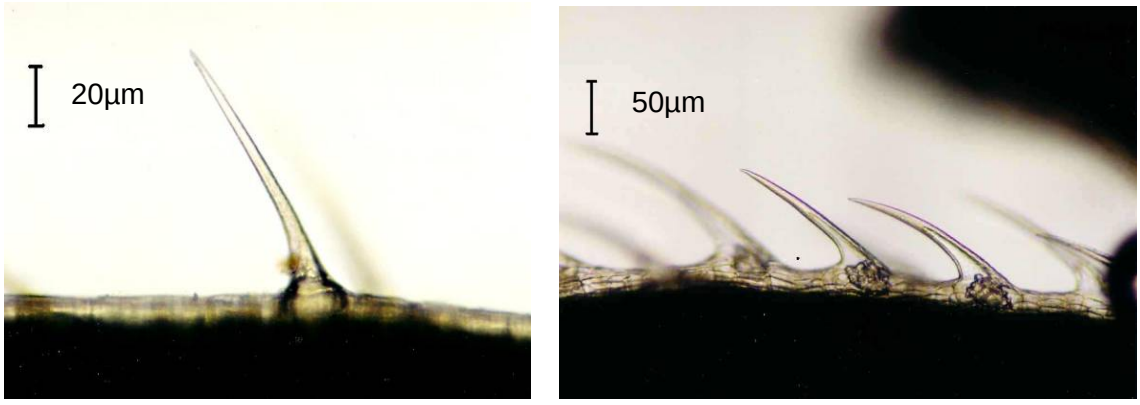
An example of this type is shown in Figure 3.3a. The trichome is seen to have developed from a cell within the epidermis. These simple trichomes, also known as covering trichomes, were the first to appear. These were initially observed on the surface of cotyledons immediately after germination. This form continued to develop in abundance on the underside of leaves (and to a much lesser extent on the upper

surface) throughout the plant's life. These trichomes were typically orientated to face towards the distal part of the leaf, bract or bracteole and in many instances would lie almost flat on the surface. This pubescence of trichomes would cover the underside of the leaf with a layer of trapped air, thereby reducing water loss and providing some insulation against extreme temperatures (Rodriguez *et al.,* 1984).

3.5.1.2 Cystolythic trichomes

This type of trichome is shown in Figure 3.3b. Specimens were first observed on the upper surface of the initial pair of true leaves on a cannabis seedling. Always pointing towards the distal part of the leaf, these trichomes gave the upper surface a texture that was rough to the touch.

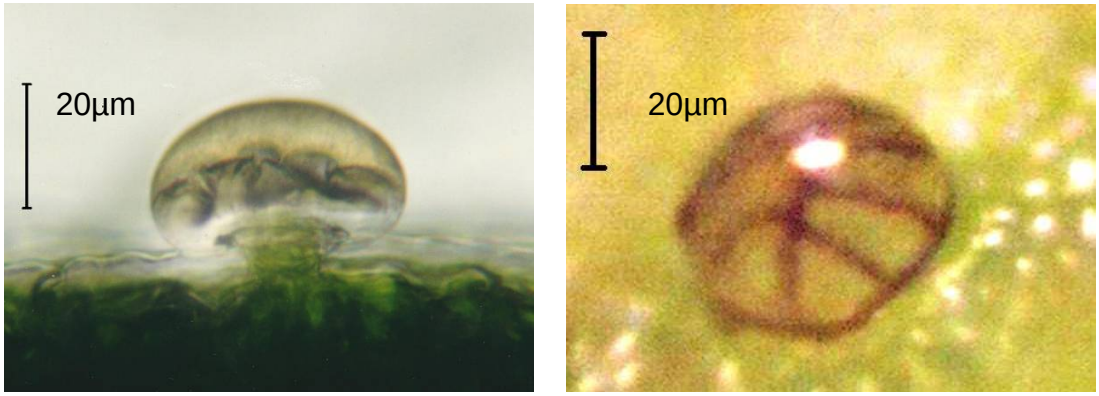
At the base of each trichome is a cystolyth. These concretions are common throughout the plant kingdom and are typically formed from calcium oxalate or calcium carbonate crystals. Those found in *Cannabis* are of the latter type (Dayanandan and Kaufman, 1976; Evans, 2002). These tough trichomes would presumably reduce the palatability of the foliage to leaf-eating predators. Histochemical chemical staining of these trichomes with *fast blue* occasionally resulted in pigmentation of these organelles, but the presence of phenolic substances on these trichomes was attributed to contamination from leaking capitate stalked trichomes. This supposition was supported when the vital stain *tetrazolium red* was used. No reduction of tetrazolium was observed apart from in the cells immediately surrounding the cystolyth, where respirative activities accompanied the formation of these concretions.



(a) (b)

Figure 3.3. (a) Unicellular non-glandular trichome. The sample is temporarily mounted under hemp oil and viewed in transmitted light; (b) Cystolythic trichomes observed on the leaf margin of a young leaf. The sample was temporarily dry-mounted and viewed in transmitted light. Cystolyths (concretions of calcium carbonate) are visible at the base of each trichome (Potter, D..J.).

3.5.1.3 Capitate sessile trichomes (more commonly simply called sessile trichomes)



(a) (b)

Figure 3.4. (a) a capitate sessile trichome observed on the edge of one of the first pair of true leaves of a cannabis seedling. The specimen was temporarily dry-mounted and viewed using both transmitted and incident light; (b) a sessile trichome on a leaf surface stained with Fast Blue. The still-wet sample was temporarily dry-mounted and viewed using incident light (Potter, D..J.).

Apart from on the cotyledons and the supporting hypocotyl, sessile trichomes were observed on all other aerial surfaces throughout the plant's lifespan. The example shown in Figure 3.4a was a very rare find, being situated on the margin of one of the first pair of monofilous leaves of a cannabis seedling. This location enabled the specimen to be observed in profile, without the need of microtome sectioning.

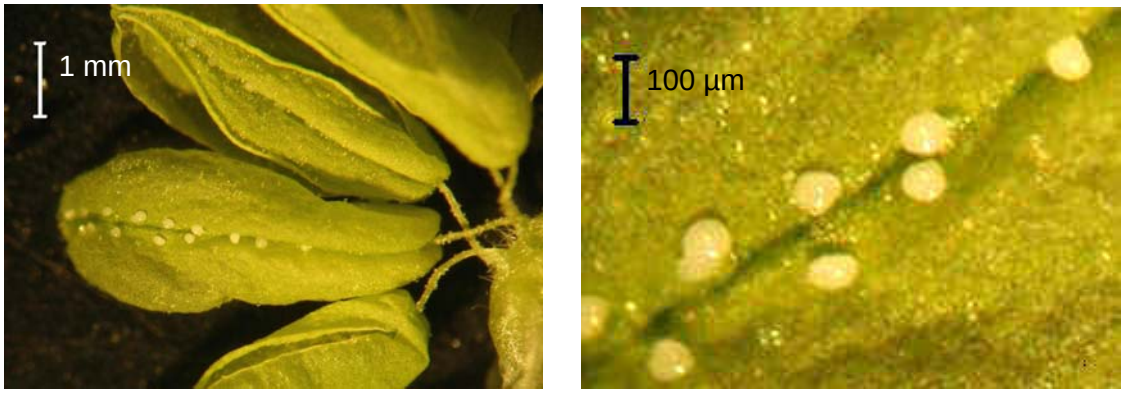
Those previously studying cannabis trichomes have referred to this type as sessile (Latin *sessilis* – sitting) or capitate sessile (Latin *caput* – head). By definition this type does not have a stalk. The trichome is in fact connected to the mesophyl cells via a stalk cell, but this is hidden beneath the trichome resin head. The stalk cell was seen to contain chloroplasts, enabling some photosynthetic activity. This form of trichome is found in many other plant families and, due to its flattened shape and short stalk, is often referred to as a 'peltate' trichome (Latin *pelta* – a short-handled hand-held round shield). According to some plant physiologists, the development of this structure from sub-epidermal cells defines this structure as an 'emergence' rather than a 'trichome' – the latter always developing from an epidermal cell. However, as stated earlier, most authors regard both structures as 'trichomes' (Werker, 2000).

The glandular head (or resin head) incorporates a disc of secretory cells at the base. These appear to totally lack chlorophyll. Above the secretory cells, and below the trichome's outer membrane, is a chamber within which the secretory cells sequester a resinous mixture that includes cannabinoids and essential oils (Mahlberg *et al.,* 1984).

Mature sessile trichomes are reported to typically have eight secretory cells within the disk. When the trichomes were viewed from the side, as in Figure 3.4a, it was not possible to confirm this. When illuminated and viewed from overhead the disk of secretory cells was also difficult to observe. This problem was exacerbated by the fact that the glandular head itself acted as a powerful convex lens. As a result it was difficult to gain an undistorted view of structures with the glandular head. As the sessile trichomes matured, the interior of the structure would typically turn translucent or opaque white, further hampering observations of internal structure. Trichomes would sometimes turn brown in very mature or aged samples. A clearer confirmation of secretory cell numbers was achieved by immersing the cannabis leaf in fast blue stain for ten minutes and then rinsing in water immediately prior to examination. Trichomes varied in the speed at which the stain was absorbed and assimilated. Some samples exhibited very little staining while others became totally red, allowing no visible differentiation of tissues. In ideal situations only the membranes of the secretory cells were stained, allowing a clearer view of the trichome's internal structure (Figure 3.4b). Their function is not known but across the Plant Kingdom their role is guessed to be the protection of the plant tissue against predators.

3.5.1.4 Antherial Sessile Trichomes

Sessile trichomes were also observed on cannabis anthers. The observations supported the theory of Fairbairn (1972) that these 'antherial sessile trichomes' were a distinct form of trichome. These antherial trichomes were different from all other sessile trichomes by virtue of their larger size (Figure 3.5). With a diameter of approximately 70-80µm these unique sessile trichomes were significantly larger than those located elsewhere. Sessile trichomes were also generally present on the calyx surrounding these anthers, but these were of the more normal smaller form. Very similar antherial trichomes are observed in the furrows of the anthers of the male hop *Humulus lupulus* L. (Neve, 1991a).



(a) (b)

Figure 3.5. (a) a row of antherial sessile trichomes showing their normal distribution in the furrow of a cannabis anther. These anthers were captured in incident light through a low-power microscope. (b) closer view of antherial trichomes (Potter, D..J.).

3.5.1.5 Capitate Stalked Trichomes

These trichomes were generally abundant on the calyx, bracteoles, bracts and accompanying petioles of female plants. Although sufficiently rare on male plants to be thought totally absent by some researchers (Hammond and Mahlberg, 1977), their existence has been confirmed by others (Dayanandan and Kaufman, 1976). During studies for this study, capitate stalked trichomes were only found on males of a few varieties and these were restricted to the filaments bearing the anthers. Capitate stalked trichomes were the most complex. As shown in Figure 3.6, they developed a resin head, similar to that of the sessile type, but in mature specimens this was surmounted on top of a multicellular stalk. As experienced by previous workers (Mahlberg *et al.,* 1984) it was not always easy to distinguish between sessile and immature glandular stalked trichomes, where the stalk was yet to form. Observations confirmed the findings of others (Ledbetter *et al.,* 1975) that resin heads on capitate stalked trichomes are typically 70-100 µm in diameter, compared to sessile types which were more typically 40-50 µm. The stalk consisted of two distinct cell types. Hypodermal cells, at the core of the stalk, formed an active channel through which nutrients could be transported to the glandular head from the phloem. This was surrounded by a single layer of outer epidermal cells, which was a continuous extension of that covering the bract or bracteole surface.

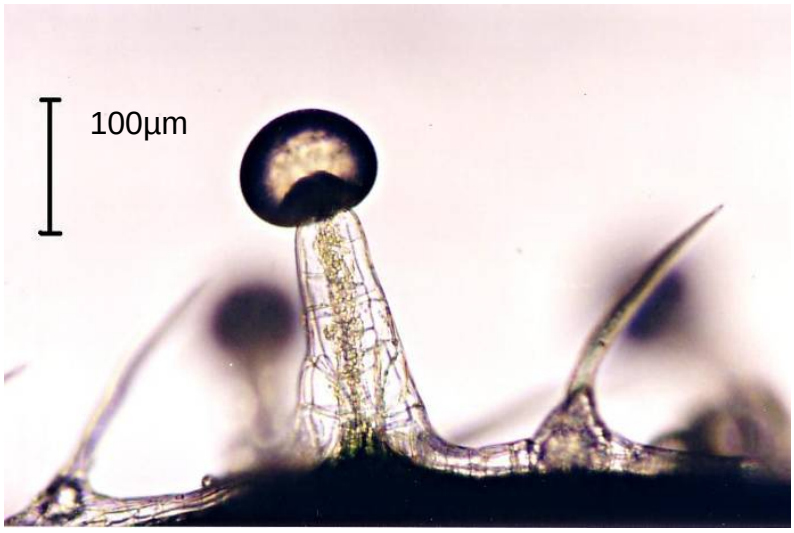


Figure 3.6. A capitate stalked trichome (centre) between two cystolythic trichomes. The specimen is temporarily dry-mounted and illuminated from below. The secretory cells are out-of-focus due to the optical distortion within the glandular head (Potter, D..J.).

Nineteenth-century studies, using the light microscope, identified a disk of secretory cells at the base of the resin head (Briosi and Tognini, 1894). More up-to-date electron microscope studies have been performed which examined the morphology of this feature (Mahlberg *et al.,* 1984; Mahlberg and Kim, 1991; Kim and Mahlberg, 1991, 2003). The secretory cell disk was readily found during light microscope studies for this thesis, albeit initially in the form of a poorly defined mass of translucent material at the base of the glandular head. Viewing techniques had to be adjusted to identify the individual secretory cells within the disk.

As with the sessile type, the resin head on capitate stalked trichomes would swell during the early stages of development, as the secretory cells became active. The contents of the resin head were clear during the earlier stages of development, but would become opaque-white in older specimens. In most cases this would not occur until trichomes were at least four weeks old, but the rate of loss of transparency was a genotypically-dependent characteristic. In CBG-dominant clones (eg M280, Figure 3.13) glandular heads would become a dense opaque-white when just a few days old. This was an unusual example of where the microscope could be used to help identify a cannabis plant's chemotype. Further ageing of most genotypes would sometimes result in the resin heads turning brown. This colouration was often seen to commence within the disk of secretory cells, and was possibly due to necrosis of these now inactive tissues. This browning would continue after plants had been harvested and dried.

Viewing cellular structures within the trichome and surrounding tissues was frequently hindered by their refractive properties. This was especially the case when viewing

unmounted specimens. The interior of the glandular resin head was particularly difficult. Although the tissue was crystal-clear during the early stages of development, as observed with sessile trichomes the glandular head acted as a powerful convex lens. When illuminated from below the transmitted light was refracted by the glandular head contents. This could lead to a distorted virtual image being formed, which appeared to be located outside of the structure, as in Figure 3.7.

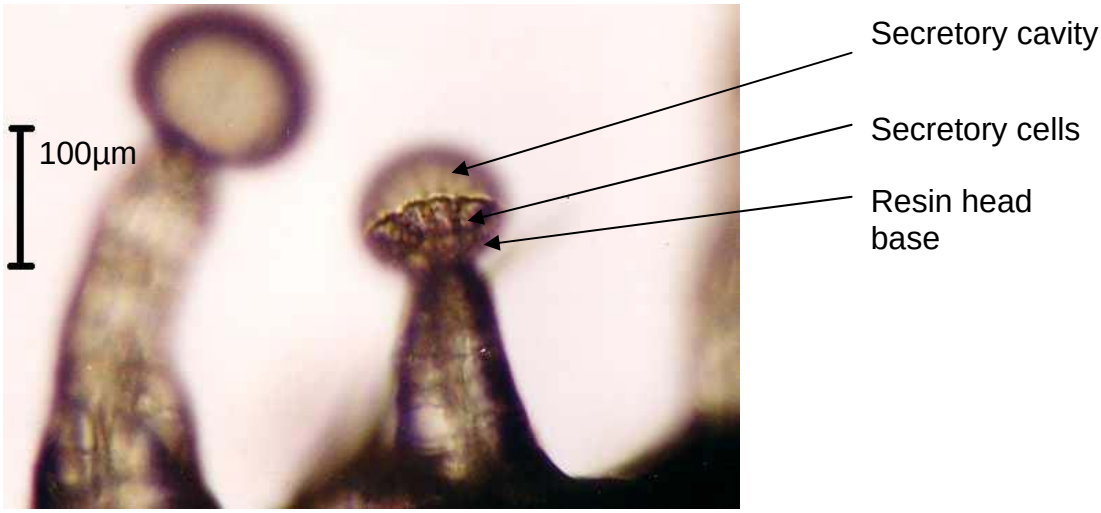


Figure 3.7. Two dry-mounted capitate stalked trichomes viewed in transmitted light. Most of the features are out-of-focus. In the right-hand trichome, a crisp view of cells within the secretory cell disk appears as an in-focus image. However these appear to be located outside of the trichome structure, due to the refractive properties of the resin head (Potter, D..J.).

Mounting the specimen in an appropriate fluid reduced the aberration, as this decreased the difference in refractive index between the specimen's contents and that of the surrounding medium, the refractive index (RI) of air being 1.0. Initial tests found water to be a poor mounting medium. Air bubbles frequently became trapped between the hydrophobic waxy plant surfaces and the water. Possibly due to its refractive index (1.33), image clarity was relatively poor when samples were mounted in water. Glycerol (1.47), hempseed oil (1.47) and conventional immersion oil (1.51) have similar refractive indices to those of the main terpenes within the resin head (myrcene 1.48, trans-caryophylene, 1.50) and, possibly because of this, these were found to be more suitable mounting media. A 70% v/v aqueous solution of glycerol was less viscous and sometimes easier to use. This also offered a slightly lower RI of 1.44.

With appropriate lighting, the disk of secretory cells was readily observed at the base of the glandular head, as shown in Figures 3.8a. When dry mounted, the secretory cells were poorly defined and appeared dark and translucent. When mounted in glycerol, more details of the secretory cell disk could be observed. Viewed in profile, the disk

appeared as an undulating semi-opaque conglomeration of cells (Figure 3.8b). An illustration of a trichome viewed from the same angle, as drawn by Briosi and Tognini (1894), is included for comparison (Figure 3.8c). In all three Figures 3.8a-c, the stalk is seen to include an inner channel of hypodermal cells surrounded by an outer layer of epidermal cells. At the uppermost extremity of the hypodermal channel, Figures 3.8b and c show a more opaque 'basal cell'. The Briosi and Tognini illustration also shows this basal cell to be surmounted by two of the four stipe cells, the distal surface of which acts as a base for the secretory cells. The stipe cells cannot be seen clearly in this photomicrograph (Figure 3.8b).

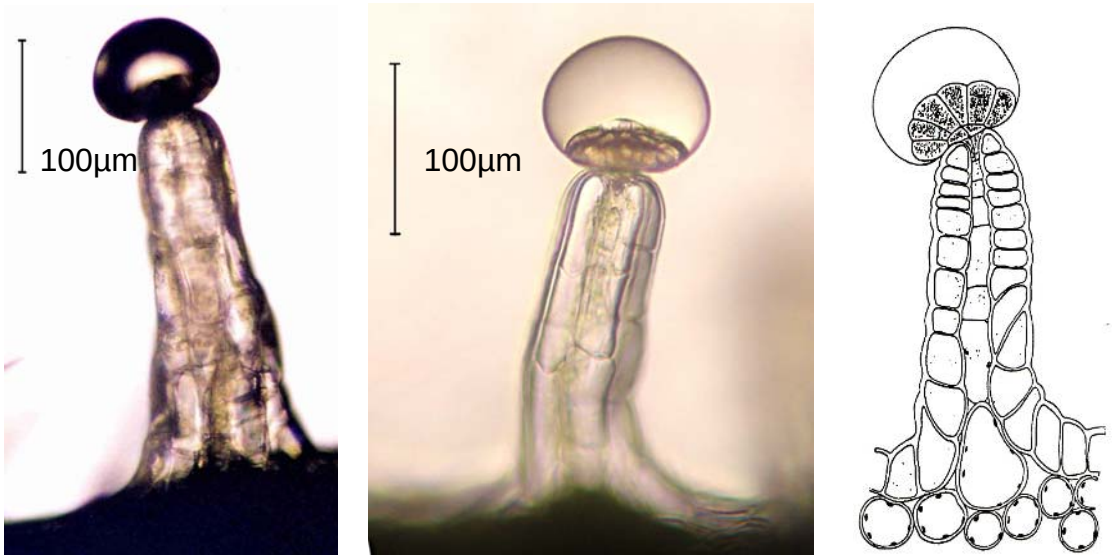


Figure 3.8. (a) A temporarily dry-mounted capitate stalked trichome viewed in transmitted light. An irregular arrangement of poorly-defined secretory cells is visible at the base of the glandular head (Potter, D..J.); (b) A capitate stalked trichome, temporarily mounted in glycerol and viewed in transmitted light (Potter, D..J.), and (c) an illustration of a capitate stalked trichome on *Cannabis sativa* by Briosi and Tognini (1894).

While observing capitate stalked trichomes mounted in oil, the secretory cells were perceived to be completely clear structures. These were located above a layer of more opaque tissue (Figures 3.9a and b). Transmission electron microscope (TEM) studies have shown the distal part of the secretory cells to contain extensive areas of hyaline tissue (Kim and Mahlberg, 2003), which may correspond with the clear secretory cell tissue viewed here. Related TEM studies also identified increased levels of thickening between the secretory and stipe cells (Mahlberg and Kim, 1991), and similar thickening

may explain the presence of the apparently-opaque tissue seen at the base of the resin head in these studies.

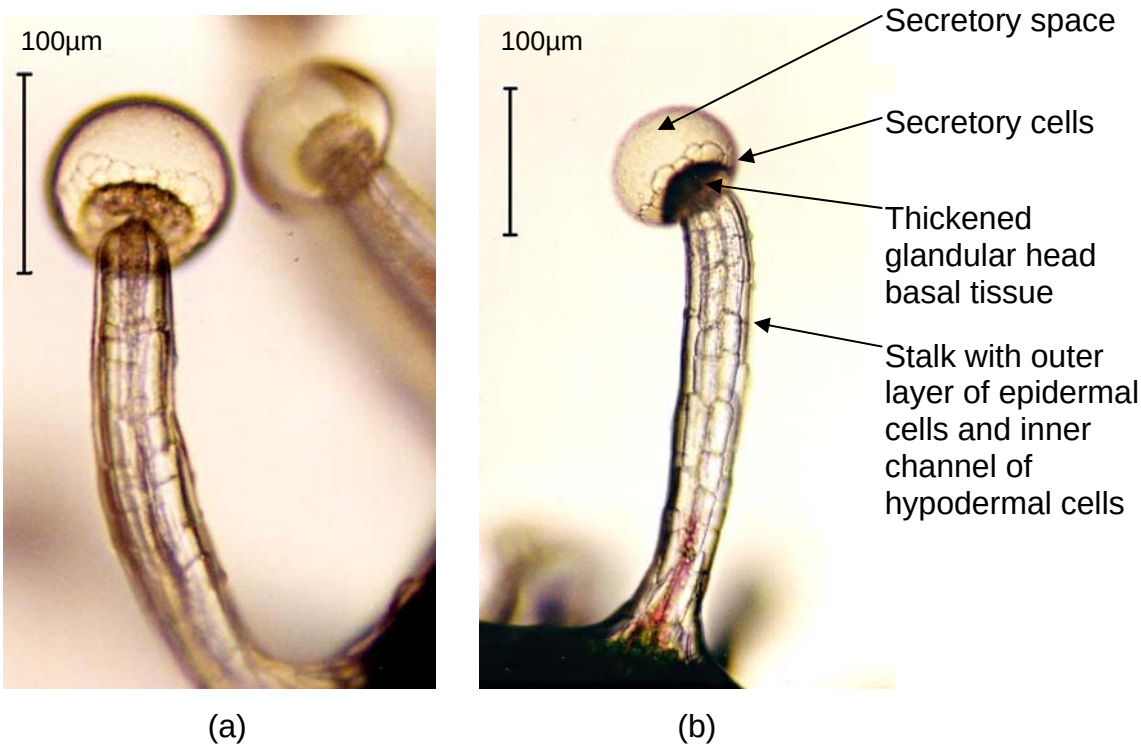


Figure 3.9. (a and b). Similar sized capitate stalked trichomes temporarily mounted under hemp oil. The samples are viewed in transmitted light. Possibly because of a similarity in the refractive index of the oil and the secretory cell contents, these cells appear clear. The outer membrane at the base of the glandular head appears dark and opaque (Potter, D..J.).

TEM studies of the resin head of capitate stalked trichome resin heads have also indicated that the secretion produced by the secretory cells enters the secretory cavity in the form of so-called vesicles (Mahlberg *et al.,* 1984). These vesicles are separated by a network of fibrils, which have a wall thickness approximately half that of the secretory cell membrane. Studies of untreated specimens for this thesis, using the STE light microscope only rarely gained a clear view of the vesicles. Indeed it was initially postulated that the clusters of clear organelles within the secretory head in Figure 3.9a and b were not secretory cells, but secretory vesicles. These would have been produced from secretory cells, which were initially perceived to be the dark tissues at the base of the secretory head. Although this interpretation of the observation is still possible, the size of the clear structures appears too large for them to be vesicles, which electron microscope studies have shown to be a few microns in diameter (Mahlberg *et al.,* 1984). While viewing these secretory cells from directly above the trichome, using 70% v/v glycerol as a mounting medium and with light being transmitted from below, more credible identification of secretory vesicles was

sometimes possible (Figure 3.10). Illuminated in this manner, the secretory cells and the secretory vesicles appeared transparent. To maximise the sharpness if the image within the optically-distorting secretory head, the microscope was initially focused on the subject and the condenser lens then adjusted to gain maximum clarity. Appropriately focused, the reported tally of sixteen radially-arranged secretory cells could sometimes be counted. As shown in Figure 3.10, when viewed in profile, the disk of secretory cells appeared more opaque.

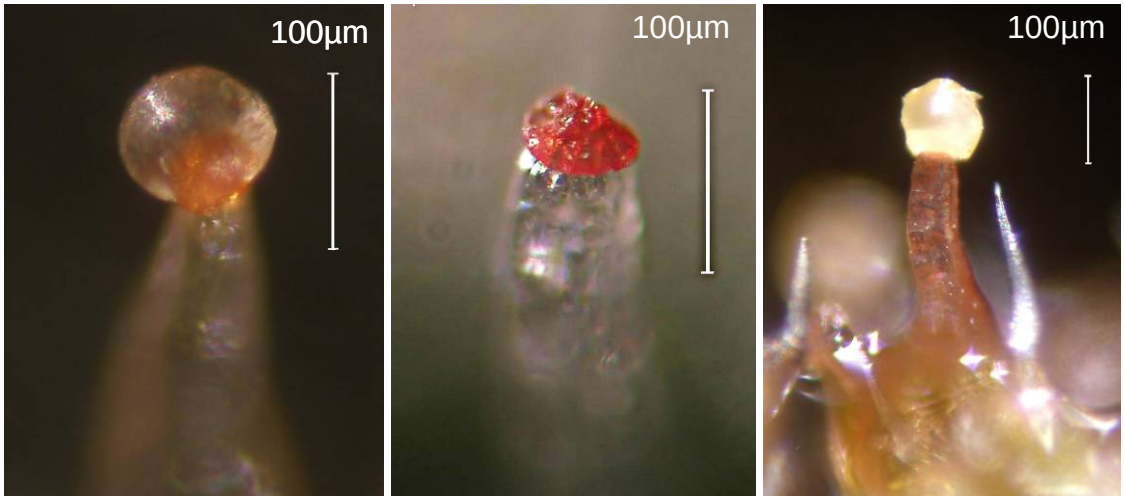
Figure 3.10. A contrasting pair of resin heads on capitate stalked glandular trichomes, naturally orientated to allow sideways-on (left) and overhead views (right). The specimen is temporarily mounted in a 70% v/v aqueous solution of glycerol and illuminated from below (Potter, D..J.)..

Mahlberg *et al.* (1984) stated that secretory vesicles form above the secretory cells and migrate through the secretory head, depositing some of their contents on the secretory cavity's outer membrane, thereby maintaining its strength as it inflates. In their studies the outer cuticle of the secretory head was seen to increase eight-fold in thickness as the trichome developed. These workers used THC monoclonal antibodies to detect the presence of THCA within the secretory head. THCA was only detected when attached to the vesicle wall and none was discovered within the vesicle interior. If THCA was indeed restricted to the vesicle surface, it would not be possible to achieve the THCA concentrations of over 40% w/w found in some cannabis resin samples (Potter *et al.,* 2008; Hardwick and King, 2008).

The metabolic activity within the secretory cells disk has been well studied by many researchers and is still the cause of some debate. The biosynthesis of monoterpenes

and sesquiterpenes, within the membranes of secretory cells, has been identified as occurring in plastids and endoplasmic reticulum respectively (Croteau *et al.,* 1984). Mahlberg *et al*. (1984) suggested that these plastids were also involved in cannabinoid biosynthesis. Recent research shows that CBGA, the biosynthetic precursor of THCA and other cannabinoids, and THCA synthase enzyme are found within the secretory cavity (Sirikantharamas *et al.,* 2005). This suggests that the secretory cavity is not just the site for the accumulation of cannabinoids, but also the site of THCA biosynthesis. The previous theory that THCA is biosynthesised in plastids within the secretory cells is further weakened by the observation that THCA and CBGA are cytotoxic (Sirikantharamas *et al.,* 2005). By sequestering the cannabinoids in the secretory cavity the secretory cells would be protected from damage. The enzymes for cannabinoid synthesis within the secretory cavity would require water and molecular oxygen to function. As the hydrophobic terpenes accumulate within the vesicles, the oxygen and water would most likely be transported via the vesicle fibrillar wall. However, alternative studies suggested that CBGA indeed may be synthesised in the secretory cells, but further investigations are needed (Taura *et al.,* 2007).

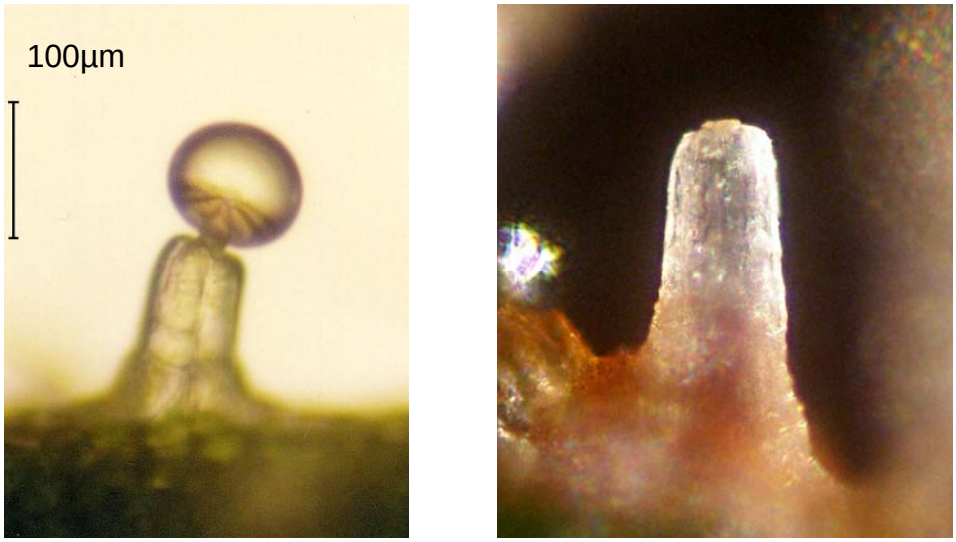
The secretory cells were confirmed as being metabolically highly-active sites by the use of the vital stain *tetrazolium red* (Figure *3*.11a). The cells were the first to take up the stain and develop a red colour. A 1% solution stained most trichomes within one hour, especially in the case in less-mature trichomes where the glandular head contents were still clear. When the glandular head's outer membrane was removed by abrasion, to expose the disk of secretory cells, the secretory tissue stained red within a few minutes (Figure *3*.11b). The glandular cells within intact secretory resin heads of more mature trichomes were often unstained, suggesting that metabolic activity had ceased. However, detailed study of these cells in more-mature trichomes was obscured by the age-related increasing opacity of the glandular head (Figure *3*.11c). As this figure also shows, prolonged exposure to *tetrazolium red* resulted in staining of the trichome stalk, through which metabolites would be transported. Within the stalk's hypodermis, the phloem transport of sugars to the secretory cells would be via sieve tubes. Although phloem transport requires metabolic energy phloem transport is passive, but energy is needed to maintain the living condition of these sieve tubes (Clifford, 2004). Respiration would also continue in the surrounding epidermal cells, resulting in some reduction of the stain.



(a) (b) (c)

Figure 3.11. (a) Secretory cells stained red, within the glandular head, after thirty minutes in 1% tetrazolium red; (b) A capitate stalked trichome with glandular head removed by slight abrasion. After thirty minutes in 1% tetrazolium the disk of secretory cells is stained bright red; (c) A mature capitate stalked trichome between two non-glandular cystolythic trichomes, viewed after twelve hours in 0.1% tetrazolium solution (Potter, D..J.).

As the trichomes aged, it was common for the resin head to become detached from the stalk. This would usually arise as a result of the entire head parting with the stalk (as commenced in Figure 3.12a). In this photomicrograph the epidermal cells have pulled away from the resin head, the hypodermal cells maintaining contact via a narrow channel of stipe cells, which are still connected to the disk of secretory cells within the resin head. In Figure 3.12b the resin head has completely detached and been removed. The stipe cells are just seen protruding from the top of the stalk. Less frequently, the resin head would detach as a result of a fissure apparently forming above the secretory cell disk (Figure 3.13).



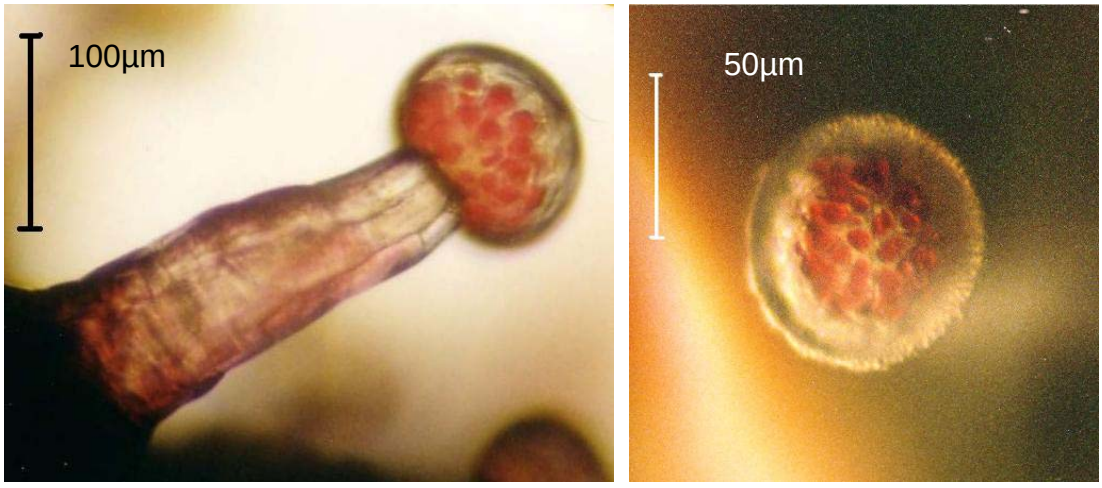
(a) (b)

Figure 3.12. (a) A capitate stalked trichome temporarily dry-mounted and viewed in transmitted and incident light. The glandular head has become partly detached from the stalk to expose the stipe cells, which connect the disk of secretory cells to the hypodermal cells within the stalk; (b) The stalk of a capitate stalked trichome after detachment of the resin head. The specimen was dry-mounted and illuminated with incident light. The stipe cells can just be seen protruding from the top of the stalk (Potter, D..J.).

Figure 3.13. Separation of the glandular head during (left) and after (right) the appearance of a fissure above the secretory cells. The example shown was observed on (M280). The specimen was viewed in incident light (Potter, D..J.).

Figures 3.14a and 3.14b show an alternative view of a capitate stalked trichome before and after detachment of the resin head. Many of the cells in this clone are naturally pigmented red, some of which are on view within the secretory head (Figure 3.14a). During secretory head detachment, the base of the secretory cell disk has remained

attached to the stalk, but the secretory cells have been detached along with remainder of the secretory head (Figure 3.14b). The secretory head tissue remaining attached to the stalk would appear to be formed of thickened resin-head cuticle. In an earlier publication it was stated that the red structures within the secretory head were the secretory cells (Potter, 2004). With the improved knowledge of trichome morphology since gained, it would now appear that these flavonoid-pigmented structures were not secretory cells but the vessels carrying carbohydrate and water to the secretory cells. These appear embedded in and surrounded by a dense opaque material. A substantial proportion of the weight of the resin head clearly has to be attributed to this rigid basal material. It is quite likely that much of this material is made of cutin, suberin or both. The lateral walls of glandular trichomes in many species become thickened with this material, forming a so-called impermeable casparian strip. This prevents apoplastic flow of secretory products down the trichome stalk (Werker, 2000).



(a) (b)

Figure 3.14. (a) An intact glandular stalked trichome of a naturally pigmented clone M186, with coloured cells visible within the resin head. The sample was temporarily mounted in hemp oil; (b) A direct overhead-view of the stalk of a capitate stalked trichome on clone M186 after resin head detachment. The resin head has become detached leaving thr base of the secretory head attached to the stalk. The sample was temporarily mounted in oil (Potter, D..J.).

Steady views of the outer surface of the base of more-mature secretory heads were made possible by trapping the structures on the surface of adhesive tape. (Attempting to remove resin heads from very immature capitate stalked trichomes usually ruptured the thinner outer membrane, causing spillage of the contents.) The scars of attachment to the four stipe cells were readily observed (Figure 3.15). This is surrounded by the apparently-encrusted material of the secretory head outer wall. It would appear from these studies that there are three methods of secretory head

detachment. In the vast majority of cases it would result from a split occurring in the basal or stipe cells. Less commonly however, some of the secretory head could remain attached to the stalk, and this may or may not include the secretory cells. Whether or not the secretory cells are included in the collected material, this would not affect the balance of the main secondary metabolites, as research suggests that the secretory cells contain no cannabinoids (Mahlberg *et al*., 2004; Sirikantaramas, 2005; Taura *et al.,* 2007).

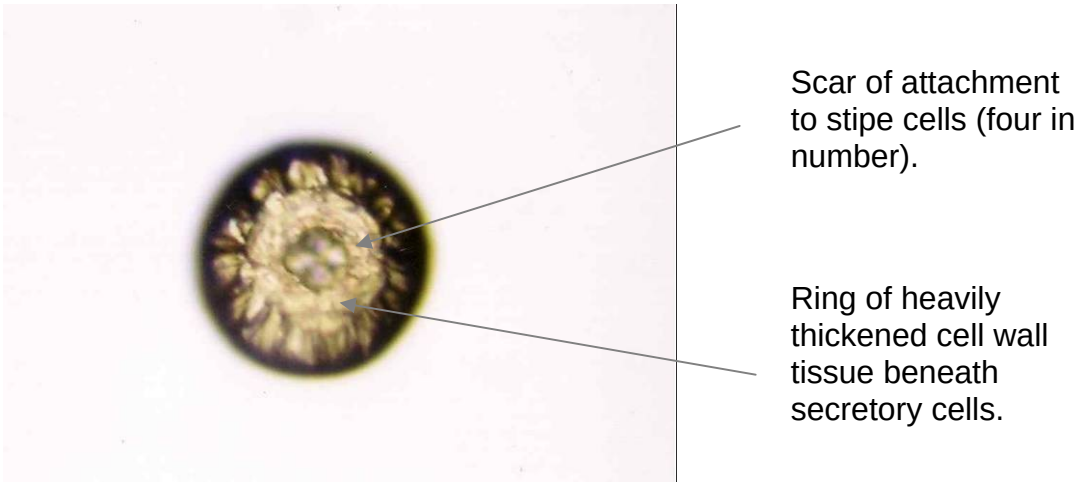
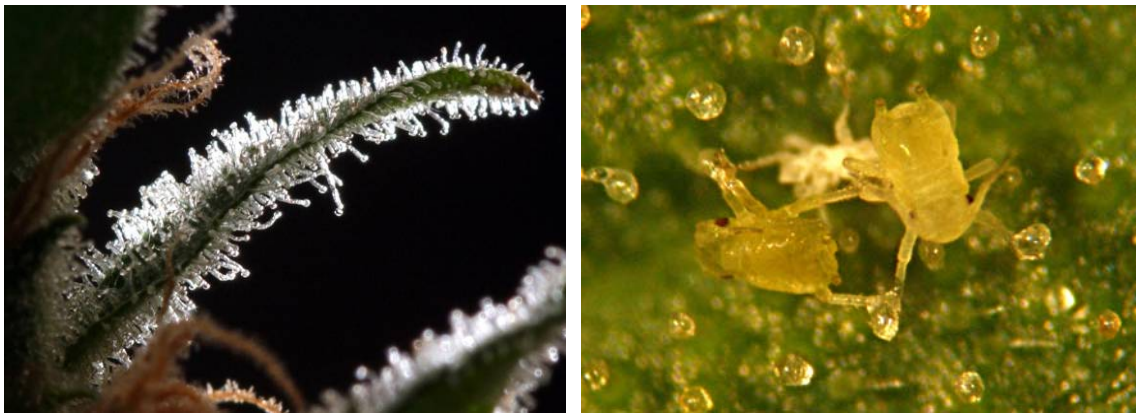


Figure 3.15. A detached resin head (approximately 100 µm diameter) from a capitate stalked trichome, viewed from below to gain a clear view of the scar where the stipe cells were originally attached. The head had been trapped on the surface of clear adhesive tape (Potter, D..J.).

On the female plant's calyx, bracteoles, bracts and associated petioles capitate stalked trichomes would commonly form a dense pubescence (Figure 3.16a), which would act as a physical barrier to small phytophagous insects. By trapping a layer of air close to the surface, it would also provide some protection against desiccating cold winds (Mahlberg *et al.,* 1984). By reflecting infra-red light a dense trichome pubescence has cooling properties and, being equally effective across the complete light spectrum, it also reflects ultra-violet (Roberecht and Caldwell, 1980). Phenolic resins like the cannabinoids have also been shown to offer UV protection (Rhodes, 1977). This is especially welcome in floral structures housing gametophytic tissues, which are susceptible to damage by UV-B radiation (Caldwell *et al.,* 1983). Although glandular trichomes never exhibited a complete cover on bracts and bracteole tissue, UV protection could never be complete, but any degree of protection would improve survival chances.

Struggling insects were frequently found trapped to the resin heads of capitate stalked trichomes, thereby inhibited from further feeding and reproduction (Figure 3.16b). This defensive role of trichomes is observed in many other plant species, e.g. Alfalfa weevil *Empoasca fabae* on lucerne *Medicago sativa* (Shade *et al*., 1975) and other insect species on *Pelargonium* spp. (Harman *et al.,*. 1991).The most common victim observed struggling on *Cannabis* was the cotton melon aphid *Aphis gossypii*. When attacked by predators, *Aphis gossypii* emits an alarm pheromone to warn other others of danger (Byers, 2005). It is possible that a trichome-ensnared aphid responds similarly. One of the most common pests of cannabis – the tobacco thrip *Thrips tobaci* - would also become trapped. It too is capable of emitting an alarm pheromone (Anathakrishnan 1993). If this theory is correct, the loss of a few trichomes to insects could discourage a more extensive attack. Restricted allocation of capitate stalked trichomes to floral tissue is widespread throughout the Plant Kingdom, where plants optimise investment in defence by allocating secondary metabolites to tissues in direct proportion to their value (Herms and Matson, 1992). It was notable that sessile trichomes played no part in insect entrapment, suggesting that these had a different function. The cannabinoids CBGA and THCA have been shown to cause apoptosis in insect cells, and it has been suggested that this is an important defensive role for cannabinoids in capitate stalked and sessile trichomes (Sirikantaramas *et al.,* 2005).

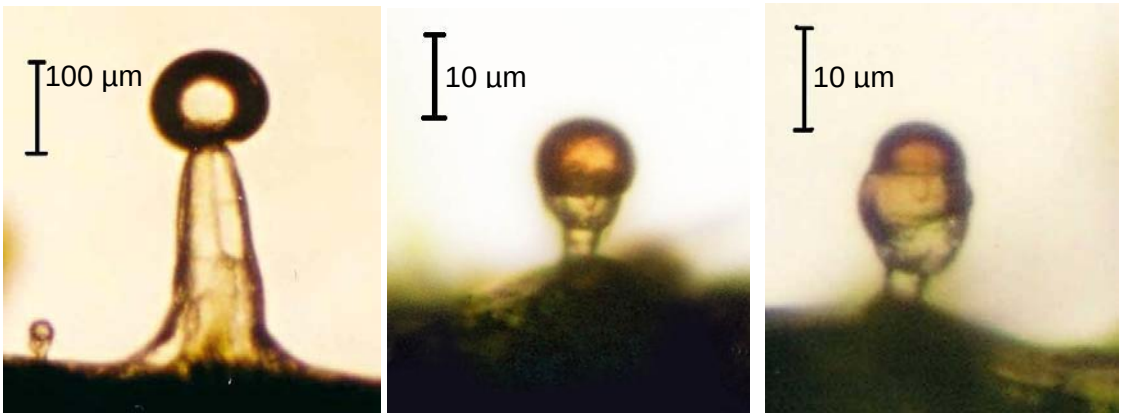


(a) (b)

Figure 3.16 (a) A dense pubescence of glandular stalked trichomes on a bract within a cannabis female inflorescence. The specimen was illuminated from behind and photographed with a tripod-mounted camera incorporating a macro lens. The orange/brown structures are senesced stigmas; (b) two young cotton-melon aphids *Aphis gossypii*. All six legs on each specimen are irreversibly adhered to the resin heads of capitate stalked trichomes (Potter, D..J.).

3.5.1.6 Bulbous Trichomes

With a diameter of approximately 10-20 µm, these were the smallest of the glandular trichomes (Figure 3.17a). First seen on the stem and the lower leaves, these were widespread across the entire surface of the aerial part of the plant. Connected to the epidermis by two cells (the top one much larger than the lower) these would produce a simple spherical glandular head (Figure 3.17b) or a rarer complex multi-compartmented glandular head (Figure 3.17c). Their function is not known. It was notable that the CBG chemovar, shown in Figure 3.13, produced opaque white capitate stalked and sessile trichome resin heads. This opacity is attributed to the CBGA. The bulbous trichomes on this chemovar were clear or brown, as in Figure 3.16b. This suggests that, in this genotype at least, the bulbous trichomes do not contain significant concentrations of the cannabinoid.



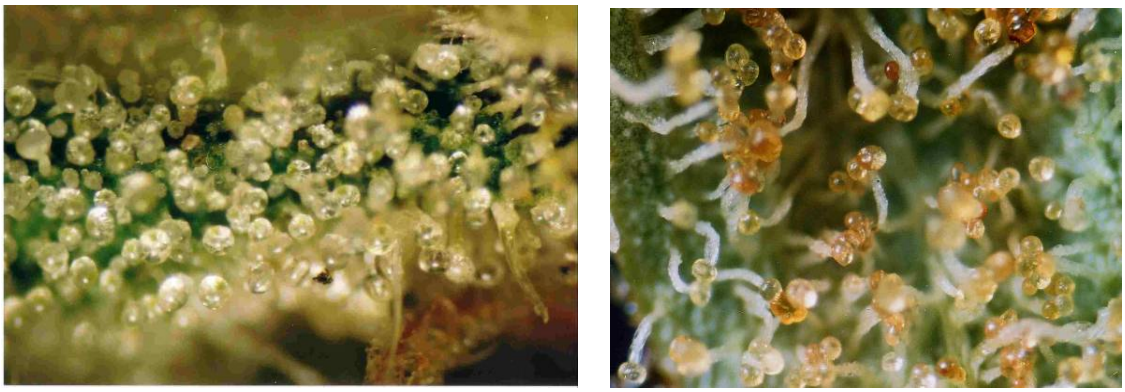
(a) (b) (c)

Figure 3.17 (a) A small bulbous trichome (left) alongside a fully developed glandular stalked trichome. The contrast in resin head diameter (10 µm v 100 µm) is clear; (b) a simple bulbous trichome and (c) a complex bulbous trichome. These are 10-15 µm in diameter. These samples were temporarily dry-mounted and viewed in mixed transmitted and incident light (Potter, D..J.).

3.5.1.7 Effect of age and storage on glandular trichome colour

Glandular trichome resin heads were observed to turn brown as the plant aged, as previously reported by Turner *et al.* (1977) and Mahlberg *et al.* (1984). Bulbous trichomes were the first to discolour, as seen in Figures 3.16b and c. In the glasshouse this browning would rarely be seen on capitate stalked trichomes that were less than five weeks old. The colouration continued during storage at ambient temperatures. The clear colouration of freshly harvested floral material is contrasted with the brown colour of a three-year old sample in Figures 3.18a and 3.18b. The width of the

glandular stalk in the dried sample has narrowed during drying due to the collapse of the epidermal cells. This has also resulted on the stalks losing their turgid upright appearance.



(a) (b)

Figure 3.18. (a) Clear glandular stalked trichomes on freshly harvested young cannabis floral tissue; (b) Brown trichomes on three-year old stored cannabis (Potter, D..J.).

3.5.2. Effect of glandular trichome array on the secondary metabolite content of plant tissues

The densities of capitate stalked and sessile trichomes counted on the proximal and distal sections of bracts are shown in Figure 3.19.

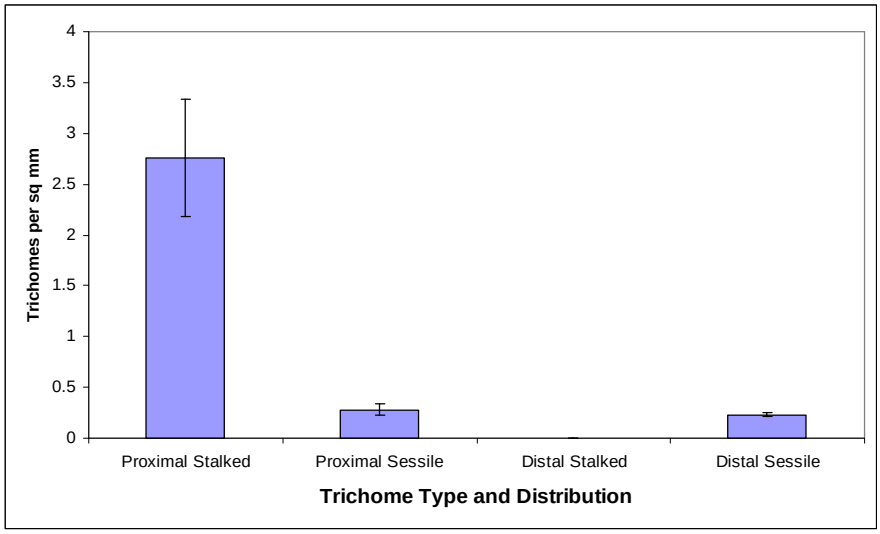


Figure 3.19 The mean density of capitate stalked and sessile trichomes (± 1SE) (upper and lower surfaces combined), on each of three high-THC cultivars M3, M6 and M7. On each clone twenty randomly selected fields were counted on both the upper and lower surface in the proximal and distal areas.

This confirmed that the bract dissection method had been successful in separating the samples into proximal sections, where capitate stalked trichomes were abundant, and distal sections where they were absent. Despite the polarisation of capitate stalked trichome populations, the density of sessile trichomes was found to be similar in proximal and distal areas. It was also noted that glandular stalked trichomes were in the greatest numbers on the upper surface of the bract in this variety. Studies with other varieties (not reported here) showed trichome density to be greatest on the underside of the bract.

In all three clones analyses of variance showed that distal tissue had a significantly higher proportion ($p < 0.001$) of CBC within the cannabinoid profile. Figure 3.20 showed that the capitate stalked trichomes in the proximal area outnumbered sessile trichomes by a factor of approximately five. Individual capitate stalked trichome resin heads have a volume approximately eight times greater than that of a sessile trichome. Combining these factors, the potential difference in volume of the capitate stalked and sessile populations is therefore a factor of forty. The findings of previous workers suggest that the difference in quantity of cannabinoid stored could be even greater, as capitate stalked trichome contents were also found to have a higher cannabinoid concentration (Turner *et al.,* 1977). This suggests that, on tissues that have abundant capitate stalked trichomes, the sessile trichomes population would have minimal influence on the overall cannabinoid potency and profile of that tissue.

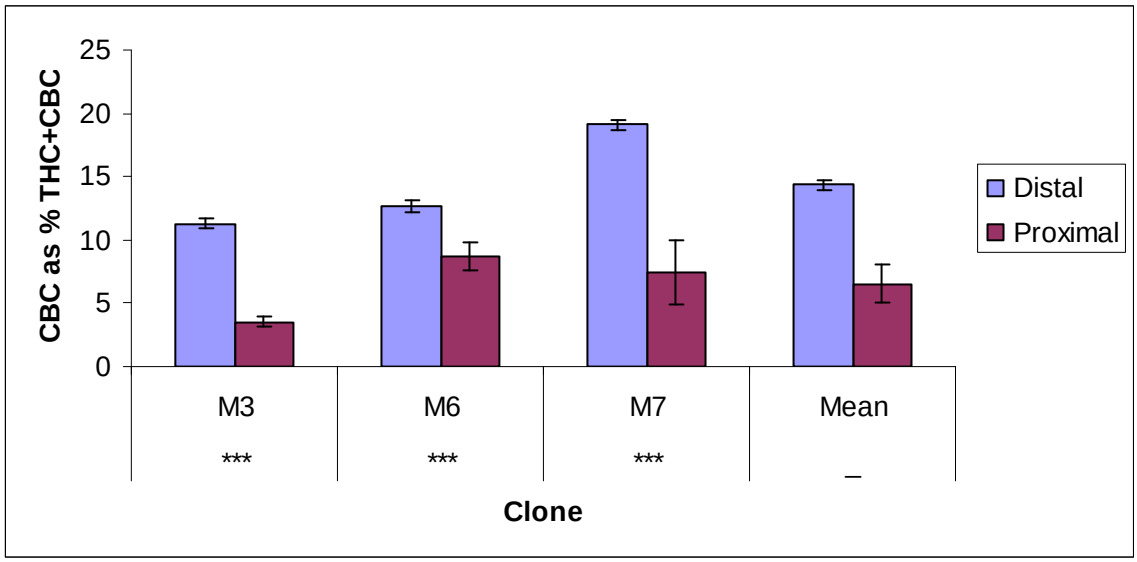


Figure 3.20. The proportion of CBC expressed as % of THC+CBC in the cannabinoid profile of proximal and distal tissue of bracts from each of three high-THC clones. (Error bars on clones represent ± 1SD, and on the mean ± SE). In all three clones the difference was highly significant (ANOVA, *** denotes $p < 0.001$).

Producers of illicit sinsemilla cannabis commonly recognise that distal bract tissue has a much lower cannabinoid content. It is common practice to remove the distal part of the less resinous bracts. The material that remains is consequently reduced in weight, but its overall potency is increased. Illicit producers commonly refer to this process as 'manicuring' (Potter, 2004). The practice has also been followed by producers of pharmaceutical grade medicinal cannabis in the Netherlands (Institute of Medical Marijuana, 2008). In pharmacognosy, the removal of less desirable material from a pharmaceutical feedstock is sometimes called 'garbling' (Tyler *et al.*, 1988). This study suggests that, where bracts only exhibit glandular stalked trichomes on the proximal tissue, removal of the distal tissue will indeed increase the potency of the material that remains. The cannabinoid profile of the remaining material will be minimally affected, because of the relatively small influence of the associated sessile trichome population.

3.5.3 Effect of capitate stalked trichome density and colour on cannabinoid content and profile.

The mean THC content was calculated for all sinsemilla samples attracting the same score for trichome density. The vast majority of samples attracted scores on the first five points of this 1-9 scale (76% being awarded a score of 2 or 3) and meaningful standard error calculations were only possible on values in this range (Figure 3.21).

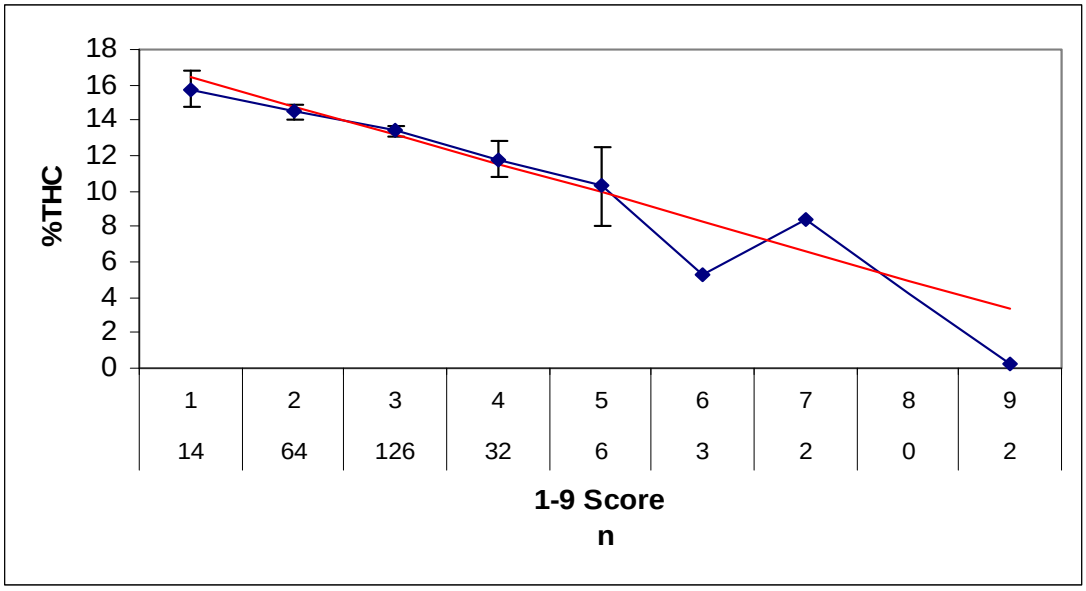


Figure 3.21 The correlation between capitate stalked trichome density (visually assessed 1-9 scale, 1 = high, 9 = low) and the overall THC content of the sample. Values shown are the mean % w/w THC content (± SE only where n > 5) of all samples for each density score. Regression line is shown in red. The regression model is:- % THC = 18.051 – 1.629 * Density Score. ($p < 0.001$, $R^2 = 0.17$).

The results show that there is clearly a significant tendency for plant samples with greater capitate stalked trichome densities to have a higher cannabinoid content. In plants with a less dense pubescence, high cannabinoid contents are not simply maintained by more secondary metabolite being sequestered in each trichome. A high density of glandular trichomes is therefore a very useful visual parameter upon which a judgement can be made of the cannabinoid content of dry plant material. However, the low R^2 value shows that this is only an approximate guide when assessing samples of variable genotype and provenance.

The relationship between trichome colour and cannabinoid content is shown in Figure 3.22. The data were more evenly spread than those from the corresponding trichome density study. The slope of the regression line shows that there is a weak but significant tendency for darker coloured trichomes to be associated with low-potency cannabis samples. Turner *et al.* (1977), comparing the THC content of colourless and brown capitate stalked trichomes of a single clone, reported that brown trichomes contained less THC per trichome. However, there were no accompanying statistical analyses to clarify the significance of the reported finding.

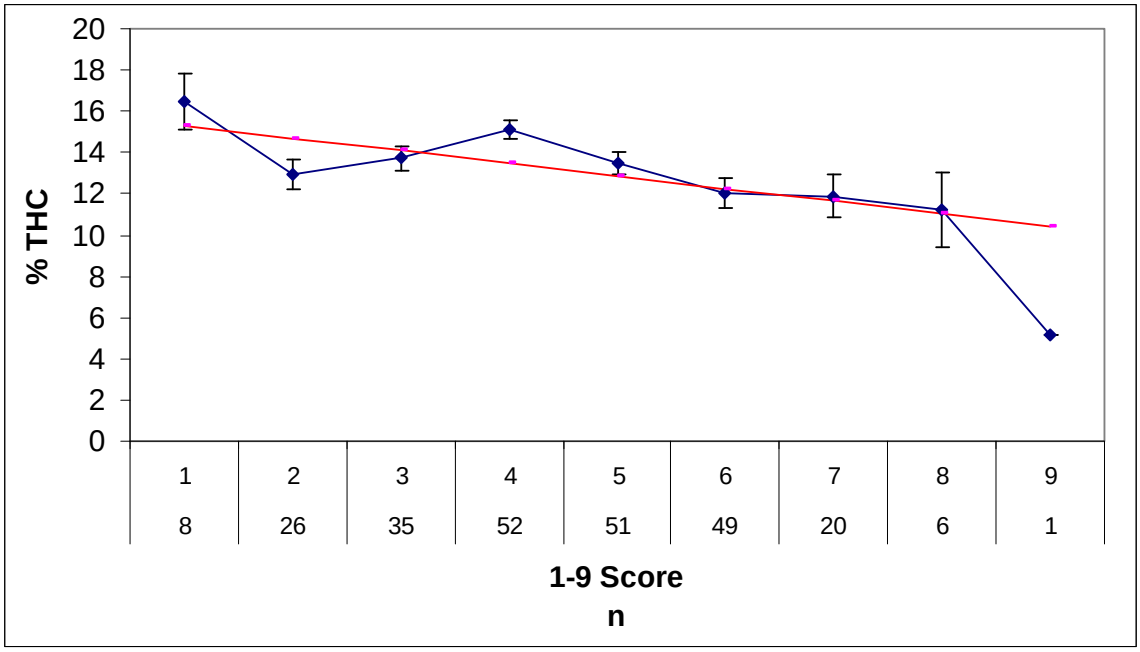


Figure 3.22. The correlation between capitate stalked trichome colour (visually assessed using a 1-9 scale, 1 = clear, 9 = darkest brown) and the overall THC content of the sample. Values shown are the mean % w/w THC content (±SE) of all samples for each colour score. Regression line is shown in red. The model for this is: - Percent THC = 15.990 – 0.587 * Trichome Colour Score ($p < 0.001$, $R^2 = 0.053$).

The pigment causing this brown colouration is not known. Commencement of browning was commonly observed in the region around the secretory cells and could be due to oxygen entering the resin head via the scar tissue (Figure 3.13) when it becomes detached from the epidermal cells on the trichome stalk, as shown in Figure 3.12a. Alternatively it could be caused by the biproducts of catabolism of the secretory cells contents.

The mean average CBN content of the samples awarded each score is shown in Figure 3.23. For those samples where CBN contents were very low and below the minimum detectable level of 0.10% a nominal value of 0.05% was used.

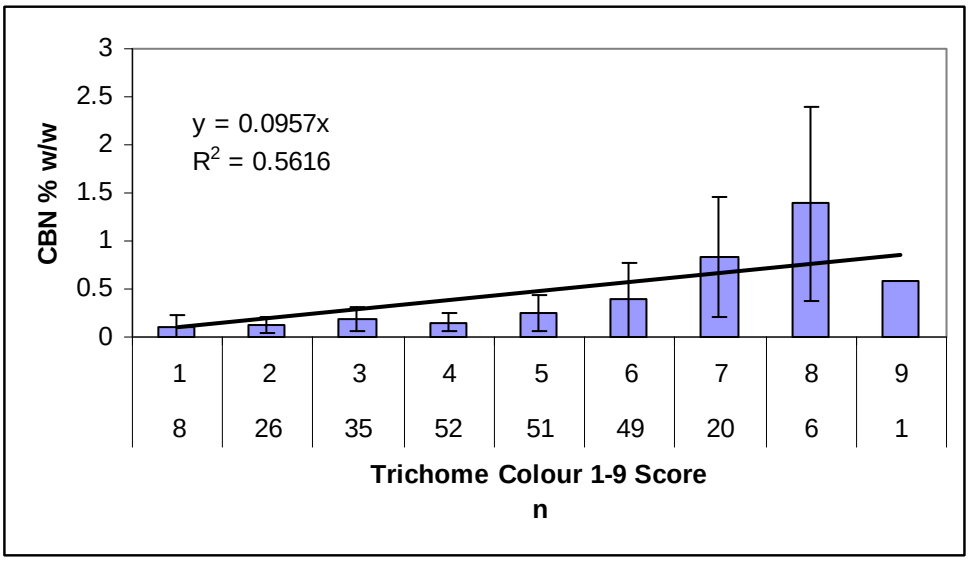


Figure 3.23. The mean CBN content (± SD) of populations of sinsemilla samples awarded each of the 1-9 ratings for trichome colour 1 = clear, 9 = darkest brown. All samples were seized by police in 2004/2005.

There was a trend for samples with darker trichomes to have a higher CBN content but the data was extremely variable, as shown by the large standard deviations. The degree of THC catabolism was assessed by expressing the CBN as a proportion of the CBN+THC total for each sample (i.e. CBN/(THC+CBN). The correlation between trichome colour and CBN/(THC+CBN) is shown as a scatter-graph in Figure 3.24. This shows that very little CBN existed in clear or white trichomes. A high level of CBN formation was restricted to plants with brown glandular trichomes, but dark coloured trichomes could still be devoid of this catabolite. Six values were identified as clear

outliers, according to the ISO recommended Grubb's Test (Miller and Miller, 2005) and excluded.

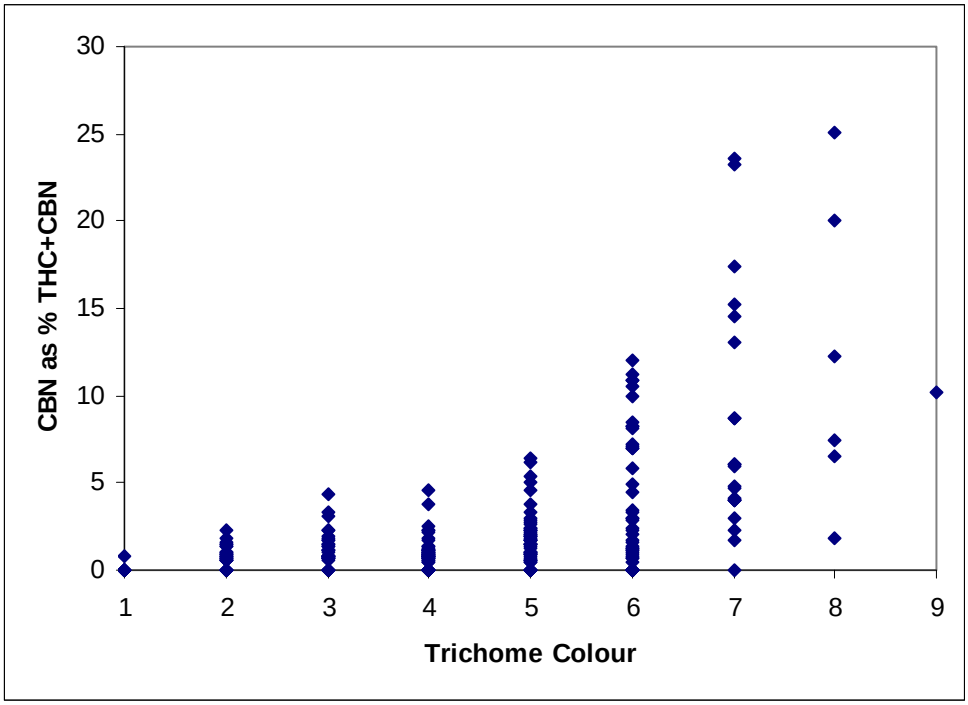


Figure 3.24. The variability in degree of THC catabolism to CBN as related to their colour. Of 249 original samples 6 were rejected as outliers and not included here. Trichome colour was assessed visually and scored on a 1-9 scale where 1 represents totally clear and successively higher scores denote an increasing opacity and darkening in colour.

The comparison between trichome colour and CBN content showed that the lower average potency in darker colour trichomes could be almost entirely due the THC having catabolised in these samples. There was a greater likelihood of finding CBN in brown coloured trichomes but its presence was not guaranteed. This shows that if a pharmaceutical company demands cannabis material containing minimal levels is CBN, a brown colouration does not immediately imply rejection of the sample.

It appears that the conditions required for the oxidative catabolism of THC to CBN are not the same as those causing the brown colouration. Experienced observation of many samples has shown that this pigmentation is not reversible and the colour stabilises. Indeed, this colour was seen to have been maintained in trichomes photographed while contributing to studies on an archaeological cannabis sample discovered in an ancient Chinese tomb. Carbon dating estimates the age of the sample in Figure 3.25 as 2700 years old. It is accepted that additional catabolic reactions would have occurred over time and possibly had an influence on the colour,

but catabolism appeared to have stabilised and cannabinoids were still present in these trichomes (Russo *et al.,* 2008).

Figure 3.25. Aged brown sessile glandular trichomes on 2700 year old cannabis. The sample is illuminated with incident light and photographed digitally. The pubescence of unicellular non-glandular trichomes is also clearly visible (Potter, D..J.).

3.5.4 Effect of photosynthetic ability, or lack of ability, on cannabinoid biosynthesis in sessile trichomes on variegated leaf tissue.

The potency of the leaf tissue samples in these tests is shown in Table 3.4. On a dry weight basis, yellow leaf material was clearly more potent than green. This would have been influenced by the fact that green tissue weighed 33% more per unit area, probably due to the production and storage of starch in these tissues following photosynthesis. By assessing potency in terms of cannabinoid per unit area, the effect of starch accumulation would be removed. Assessed this way, the yellow material was still significantly more potent ($p < 0.05$), although the difference was much smaller. Microscope observations suggested that this difference was not due to differences in densities of sessile trichomes on yellow and green tissue, but an accurate count was not possible because of the density of non-glandular trichomes obscuring the view. The earlier work by Crombie (1977) had shown that cannabinoid biosynthesis still continued in 'albino' tissue. This study went further and suggested that cannabinoid biosynthesis was totally unrelated to the immediate tissue's ability to photosynthesise. However, the rate of biosynthesis in these tissues would be affected by the overall photosynthetic-ability of the plant.

The biosynthesis of terpenoids has a higher energy requirement than most other primary and secondary metabolites, because of the extensive level of chemical reduction within these compounds (Gershenzon, 1994). The energy for this biosynthesis would be derived from light energy, captured within the chloroplast. During periods of light exposure, some chloroplasts would be directly illuminated and others would be in varying degrees of shade. This study showed that plant tissues devoid of chlorophyll, and thereby unable to photosynthesise, could support the biosynthesis of cannabinoids within their own glandular trichomes. The carbon source required for cannabinoid biosynthesis would have been produced elsewhere within the plant and then translocated to these trichomes. This demonstrates that in a normal growing environment, where some parts of the plant are in full sun and others in varying degrees of shade, all aerial parts the plant will be able to synthesise cannabinoids. The resultant increase in trichome content uniformity is fortunate for the grower of cannabis for the pharmaceutical industry.

	Green Tissue	Yellow Tissue	ANOVA *p* value
Potency by Weight Test 1. THC % w/w dry (n = 3 bulked samples)	1.51 (± 0.26)	2.30 (± 0.26)	0.008
Potency by Weight Test 2. THC % w/w dry (n = 2 bulked samples)	1.18 (± 0.08)	1.92 (± 0.08)	0.005
Potency by Area Test 2. THC g m^{-2} (n = 2 bulked samples)	0.50 (± 0.03)	0.61 (± 0.01)	0.038

Table 3.4. The potency (THC content) of yellow and green leaf tissue of the variegated cultivar G60-M55 assessed in each of two tests (± SD). The potency in the second test is also shown as a weight of THC per unit area.

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Mahlberg *et al.* (1983) compared the cannabinoid content of capitate stalked trichomes on the upper and lower surfaces of cannabis bracts and found those on the upper surface to be more potent. This was attributed to the upper surface receiving more light. This study with variegated cannabis weakens that argument, showing that the ability of leaf tissue to photosynthesise has minimal effect on its ability of the local glandular trichomes to synthesise cannabinoids.

3.6 CONCLUSIONS

While performing a detailed study of cannabis trichomes, Dayanandan and Kaufman (1976) stated that the study of these organelles was essential to understand the biogenesis, distribution and function of the different cannabinoids. The research reported in this chapter clearly showed how the distribution of THC and CBC differed. The subcellular details of cannabinoid secretion were also shown in greater detail than perhaps previously captured with light microscopes.

Within the pubescence on *Cannabis sativa* L there are six forms of trichome, of which only five are found on the female plant. With appropriate mounting and staining, it was shown that what was believed to be a previously unavailable undistorted views of the internal structures of these trichomes could be differentiated to a high degree with the use of low cost microscopes. The use of these mounting media could facilitate future further studies of these structures.

It was shown that the cannabinoid profile of bract tissue appears to be affected by the ratio of so called ‘capitate stalked’ and ‘sessile’ trichomes present. This suggests that the two types of trichome had differing cannabinoid profiles. By separating tissues with differing ratios of capitate stalked and sessile trichomes, it was shown that the grower had the capability to produce samples with significantly different cannabinoid profiles. The research also showed that insects were regularly ensnared by glandular stalked trichomes but never by capitate sessile trichomes. This was a clear indication that these two trichome forms differed in function. It was hypothesised that this was possibly attributable to a difference in secondary metabolite content. The observed contrasting sizes and densities of glandular stalked and sessile trichomes suggested that in floral material, exhibiting both trichome types, the capitate stalked trichome population had a potentially greater influence on cannabinoid profile than the sessile trichome population.

Vital staining techniques provided supportive evidence that that the secretory cells within the trichome head were the area of greatest metabolic activity. Novel studies with variegated cannabis also showed that secretory cells within leaf tissue lacking chlorophyll suffered no reduction in cannabinoid synthesis ability. In fact a small but significantly higher concentration of cannabinoid (weight per unit area) was found in tissue lacking chlorophyll. Although the overall ability of the plant to biosynthesise cannabinoids was likely related to the total amount of energy available to the plant, this study implied that where part of a plant was in shade, this did not affect its ability to

biosynthesise cannabinoids ability. Thus partial shading did not increase the variability of plant tissue cannabinoid contents.

As glandular trichomes age it was observed that there is a colour change, the initially clear resin head first turning white and then brown. On feedstock exhibiting brown trichomes there is an increased likelihood, but not a guarantee, that there will be a reduced THC content and a raised content of the THC catabolite CBN. A high density of capitate stalked trichomes on the female floral tissue indicates an increased probability, but not a guarantee of high cannabinoid content.

As the capitate stalked form matures, the resin head is increasing likely to detach itself from its stalk. When this occurs, the secretory cells are usually retained within the resin head, but they are sometimes left attached to the stalk. This mode of detachment would be expected to affect the proportion of waxes, proteins and other unidentified ingredients within any enriched trichome preparations made.

Having assessed the form and distribution of the trichomes, Chapter Four looks in detail at the chemical content of the capitate types. It also assesses how this alters as trichomes age. The knowledge gained would help the grower identify the optimum time for harvest and evaluate the possibly of producing feedstocks by removing these trichomes from the plant.

Chapter 4. The Function and Exploitation of Secondary Metabolites from Glandular Trichomes of *Cannabis sativa* L.

4.1 INTRODUCTION

Across the Plant Kingdom, glandular trichomes are responsible for the biosynthesis and/or sequestration of a vast range of secondary metabolites. Of these the terpenoids, which includes the cannabinoids and cannabis-related terpenes, are the most common and structurally diverse compound group, and these are spread throughout many plant families (Kelsey *et al.,* 1984). Other carbon, hydrogen and oxygen based categories of secondary metabolites are also found, but are restricted to the glandular trichomes of smaller ranges of species. Examples include the methylated flavonoids of the creosote bush *Larrea tridentata* (Thompson *et al.,* 1979) and the quinones found in the semitropical plant family Hydrophyllaceae (Kelsey , 1984).

The total number of secondary metabolites found in plants has been estimated at approximately thirty thousand. Of these, about 40% contain nitrogen, of which all but a thousand belong to the alkaloid group (Acamovic and Brooker, 2005). Of the secondary metabolites found in glandular trichomes, the proportion that contains nitrogen is much less than 40% (Wagner *et al.,* 2004). Examples include histamine, which is synthesised within the stinging glandular trichomes of nettles *Urtica spp.* Another example of great importance to world health is the alkaloid nicotine, which is abundant in trichomes of some cultivated varieties of tobacco *Nicotiana tabacum.* Although sequestered in trichomes, this alkaloid is actually translocated there from the roots where it is biosynthesised. Trichomes of the velvet bean *Mucuna pruriens* also contain alkaloids and additionally produce serotonin (5-hydroxytryptamine) (Ghosals Singh *et al.,* 1971).

The previous chapter described the different forms of non-glandular and glandular trichomes in *Cannabis sativa* L and compared their differing functions. It also reported studies which supported previous researchers' findings (Turner *et al.,* 1977, Mahlberg et al., 1984) that capitate stalked and sessile trichomes differed in their cannabinoid profile. It was shown that by segregating plant tissues, according to their trichome pubescence characteristics, it was possible to produce feed-stocks with significantly different cannabinoid profiles. This chapter looks in greater detail at the secondary metabolite content of these trichomes. It was hypothesised that, if the sessile and capitate stalked trichomes could be removed from the plant and then separated, it might be possible to provide feedstocks with more closely controlled cannabinoid

profiles. Separating trichomes that were at different stages of maturation may also have benefits.

The removal of glandular trichomes from living *Cannabis* plants is common practise, being a major part of cannabis resin manufacture. Many centuries-old cultural methods are practised, but few are of relevance to the modern pharmaceutical industry. One method of resin manufacture includes manual rubbing of the inflorescences, resulting in the trichomes adhering to the hands (Cerniak, 1985). British physician W.B. O'Shaughnessy, who introduced cannabis to Western medicine from India, reported that workers dressed in leather would walk through the crop and then scrape off the resin that adhered to their clothing (Samuelsson, 1999). Both techniques rupture the trichomes, accelerating the loss of the more volatile components and the oxidation of others. The materials also become heavily contaminated with other plant fragments. More common methods of trichome removal for resin production are based upon the sieving of dried cannabis plants. This practise is utilised in Morocco, which supplies the vast majority of cannabis resin entering the UK (UNODC, 2006). Plants are dried and sometimes left for many weeks before processing, by which time the trichomes will have lost much of their more volatile terpene content. Techniques used to make so-called 'modern hashish' (Clarke and Watson, 2007) perhaps offer greater opportunities for collecting undamaged glandular trichomes, with more of their secondary metabolites intact. In one method, cannabis material is simply agitated in cold water and the dislodged capitate stalked trichomes collected using sieves. Jansen and Terris (2002) reported that, with a Dutch Government subsidy, this technique had been adapted to make hashish for pharmaceutical research purposes. Separation of capitate stalked and sessile trichomes was not reported. For the remainder of this thesis, the terms hashish and resin are avoided when referring to products made for scientific as opposed to recreational purposes. The term enriched trichome preparation (ETP) is preferred.

As well as the cannabinoids, most of the monoterpenes and sesquiterpenes found in *Cannabis* are also located in the glandular trichomes (Malingré, *et al.,* 1975; Turner *et al.,* 1980). These are generally present in much smaller quantities than the cannabinoids, and this hinders their detailed study. Studies of the terpene content of cannabis trichomes are few and have involved the steam distillation of intact cannabis tissue as the source material (Mediavilla and Steinemann, 1997). The bulk collection of intact cannabis trichomes would clearly facilitate more detailed terpenoid analysis by providing a richer source of material for analysis. To enable this to happen, research was needed to assess the feasibility of removing and then segregating large quantities

of intact glandular trichomes from cannabis. A number of techniques have been employed to remove intact glandular trichomes from other species. These have been reviewed by Wagner *et al.* (2004). Techniques included removal of individual trichomes with forceps, shaking in aqueous solution with an abrasive (e.g. sand, fine glass beads or powdered dry ice) and the gentle brushing of fresh or frozen tissue. In some cases different trichome forms have subsequently been separated by sieving and/or centrifugation, e.g. separation of bulbous and peltate trichomes of sweet basil *Ocimum basilicum* (Gang *et al.,* 2001). All these collection methods produce extremely small trichome samples. In contrast, removal of glandular trichomes on an industrial scale is currently performed in the hop (*Humulus lupulus*) industry. By agitating plant material in extreme cold temperatures, the sessile glandular trichomes (known commercially as lupulin) are dislodged and separated from the plant (Rigby, 2000).

The previous chapter confirmed the findings (Ledbetter and Krikorian, 1975) that the four types of glandular trichome found in *Cannabis*, namely bulbous, capitate sessile, antherial sessile and capitate stalked, had diameters of approximately 10 µm, 50 µm, 80 µm and 100 µm respectively. Based on their comparative spherical dimensions a bulbous trichome would have a potential secondary metabolite content approximately one hundred and twenty five times less than that of a capitate sessile trichome. Previous workers have expressed difficulties in working with bulbous trichomes because of their small size, and evidence for the existence of cannabinoids in these trichomes is lacking. Regular observations of bulbous trichome populations, while studying other trichome forms for this thesis, suggested that the population densities of bulbous trichomes were similar to those of sessile trichomes. This supported the observation of previous researchers (Turner *et al.,*1978; Mahlberg *et al.,* 1984). This, combined with the minute size, suggests that bulbous trichome contribute little to the overall secondary metabolite content of cannabis. Bulbous trichomes were not studied further for this thesis.

4.2 AIM AND OBJECTIVES

A series of studies were performed with the aim of separating, purifying and analysing substantial quantities (>1g) of intact glandular trichomes from cannabis. It was envisaged that trichome filtrates derived from cannabis tissues of differing chemotype, stage of maturity and location on the plant might exhibit different secondary metabolite profiles. As progress was made the following sequence of objectives was developed: -

4.2.1. To collect and separate intact capitate stalked and sessile glandular trichomes from fresh floral material.

4.2.2. To isolate intact sessile glandular trichomes from vegetative material.

4.2.3. To collect sessile trichomes from foliage of a high-CBC chemotype as a means of isolating the minor cannabinoid CBC

4.2.4. To examine the ontogenetic changes in terpenoid content of glandular trichomes during plant maturation.

4.3 MATERIALS

4.3.1 Germplasm

Clone	Chemotype	Variety Name	Supplier
G1-M3 G2-M6 G5-M13 G5-M16 M240	High-THC High-THC High-CBD High-CBD High-CBC	Guinevere Galina Grace Gill Unnamed	GW Pharmaceuticals Ltd, Porton Down Science Park, Salisbury, Wiltshire.

Table 4.1 Name, source and chemotype of clones used.

4.3.2 Apparatus

Kenwood HM 220 Food Mixer	Dixons Ltd. Internet purchase.
25 µm, 43 µm, 73 µm and 220 µm 'Bubblebag' Sieves	EveryoneDoesIT Unit D1, Phoenix Industrial Estate, Rosslyn Crescent, Harrow, HA1 2SP
Ebb and Flood Irrigation Tank 2.0 x 0.7 x 0.4 m	Bridge Greenhouses Ltd., Chalk Lane, Sidlesham, Chichester, PO20 7NQ

Table 4.2 Miscellaneous items and commercial sources.

4.4 METHODS

4.4.1 Separation of sessile and capitate stalked trichomes glandular heads from mature fresh floral material.

In the first of a series of investigations, this test adapted a method of removing trichomes from cannabis material described by Jansen and Terris (2002). The high-THC clone G1 M3 was used for this study. Approximately 500 g of fresh floral material was removed from a batch of fully mature plants. These had been in flower for eight

weeks and were at the normal stage for harvesting. Using scissors, the material was cut into pieces, each being no greater than approximately five grams. Six randomly selected pieces were retained for analysis. The material was then plunged immediately into twenty five litres of slurry containing tap water and crushed ice. The mixture was gently mixed for ten minutes to allow the plant material to cool and then agitated for ten minutes using a Kenwood HM220 domestic food-mixer operating at maximum speed. Manual examination confirmed that the glandular trichomes lost their sticky texture at low temperatures. A large proportion of the trichome glandular resin heads were dislodged from the plant by this process. Being denser than water they sank and readily separated from the pulp when poured through a fine sieve (220 µm approximate mesh). The resin heads passed through the mesh and the 'spent pulp' was retained. The resin heads are then efficiently separated from the bulk of the water by pouring the suspension through a 73 µm sieve and then a finer 25 µm sieve. In theory, the resin heads from glandular stalked trichomes (reported typical diameter 75-100 µm) should have been trapped on the 73 µm sieve, sessile trichomes (typically 50 µm) falling through and being caught on the 25 µm mesh.

The resin heads collected from each sieve were removed and a few milligrams taken for microscope study. The remainder was frozen while awaiting chemical analysis.

4.4.2 Bulk-Production of Pure Sessile Trichome Preparations.

Five kilograms of leaves were removed from plants of the high-THC clone G1 M3. The plants had been grown for three weeks in continuous lighting (75 Wm^{-2} PAR) at 25ºC, and were therefore still in the vegetative phase and devoid of capitate stalked trichomes. The collected material was thoroughly mixed and approximately twenty grams frozen (-20ºC) pending analysis by GC (Appendix 2). The 5 kg of foliage was thoroughly mixed in a shallow tank with 200 litres of iced water for thirty minutes using a domestic food mixer at maximum speed. The bulk of the treated foliage was then manually removed and the liquid remains drained through 220 µm and 25 µm sieves. The material collected on the second sieve was examined for purity, using a microscope, and a sub-sample (approximately 1 g) taken for cannabinoid assay. The remaining material was frozen prior to steam distillation and qualitative chemical analysis by GC at Botanix Ltd. A batch of plants (≈ 300) of the same clone was also grown to full maturity, then harvested and dried for routine drug production. Six random subsamples (approximately 5 g) of the stripped leaf and foliar material were thoroughly mixed prior to analysis by GC (Appendix 1).

4.4.3 Production of a cannabichromene-rich sessile trichome preparation.

The production of a CBC-rich sessile trichome preparation was attempted, using the above trichome collection method. The plant material utilized was stripped foliage of the CBC-rich clone M240. These plants had been grown vegetatively for four weeks in the same regime.

4.4.4 Ontogenetic changes in Secondary Metabolite Content of Glandular Trichome Contents.

Two cultivars were selected for this study; high-THC clone G2 M6, and high-CBD clone G5 M13. Plants were grown according to the routine methods for the propagation of the medicinal cannabis crop. Ten randomly selected plants of each cultivar were removed from the crop after three weeks of vegetative growth. Further samples were made on each of five harvest dates - six, seven, eight, nine and ten weeks after the induction of flowering. The foliage and floral material from each batch was stripped from the stem. This was thoroughly mixed and six subsamples (approximately 10 g each) were taken. All materials were frozen at -20ºC pending analysis. Stems were discarded. The six subsamples were subsequently analysed for cannabinoid content by GW Pharmaceutical Ltd using GC (Appendix 1).

The main samples were distilled by Botanix Limited according to their Standard Operating Procedure for the Determination of Volatile Hop Oil (Institute of Brewing Method). Each was ladled into a one-litre round bottom flask with a B55 neck. A few anti-bumping granules were added before connecting each one to a 'British Pharmacopoeia Still' using a B55/34 adaptor. The flasks were then heated using a heating mantle and the contents distilled for three hours. Employing this method, Howard (1970) showed that the extraction of essential oils from hop lupulin (glandular trichomes) was reliably complete in three hours and indeed no further distillate was seen to be collected after this time. During this period the flow of condensate was controlled to cause minimum disturbance to the oil in the trap. At the end of three hours the volume of oil was recorded and decanted. These oil fractions were analysed for qualitative terpene content by Botanix Ltd. The quantitative content of a small range of terpenes within this mixture was determined by GC at GW Pharmaceuticals Ltd (Appendix 2).

4.5 RESULTS AND DISCUSSION

4.5.1 Separation of Sessile and Capitate Stalked Trichomes Glandular Heads from mature fresh floral material

Table 4.3 shows the cannabinoid content of single trichome preparations collected from the fine and coarser sieves and a bulked sample of plant material before and after trichome collection. Three sub-samples of each material were taken for analysis. The standard deviations therefore only indicate the uniformity of the preparations and the precision of the analytical method.

Sample Details	% of each cannabinoid in the cannabinoid total ± SD			% w/w total cannabinoid ± SD
	CBC	CBG	THC	
Sessile trichome rich filtrate - 25 µm sieve	1.44 ± 0.02	1.19 ± 0.04	97.37 ± 0.05	37.00 ± 7.26
Capitate stalked trichome rich filtrate - 73 µm sieve	0.93 ± 0.02	0.86 ± 0.03	98.21 ± 0.02	58.70 ± 4.29
Starter Material	1.13 ± 0.14	1.30 ± 0.05	97.58 ± 0.16	11.50 ± 0.34
Spent Pulp	1.07 ± 0.18	1.21 ± 0.03	97.72 ± 0.19	7.30 ± 1.53

Table 4.3. A comparison of the cannabinoid profile and content of fresh cannabis floral material (clone G1 M3) and sieved trichome filtrates made there from. One sample of each fraction was produced. Analyses show mean analytical results of three subsamples (±SD). Also shown is the cannabinoid content of the floral material before trichome extraction and the spent pulp after extraction (n=3).

Microscope analysis confirmed that most of the material caught on the 73 µm sieve consisted of the larger resin heads from capitate stalked trichomes. The material caught on the 25 µm sieve appeared to consist predominantly of the smaller sessile trichome resin heads. However, the resin heads from some immature capitate stalked trichomes may have also passed through the 73 µm sieve and been included here. The material collected on the finer 25 µm sieve had a much lower cannabinoid content (37% w/w) than that removed from the coarser 73 µm sieve (59% w/w). This supports previous observations in a previous study that showed the cannabinoid concentration in individually collected sessile trichomes to be lower than that of the capitate stalked form (Turner *et al.,* 1978, 1979).

The sessile trichome rich filtrate on the finer sieve had a notably higher proportion of the cannabinoid CBC. The result supports the finding of the previous chapter, suggesting that the two trichome forms appear to have different cannabinoid profiles. However, in that earlier study, the comparison was made between capitate stalked and sessile trichome populations from individual bracts, and the two trichome forms were of the same age. In this later study with sieved trichome samples, the average age of the sessile trichomes would have been greater than that of the capitate stalked trichomes. This is because the sessile form would have been produced throughout the lifetime of the plant, whereas the bulk of the capitate stalked trichomes would have been formed within the last few weeks of growth. It has been shown that, in most chemotypes, the proportion of CBC within the cannabinoid profile of the whole plant drops sharply during the life-time of the plant (Shoyama *et al*., 1975; de Meijer *et al*., 2009). The drop is greatest during the first weeks of growth when no capitate stalked trichomes are present, and the decrease in CBC proportion thus cannot be attributed to a changing balance of sessile and capitate stalked trichomes. The higher proportion of CBC in the filtrate on the finer sieve would have thus likely been due to two factors: -

i) It contained more sessile trichomes, which have been shown to have a higher proportion of CBC to capitate stalked trichomes of the same age, and

ii) The trichome on this sieve contained a higher proportion developed within the first weeks of growth when the proportion of CBC within the whole plant was much higher.

Table 4.3 also shows that there was a higher proportion of CBG in the cannabinoid profile of the material collected on the finer sieve. It is not possible to state if this is due to direct differences in the cannabinoid profile of sessile and capitate stalked trichomes, or due (at least partly) to the younger average age of the capitate stalked trichome-rich material. (Younger material would reasonably be expected to contain a higher proportion of trichomes within which less of the GPP and olivetolic acid had been fully converted into THC via the intermediate in question – CBG.) In the previous chapter, where the cannabinoid profiles of similar aged trichome populations on proximal and distal bract tissue were compared, CBG was not present in detectable quantities in all samples. A comparison with that test is therefore not possible. Even though the reasons were not totally clear, from this test it was important to note that separating trichomes with sieves appeared to offer a means of producing phytopharmaceutical feedstocks with a favorably altered cannabinoid profiles.

4.5.2 Isolation of intact sessile glandular trichomes from vegetative material

This test clearly demonstrated that intact sessile trichome resin heads could be readily collected from non-flowering cannabis vegetation. This, and the previous test, overcame concerns that the technique might only be efficient on capitate stalked trichomes which, by virtue of their greater size and upright shape, would experience greater shearing forces from the agitated iced-water. A sub-sample of this preparation is shown in Figure 4.1.

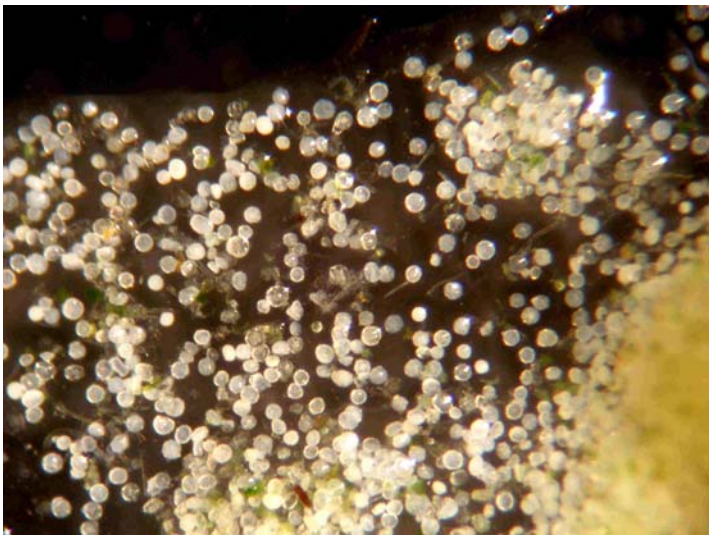

Figure 4.1. A smeared sample of sessile glandular trichome resin heads prepared from vegetative foliage of high-THC clone G1 M3. The freshly captures specimens were collected from the surface of a 25 µm sieve and are free-floating in water. A few pieces of leaf fragment are also present as a minor contaminant.

It can be seen that there was some minor contamination of this material, with fragments of green leaf tissue. The proportion of resin heads within this filtrate was higher than that generally produced when collecting resin heads from capitate stalked trichomes. The latter would be routinely contaminated with capitate glandular trichome stalks (which are not present on foliage) and cystolythic trichomes (which more readily pass through the coarser sieve). The cannabinoid content of materials collected from both the 25 µm and 45 µm sieves were analysed. In Table 4.4, this is compared to the cannabinoid content of the combined foliar and floral material collected from mature plants of the same cultivar. Although THC was still the dominant cannabinoid in the sieve contents, it can be seen that the proportion of CBC within the cannabinoid profile was greatly increased. The results show the content of trichome preparations collected from 45 µm and 25 µm sieves. For comparison, the cannabinoid content is shown in

aerial material stripped from the stems of 3 week and 11 week old plants of the same cultivar. Three replicates of each sample were analysed.

Material analysed (n = three analytical samples from a single batch)	CBC % w/w Mean ± sd	THC % w/w Mean ± sd	CBC as % of THC+CBC Mean ± sd
25 µm sieve contents From 3 week old vegetative plants	4.25 ± 0.74	22.70 ± 4.36	14.98 ± 0.19
45 µm sieve contents From 3 week old vegetative plants	6.37 ± 0.31	35.31 ± 2.44	14.92 ± 0.23
Foliage – 3 week old vegetative plants	0.08 ± 0.03	0.67 ± 0.16	10.43 ± 0.89
Foliage and floral material – 11 week old plants	0.13 ± 0.01	8.61 ± 0.45	1.48 ± 0.47

Table 4.4. The relative proportions of CBC and THC in sessile trichome rich preparations made by sieving dislodged trichomes from vegetative foliage of high-THC clone G1 M3. Three subsamples of each were analysed by GC.

The CBC potency of the preparation collected on the 45 µm sieve (6.37 % w/w) was approximately eighty times greater than that of the plant material from which it was derived (0.08 % w/w). However, the preparation collected on the 25 µm sieve was less potent (4.25 % w/w). The reason for this was not known. The smaller trichomes would have a greater surface area to volume ratio and it is possible that these contained proportionally more outer membrane and vesicle-associated fibrillar material. Despite the high proportions of THC remaining in these preparations, the results indicated that the trichome separation technique does enable samples to be produced which have a favourably altered cannabinoid profile. It is notable that the proportion of CBC within the cannabinoid profile of the separated trichomes (approximately 15% w/w) was greater than that of the foliage (approximately 10% w/w). The proportion fell further as the plant matured, repeating the observation reported by de Meijer *et al.,* (2008).

The reason for the observed difference in proportion of CBC in the trichomes and the foliage from which they were collected is not clear. Two possibilities are suggested: -

1) Although the foliage was collected from three week old plants, many of the trichomes residing there would have formed earlier during the plants development.

The cannabinoid profile of these trichomes would reflect the proportionally higher CBC expected in vegetation that was less than three weeks old.

2) In the three week old foliage there would be many new sessile trichomes forming. Following the well reported ontogenetic pattern, these would have a lower proportion of CBC than their predecessors. It is possible that these were not sufficiently resilient to be dislodged intact during agitation in iced water. The filtrate would therefore be skewed towards more mature trichomes.

4.5.3 The collection of sessile trichomes from foliage of a high CBC chemotype as a means of isolating the minor cannabinoid CBC

The preparation of sessile trichomes collected from vegetation of clone M240 was dried overnight. The material proved an extremely rich source of CBC – 44% w/w. The sample also exhibited a high level of purity. As found in the previous test, the fall in the proportion of CBC in the cannabinoid profile of the enriched trichome preparation appeared to lag behind that of the plant material from which it was collected. The foliage, in which CBC accounted for 61% of the total cannabinoids, had produced a trichome filtrate within which CBC accounted for 94% of the cannabinoid total (Table 4.5). Patent protection of this procedure, as a means of producing purer sources of CBC, was applied for (GB 0806553.4.). This material was subsequently further processed to produce a CBC sample exceeding 99% purity levels, which was submitted for in-vitro pharmacological evaluation.

	Cannabinoid % w/w mean ± SD				
Sample analysed	CBC	CBCV	CBG	CBD	% Purity CBC ± SD
Trichomes	44.37 ± 4.95	0.57 ± 0.07	0.33 ± 0.04	0.60 ± 0.06	94.2 ± 0.3
Foliage	1.36 ± 0.04	-	0.62 ± 0.02	0.25 ± 0.01	61.0 ± 0.9

Table 4.5. The proportions of principal cannabinoids (±SD) found in a trichome-rich filtrate clone M240, containing only sessile trichomes. One bulk sample was prepared and four sub-samples analysed. Also shown is the purity of the CBC (expressed as a %w/w of total cannabinoids detected) within the foliage from which these trichomes were collected.

4.5.4 Ontogenetic changes in glandular trichome secondary metabolite content

The changing terpene profile of glandular trichomes collected from plants at a range of developmental stages is shown in Tables 4.6 (THC chemovar) and 4.7 (CBD chemovar).

In the high-THC plants harvested 6 to 10 weeks after being placed in short daylength, the monoterpenes accounted for between 60%-67% of the monoterpene/sesquiterpene total (when assessed by peak area). There was no clear trend against time. The trichomes from the young foliage were notable for having a much lower proportion of monoterpenes (26%) than those from the flowering material. However, it can be seen that not all monoterpenes showed the same contrasting pattern. While the proportion of α-pinene, β-pinene and limonene is similar in both, the proportion of myrcene in the profile of foliar sample is much lower. The reason for this is not known. However, it is important to note, that in some cases, a range of terpenes can be synthesised with the involvement of a single enzyme (Croteau and Johnson, 1984). As a consequence, the ratio of the terpenes produced always maintains a fixed ratio. α-pinene, β-pinene and limonene, may be linked in this way while myrcene appears not to be linked to other terpenes.

Raw material used	Foliar	Foliar and Floral Mixture				
Trichome type	Sessile	Predominantly Stalked				
Wks after planting	3	9	10	11	12	13
Wks in 12 hr days	0	6	7	8	9	10
Monoterpenes	% Peak Area					
α-pinene	0.9	0.7	0.8	0.8	0.9	1.0
β-pinene	1.7	1.5	1.7	1.9	2.1	2.2
Myrcene	14.8	47.7	51.8	46.8	44.8	44.7
Limonene	8.5	8.4	9.3	9.1	9.2	9.4
Linalol	-	2.5	3.0	3.0	3.1	3.2
Sesquiterpenes	% Peak Area					
trans caryophyllene	21.1	18.1	14.8	17.4	17.5	17.5
trans α bergamotene	-	1.9	1.7	1.9	2.0	1.9
(z)-β farnesene	9.7	3.9	3.3	3.5	3.7	3.5
α caryophyllene	16.0	7.4	6.4	7.2	7.6	7.3
(e)-β farnesene	8.6	1.7	1.6	1.8	2.0	2.0
gurjunene	4.9	2.0	1.8	1.9	2.1	2.0
Δ guaiene	2.2	-	-	-	-	-
(e)-nerolidol	3.3	4.1	3.9	4.6	5.0	5.2
Unidentified	3.5	3.1	3.2	3.8	4.3	4.6
Unidentified	1.8	3.1	2.8	3.0	2.9	2.7
α-bisabolol	2.9	-	-	-	-	-
Monoterpenes	25.9	60.9	66.6	61.6	60.1	60.6
Sesquiterpenes	74.1	39.1	33.4	38.4	39.9	39.4

Table 4.6. The terpene profile of essential oils produced by steam distillation of glandular trichomes extracted from high-THC clone G2 M6 at various stages in the plant's development. The results are the relative peak area after analysis of the essential oil by GC. Missing values occur where individual terpene contents were below the detectable limits.

Raw material used	Foliar	Foliar and Floral Mixture				
Trichome type	Sessile	Predominantly Glandular Stalked				
Wks after planting	3	9	10	11	12	13
Weeks in flower	0	6	7	8	9	10
Monoterpenes	% Peak Area					
α-pinene	-	9.1	8.0	6.8	8.0	6.7
β-pinene	-	3.7	3.1	3.3	3.6	3.1
Myrcene	16.0	41.8	39.7	50.3	49.1	51.1
Limonene	-	4.6	4.6	6.0	5.9	6.3
β-ocimene	3.0	9.0	8.4	11.0	9.9	10.3
Sesquiterpenes	% Peak Area					
Trans caryophyllene	49.8	22.5	25.6	15.1	15.9	14.4
α caryophyllene	3.2	5.9	6.8	4.2	4.4	4.2
(e)-β farnesene	12.4	2.4	2.7	2.3	2.2	2.6
Unidentified	0.6	-	-	-	-	-
Unidentified	6.2	-	-	-	-	-
(e)-nerolidol	6.3	1.0	1.1	1.1	1.1	1.2
Caryophyllene Ox	0.7	-	-	-	-	-
α bisobolol	1.7	-	-	-	-	-
Monoterpenes	19.1	68.2	63.8	77.4	76.4	77.6
Sesquiterpenes	80.9	31.8	36.2	22.6	23.6	22.4

Table 4.7. The terpene profile of essential oils produced by steam distillation of glandular trichomes extracted from high-CBD clone G5 M13 at various stages in the plants development. The results are the relative peak area after analysis of the essential oil by GC. Missing values occur where individual terpene contents were below the detectable limits.

Unfortunately, in the CBD-chemovar, the α-pinene, β-pinene and limonene concentrations were below the detectable minimum in the foliar fraction and a similar comparison was not possible. In this chemovar monoterpenes accounted for 64-68% of the terpene total in the first two harvests. However, this rose to 76-78% and remained stable for the last three weeks when plant development ceased. As with the high-THC clone, the proportion of monoterpenes was much lower in the young vegetative foliage (19%). It appears likely that the slightly higher proportion of sesquiterpenes in the

younger flowering plants was due to these having a higher proportion of sesquiterpene-rich leaves. The proportion of foliage would have fallen as floral development continued up to the eighth week after induction of flowering. The higher proportion of sesquiterpenes in less developed plants observed here mirrors a similar observation reported when a hemp crop was grown outdoors for the commercial production of essential oils (Meier and Mediavilla, 1998). A detailed evaluation of terpene production in trichomes in field-grown plants is described later in Chapter 6.

Insufficient entriched trichome preparation was available to enable analysis of the cannabinoid content. However it was possible to assess the cannabinoid profile of the original plant material. The results are shown in Table 4.8 and 4.9.

Weeks after planting	3	9	10	11	12	13
Weeks in flower	-	6	7	8	9	10
Cannabinoid	%w/w					
THC	0.40	7.92	8.94	8.92	8.92	8.33
CBC	0.06	0.14	0.13	0.12	0.12	0.11
CBG	-	0.11	0.11	0.12	0.13	0.11

Table 4.8. The cannabinoid profile of the original plant material (high-THC clone G2 M6) from which the trichome rich preparations were made. Six subsamples were combined and milled to produce one sample for analysis by GC. A missing value denotes that the cannabinoid level was below the detectable threshold.

Weeks after planting	3	9	10	11	12	13
Weeks in flower	_	6	7	8	9	10
Cannabinoid	%w/w					
CBD	0.20	2.93	3.14	3.10	3.73	3.16
CBC	_	0.16	0.16	0.14	0.16	0.13
THC	_	0.20	0.17	0.15	0.16	0.14
CBG	_	0.03	0.03	0.02	0.03	0.02

Table 4.9. The cannabinoid profile of the original plant material (high-CBD clone G5 M13) from which the trichome rich preparations were made. Six subsamples were combined and milled to produce one sample for analysis by GC. A missing value denotes that cannabinoid level was below the detectable threshold.

Plant development was seen to have ceased by their eighth week in short daylength, and plants harvested after nine and ten weeks showed very high levels of foliar

senescence. The results of the cannabinoid assay suggest that both cultivars had reached maximum potency at the end of the seventh week in short daylength. In both chemotypes, delaying the harvest beyond the eigth week of flowering appears to have no clear effect on the terpene or cannabinoid profiles. The foliage from three week old vegetative plants showed very low levels of cannabinoid and terpene, as expected.

The remarkably unchanging cannabinoid and terpene profiles, from the eighth week of the flowering period onward, suggest that the secondary metabolites are stable while sequestered in the trichome. This facilitates the task of the grower of pharmaceutical-grade cannabis, as delaying harvest does not appear to affect the specified ratio of metabolites. This is in contrast to observation in other species where the terpene profile changes due to enzymic conversion of one terpene to another. In peppermint *Mentha piperita* L. for example, menthone is converted to menthol in the latter stages of flowering. (Gershenzon, 2000) Research also showed that catabolism of monoterpenes occurs in intact glandular trichomes of mint (Croteau and Martinkus, 1979).

The much higher secondary metabolite content of floral tissue compared to the foliage is typical of most plant species. The loss of floral tissues is likely to have greater impact on a plant's ability to pass on its genes to the next generation. The Optimal Defence Theory suggests that, away from the influence of man, plants will have evolved to generally allocate secondary metabolites to tissues in direct proportion to their value. (Herms and Matson, 1992). The very different balance of monoterpenes in the sessile trichomes on the foliage and the predominantly capitate stalked trichomes on floral tissues is supporting evidence that these trichomes have different functions. Both types contain bitter sesquiterpenes which can act as anti-feedant repellents. The increased monoterpene content of capitate stalked trichomes would be expected to lower the viscosity of the contents, thereby making it more able for them to ensnare insects, as reported in the previous chapter. The monoterpenes are more volatile, and being hydrophobic they are highly persistent in the atmosphere. Insect olfactory systems are devoid of the mucous membranes found in mammals, and they are especially sensitive to such lypophylic chemicals. Monoterpenes are thereby detected by insects at considerable distances from the plant. In many cases these monoterpenes are repellent to insects, (e.g. α-pinene and ants) those insects apparently misidentifying the monoterpene as an alarm pheromone (Kelsey *et al.,* 1984). It is notable that the secondary metabolite profiles of both chemotypes (Tables 4.6 and 4.7) were dominated by the most reduced forms of terpenes – the 'true terpenes' - containing just carbon and hydrogen. These were the most costly type to

biosynthesise (Gershenzon, 1994). The slightly more oxidised monoterpene alcohols (e.g. linalool,) are more water soluble (Merck Index, 1996) and less persistent in an atmosphere containing water vapour. It is possible that it is a function of the cannabinoids, by virtue of their anti-oxidant properties, is to prevent oxidation of the monoterpenes to more soluble monoterpene alcohols.

Some of the high monoterpene content of glandular trichomes is possibly due to human influence over many centuries. The highest monoterpene contents are most likely found in plants producing high quantities of resin. These plants would have been selectively planted by growers striving for maximum resin yields. Many would also find the odour attractive and of greater demand for those using fragrant resins as religious sacraments. Focused plant breeding of cannabis for pharmaceutical use continues to make dramatic changes to the glandular trichome contents.

4.6 CONCLUSIONS

The previous chapter showed that on mature flowering plants, only a very small proportion of the total cannabinoid content is sequestered in sessile trichomes, the majority being synthesized in the capitate stalked form. On a mature flowering plant, the cannabinoid content of the sessile trichomes has an almost negligible influence on the total cannabinoid content. Comparisons of proximal and distal bract sections, with differing ratios of sessile and capitate stalked trichomes, suggested however that the former had a higher CBC content within the cannabinoid profile. By separating and then analysing sessile and capitate stalked trichomes, the research reported in this chapter confirmed this to be the case.

Although yields were comparatively small, enriched trichome preparations could be produced from foliage, within which only the sessile form was present. Agitating the foliage in cold water (<1°C) appeared to dislodge a higher proportion of the more mature sessile trichomes, within which there was a higher proportion of CBC than that of the overall sessile trichome population. Using the foliage of a chemovar, within which CBC accounted for approximately 61% w/w of the cannabinoid total, an enriched trichome preparation could be produced, within which CBC constituted 94% w/w of the cannabinoid total. The CBC content of the dried preparation was 44% w/w. This was possibly the first time that such a rich source of CBC had been extracted from cannabis material by such simple means.

In marked contrast to some other species, this chapter showed that in *Cannabis* the terpenoid content of trichomes appears to be stable at the end of the flowering period, at least in the glasshouse growing environment. The cannabinoid and terpene profile

of the phytopharmaceutical feedstock would be minimally affected by a few days alteration to the intended harvest date. This indicates that some flexibility in harvest timing is justified.

Capitate stalked trichomes appear to have a higher monoterpene:sesquiterpene ratio than the sessile type. Although only the sessile trichome form is present on foliage, floral material predominantly exhibits the capitate stalked form. As a consequence of this marked difference if trichome distribution, floral material has a higher monoterpene:sesquiterpene ratio than foliage. Any alteration to growing conditions or harvest timing, which affected the ratio of foliar and floral material, would be likely to affect the balance of mono and sesquiterpenes in the feedstock produced.

The previous chapter showed that only the capitate stalked trichome form was involved in insect entrapment. The higher proportion in monoterpenes in this form implies that the secretory head contents would consequently be of lower viscosity than those of the sessile form. If punctured by insect contact, the contents would more readily spread over the insect surface. As the monoterpenes from the punctured trichome volatalised, this would leave an increasingly viscous adhesive coating on the insect. Microscopic observations indeed showed the capitate trichome contents to have extremely adhesive qualities.

The following chapter evaluates glasshouse growing methods with the aim of enabling the reliable and uniform propagation of high yielding phytopharmaceutical feedstocks, with a high density of capitate stalked trichomes. Uniform foliage:flower ratios would appear to be vital if both materials are included in the feedstock.

Chapter 5 Indoor Propagation of Medicinal Cannabis

5.1 INTRODUCTION

To enable year-round propagation of high yielding good quality plant material for GW Pharmaceuticals, extensive research was needed to identify suitable horticultural methods. The techniques initially adopted were recommended by consultants from HortaPharm BV. Additional advice was sought from the books on cannabis horticulture then available from at least a dozen authors (e.g. Clarke, 1981; Frank, 1997; Rosenthal, 1998). Although the first of these books sourced the work of both scientific and the clandestine growers, most publications appeared to rely totally on the latter and contained minimal input and peer review from named qualified and respected scientists. Indeed the growing of cannabis was often described as more of an art than a science. Within three weeks of planting the first seed, insect damage was observed on the crop and the battle against pest and disease had begun. Growth media had to be developed that supported good root development, and fertilizer regimes had to be devised to give the correct balance of nutrients at the appropriate time in the plants' development. As the results of these studies are also of interest to illicit cannabis growers, this aspect of the research cannot be included in this thesis.

Along with the three most commonly used legal drugs in the west – alcohol, nicotine and caffeine – cannabis-derived THC is unusual, if not unique, amongst illicit drugs in that part of the enjoyment for many users is the taste and odour of the plant material from which it is sourced. In addition to raising yield and potency, many of the cannabis growing techniques for illicit cannabis were designed to improve the taste of the harvested material. This would be of little or no relevance when growing cannabis as a phytopharmaceutical, and this typifies how the aims of the illicit and pharmaceutical growers differed. The research discussed in this thesis also differs in that it investigated the effect of growing conditions on the biosynthesis of cannabinoids other than just THC. To enable the tight specification expected of a phytopharmaceutical feedstock to be met, the research reported here placed greater emphasis on crop uniformity than crop yield. There was little doubt that environmental conditions influenced the quantity of cannabinoids in various plant parts at different growth stages, as demonstrated by Fairbairn (1976), Lydon and Teramura (1987) and others. An improved knowledge of how alterations to the growing method affected the secondary metabolite profile would reduce the likelihood of unacceptable feedstock being produced. It would also increase the ability to perform a reactive diagnosis, if a batch of feedstock was produced that

was 'out of specification'. Reviewing the findings of several researchers, Raman and Joshi (1998) highlighted the magnitude of this challenge. They reported that even if genetic and environmental factors could be tightly controlled, high interplant variability in cannabinoid content could be expected between plants of the same chemotype when grown under identical conditions.

Initial advice was that plants should be propagated from cuttings rather than seed. Most researchers agreed that genetics exert the greatest control of a plant's cannabinoid profile (Beutler and Der Manderosian, 1978; Fournier *et al.,* 1987; deMeijer, 1994). The propagation of cannabis from cuttings guaranteed the genetic uniformity of all plants produced from the same source (Samuelsson, 1999). Propagation from seed however was perceived to be less labour intensive, and with appropriate research perhaps the foreseen variation could be dispelled or overcome. Research reported here compared the variability of cloned and seed-sown plants.

To produce a medicine containing a desired even balance of cannabinoids, separate THC and CBD chemotypes were routinely propagated for Sativex® production. By blending Botanical Drug Substances from these two, it could be guaranteed that the correct ratio of these cannabinoids would be delivered. It has been concluded elsewhere that the inheritance of CBD and THC chemotype was controlled by a monogenic, co-dominant mechanism (de Meijer *et al.,* 2003). A single locus referred to as *B* was postulated, with two alleles, B_D and B_T, encoding CBD- and THC synthase respectively. According to this model, the CBD chemotype had a B_D/B_D genotype at the *B* locus, and the THC predominant plant had a B_T/B_T genotype. Heterozygous B_D/B_T genotypes were available that produced both cannabinoids, thereby possibly avoiding the need to blend materials. Research reported here was performed to assess how growing methods and harvest timing affected the ratio of these two cannabinoids in B_D/B_T genotypes.

Prior to research for this thesis, large seasonal variations were seen in plant yield. In winter crop yields fell by two-thirds and the fall in cannabinoid yield was even more pronounced. In addition to the economic implications, this variability in potency may have also unacceptably affected the secondary metabolite profile of the Botanical Drug Substance (purified extract) made from that feedstock. While glasshouse temperature remained constant throughout the year, summer light levels far exceeded those in winter, and this was the suspected main cause of the yield variation. Limited growth room tests, performed in winter, showed that when summer irradiance levels were recreated, both plant yield and cannabinoid assay values returned to those typical of a summer crop. Extensive written guidance was available regarding the use of lighting

systems to support cannabis growth. Most was aimed at those growing under totally artificial lighting conditions and few described the use of electrical lighting to supplement natural daylight. Growing cannabis this way in a glasshouse had been and continues to be less common, at least in the UK (ACMD, 2008), partly due to it being too difficult to keep the activity secret and secure. The effect of lighting on cannabis growth was discussed in little detail in peer-reviewed scientific literature.

It was well recognised that the floral material of the female plant was the most potent source of cannabinoids (Fairbairn, 1976). To confirm this, various parts of some unpollinated THC chemovar plants were analysed. The THC content of dried seeds, roots, stems, leaves and inflorescences was found to be 0.0%, 0.0%, 0.3%, 0.8% and 15.2% w/w respectively. When the outer bracts and stalks were removed the THC content of the remaining inflorescence material was found to be over 18% (Potter 2004). In a separate test pollination was found to reduce THC content by more than half, with THC yield per unit area being decreased by over 75%. It was therefore essential to grow plants though to the flowering stage to obtain the highest yielding material.

Cannabis is generally a ‘short day plant’ that by definition only commences to flower late in summer, once the day length starts to fall (Clark, 1981). When the so called critical daylength is reached floral development is stimulated. More correctly, the plant is responding to the increasing length of the night, during which time a light-sensitive phytochrome protein slowly dimerises to a different form. Within seconds of light exposure the protein reverts back to the original structure. It is only when the night time is sufficiently long that a required balance of the dimers is reached to signal commencement of flowering (Halliday and Fankauser, 2003). To reliably induce flowering in most varieties of cannabis, the night-length must be greater than the critical value. The critical daylength for an individual variety is greatly affected by its geographical origin and would generally be greatest in those plants derived well away from the equator (de Meijer and Keizer, 1994). Exceptions to this response occur in plants adapted to grow in equatorial regions, where there is minimal variation in day length. Flowering in tropical cannabis plants is more closely related to plant age. In contrast, rapid flowering ecotypes are found at latitudes of 60º (Callaway, 2002) or more. These have typically adapted to survive in the very short growing season, and commence flowering early in the season irrespective of the daylength. Despite these differences in critical daylength, it has been common practise amongst illicit cannabis indoor-growers to induce plants to flower by placing them in a twelve hour daylength. The use of such a short day length to induce flowering was perceived as somewhat

ironic, as in the northern hemisphere an autumn day length of twelve hours occurs naturally at the equinox during the last days of September, when cannabis flowering would be finishing. A slightly longer day length would still initiate flowering and would result in proportionally more light energy being delivered to the plant in one day. Research reported here investigates how plant development, yield and cannabinoid profile are affected by differing daylengths of eleven, twelve and thirteen hours.

Recreational cannabis users would often harvest their plants when about 95% of the visible stigmas had senesced, but this would vary according to the variety and the grower's personal preference (Clarke, 1993). This was partly related to taste of the material as well as its potency. It was not known how closely inflorescence development stage was related to the secondary metabolite content. It was predicted that the length of the propagation period would not greatly affect the cannabinoid profile, especially of those cannabinoids that are the final products of separate biosynthetic pathways. Rowan and Fairbairn (1977) and others had shown that during the flowering phase CBD chemotypes would produce a small proportion of THC and THC chemotypes would produce a small proportion of CBD. In THC chemotypes, although substantial proportions of CBC were often present in the early stages of growth, THC dominated the profile of THC chemotypes throughout the flowering stage. However, how the state of maturity of the plant affected the relative proportions the precursor CBG and the final cannabinoids was less well studied, and was investigated in this study. Based on visual assessment, clones of the THC chemotype typically appeared ready for harvest eight weeks after being placed in a twelve hour day length. The objective of this investigation was to check that this flowering time was the optimum to achieve efficient production of THC. The investigation would also investigate how the duration of the flowering period affected the relative proportions of THC and its precursor CBG.

The methods of production of licensed medicines had to comply with Rules and Guidance for Pharmaceutical Manufacturers and Distributors 2007 as directed by the Medicines and Healthcare Products Regulatory Agency (MHRA, 2007) to ensure that they were of sufficient quality. It was perceived that GMP did not apply to the propagation phase of the production process, but did apply once the plant material was processed. Although the cannabis plants were to be grown in a glasshouse, the research described in this chapter was performed in the knowledge that the propagation phase would have to comply with the EMEA Good Agricultural Practice (GAP) Guidelines (EMEA, 2006). The use of the word *agriculture* in this quality control

system reflects that most, if not all of the existing phytopharmaceuticals in western medicines, were derived from outdoor grown materials.

5.2 AIM AND OBJECTIVES

A series of tests was performed, all of which had the aim of identifying how glasshouse growing methods could be altered to maximise plant quality and uniformity. The linked objectives were as follows:

5.2.1 To gain a greater understanding of the irradiance levels on plant yield and cannabinoid content.

5.2.2 To confirm or dispel the belief that plants propagated from clones were significantly more uniform in cannabinoid profile than plants grown from seed.

5.2.3 To ascertain how the length of flowering period affected the cannabinoid yield.

5.2.4 To ascertain how the length of flowering period affected the cannabinoid profile of THC chemovars and heterozygous plants with a mixed THC/CBD profile.

5.2.5 To compare the effect of 13, 12 and 11 hour daylengths on plant development, cannabinoid yield, potency and profile.

5.3 MATERIALS

5.3.1 Plant Propagation and Drying Materials

Materials	Source
Complete light fittings incorporating 600 watt Osram Nav T High Pressure Sodium Lamps, compatible ballasts and Silver Wing® reflectors.	Hortisystems UK Ltd Pulborough, UK
Complete light fittings incorporating 600 watt Hortilux-Scroeder High Pressure Sodium Lamps, compatible ballasts and Deep reflectors	
1000 watt Mercury Vapour MBFU lamps	
Seradix® Rooting Powder (MAPP No 1331) 0.3% w/w 4-Indol-3yl butyric acid	Certis UK 1[b] Mills Way, Amesbury, UK
Jiffy *7* Rooting Plugs	Jiffy Products International AS, Kristiansand, Norway
Thripex-Plus sachets *Amblyseius cucumeris* for biocontrol of Thrip *Thrips tabaci*	Koppert B.V. Rodenrijs, NL
Spidex *Phytoseiulus persimilis* for biocontrol of red spider mite *Tetranychus urticae*	
En-strip *Encarsia formosa* for biocontrol of white-fly *Trialeurodes vaporariorum*	
Aphipar bottles *Aphidius colemani* for biocontrol of cotton-melon aphid *Aphis gossypii*	
Ebac BD150 commercial building drier	Ebac Ltd., Durham, UK

Table 5.1. Plant Propagation Materials

5.3.2 Germplasm Details

GW Accession Number	Variety/ Clone Name	Supplier
G1-M1 G2-M7 G5- M11 G5- M13 G5- M16	Gwen Gina Gayle Grace Gill	Seed from HortaPharm BV Schimkelhavemkade 1075 VS Amsterdam, NL, used to produce clones by GW Pharmaceuticals Ltd, Porton Down Science Park, Salisbury, Wiltshire
M237	-	Clone bred by GW Pharmaceuticals (As above)
G41 to G52, G63, G91 G130 and G159	Various	Various suppliers as follows: Sensi Seeds B.V., Rotterdam, NL Natural Mystic, Rochdale, UK Nirvana, Zaandam, NL Dutch Passion B.V. Amsterdam, NL Serious Seeds, Amsterdam, NL Seedsman Ltd, London, UK

Table 5.2. Germplasm Details

5.3.3 Light Measurement and Weighing Equipment

Skye 500 Hand-held Light Sensor	Skye Instruments, Powys, UK
Kipp and Zonnen solarimeter	Priva UK Ltd, Gloucestershire, UK
Salter M323 Top Pan Balance	E&G Websales Ltd, Delfryn, Lixwm, Holywell, UK

Table 5.3. Light Measurement and Weighing Equipment

5.3.4 Growth Medium

The growth medium used for these studies was developed at GW Pharmaceuticals Ltd, Porton Down Science Park, Salisbury, Wiltshire and its precise formula has to remain proprietary information (Wilkinson 2006). The medium (or compost) primarily consisted of peat and perlite and included sufficient nutrient to maintain healthy plant growth through to harvest with no additional feeding.

5.4 METHODS

Before describing the more detailed experimental methods that apply specifically to individual studies, sections 5.4.1.1 to 5.4.1.11 describe the routine propagation and botanical raw material production processes which were routinely used and applied to most of the studies described in this chapter. Sections 5.4.2.1 to 5.4.2.11 describe more specific methods applying to individual tests within this chapter.

5.4.1 Routine Propagation and Plant Production Methods

5.4.1.1 Seed sowing and transplantation of seedlings

Seeds were sown in trays of a range of proprietary seed compost. There is no light requirement for germination and seeds were sown approximately 1 cm deep and at least 1cm apart. The compost was sufficiently deep (> 3 cm) to allow some natural downward root development prior to replanting. The compost was lightly compressed after sowing, and well watered. The temperature was maintained at 20-25°C and lighting adjusted to maintain a 24 hour daylength. Horticultural fleece was placed over the seed tray or pot until seedling emergence to reduce surface drying of the compost.

In ideal conditions seedlings were ready for transplanting approximately ten days after sowing, by which time the hypocotyls were typically around 10 cm tall and the first pair of true leaves well formed. Seedlings were teased from the seed compost and transplanted into individual pots of the appropriate growth medium. A sufficiently deep hole is prepared in this medium to allow the seedling to be lowered undamaged so that the first pair of true leaves sat 1-2 cm above the surface of the medium. The surrounding growth medium was firmed by hand, and the growth medium sufficiently watered to maintain moisture around the seedling.

5.4.1.2 Production of Cuttings (Clones)

Branches of vegetative material were removed from plants producing ample numbers of axial buds. (Figures 5.1 a-f). The branch was then cut into sections, each carrying one axial bud and retaining approximately 5cm of stem below the bud. The stem below the bud was dipped in rooting powder containing 0.3% w/w 4-Indol-3yl butyric acid and then promptly into a very moist peat plug. These plugs were placed on a bench of regularly wetted gravel under a polythene cover to maintain very high humidity. Irradiance levels under the polythene was maintained at approximately 10 – 15 W m^{-2} for 24 hours per day. Successful cuttings were normally producing sufficient roots within fourteen days to enable repotting.

5.4.1.3 Nurturing Vegetative Growth of Seedlings and Cuttings.

For the first three weeks vegetative growth was encouraged by maintaining the cannabis plants in a twenty-four hour daylength. This supported maximum growth rate by enabling continuous photosynthesis. Being 'short-day plants', flowering was naturally prevented in this regime.

Unless otherwise stated, all high-THC plants are potted in five litre pots of GW Pharmaceuticals' standardised peat-based growth medium. High CBD plants of variety G5 are potted in three litre pots of the same medium. These were closely placed pot-to-pot on the glasshouse or growth-room bench for three weeks before being induced to flower. A proportion of plants were retained in long daylength however to produce ample vegetative material for the production of cuttings.

5.4.1.4 Induction and Maintenance of Flowering

Plants were relocated, or the lighting regime adjusted, so that plants were now in a short daylength. Unless otherwise specified, this was twelve hours per day. The response to daylength change was for plants to commence flowering within seven to fourteen days. The short daylength was maintained for the entire flowering period, which typically lasts eight weeks. During this period, unless otherwise specified, crops were spaced at a density of 10 m^{-2} (THC chemotype) or 17 m^{-2} (CBD chemotype).

5.4.1.5 Biological Pest Control

The major pests encountered were red spider mites (*Tetranychus urticae*), the onion thrip (*Thrips tabaci*), white fly (*Trialeurodes vaporariorum) and* cotton-melon aphid *(Aphis gossypii)*. These were controlled by regular introduction of the predatory mite and insect species *Phytoseilius persimilis, Amblyseius cucumeris, Encarsia formosa* and *Aphidius colemani* (Malais and Ravensberg, 1992).

5.4.1.6 Harvesting

Plants were harvested by cutting the stems just below the lowest side-branch and placed on a clean surface if being temporarily stored prior to drying. As respiration could rapidly generate heat within piled fresh material, drying of harvested plants was commenced as soon as possible to avoid heat-induced catabolism.

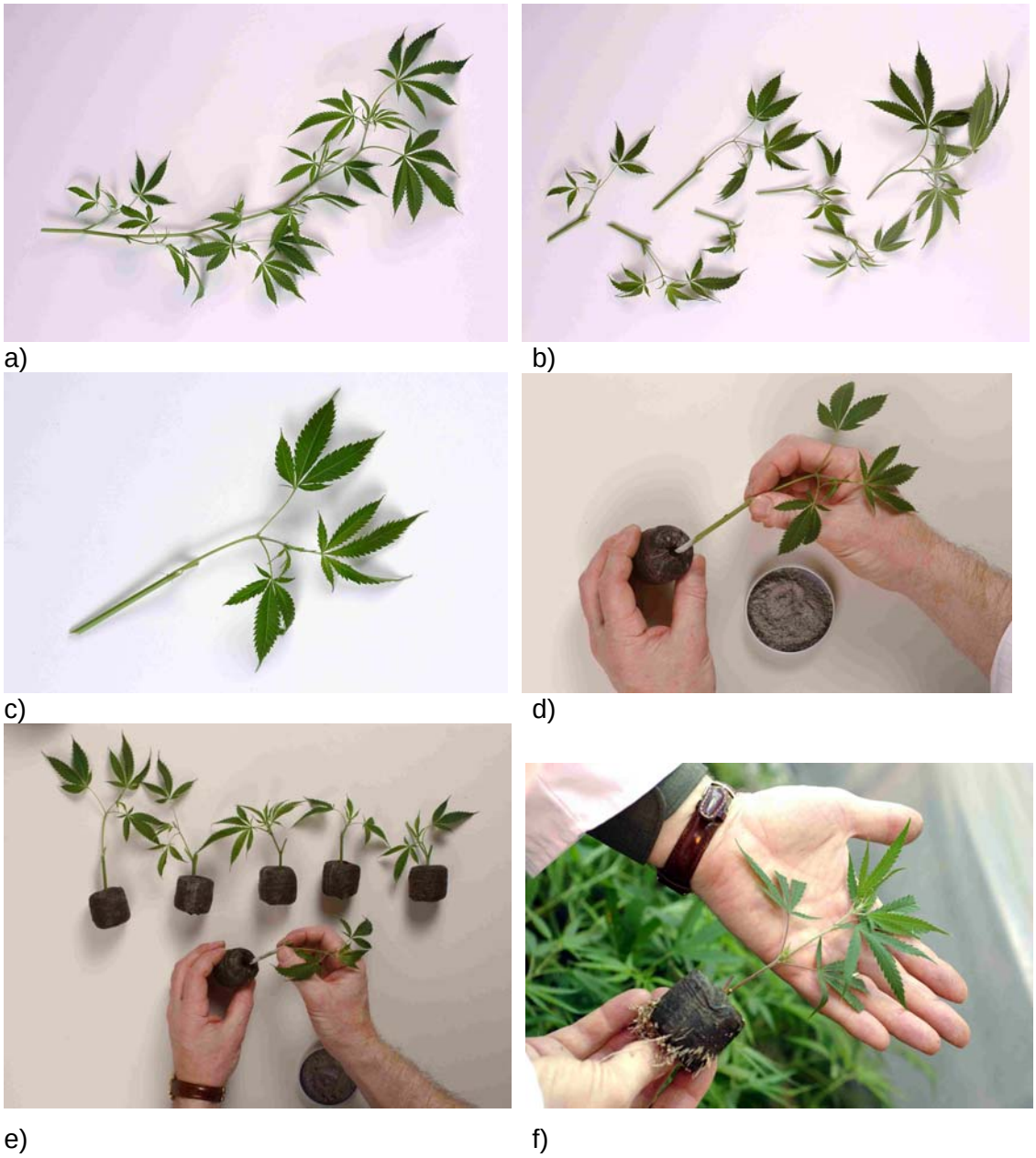

Figure 5.1. Production of cannabis cuttings. A vegetative cannabis branch (a) is cut into sections (b). Each cutting has been cut leaving approximately five centimetre of stem below a single axial bud and up to one centimetre above (c). The base of the cutting is dipped in rooting powder (d) and then placed in moist peat plugs (e). After two weeks roots are protruding from the peat plugs and the cutting is ready to be planted.

5.4.1.7 Crop Drying

Plants were hung to dry in a closed environment and industrial dehumidifiers used to lower air moisture (Figure 5.2). Horticultural fans were used to maintain air circulation. Freshly harvested material had an aqueous content of approximately 80% (w/w). The air surrounding the chamber was conditioned as appropriate to ensure that waste heat from the dehumidifiers did not raise temperatures within the chamber above 35^{o}C. The

crop was considered dry enough for storage or processing when below 15% (w/w). At his point, the crop was clearly crisp to touch, the inflorescence tissue closest to the stem feeling dry and the floral material would readily pull away from the stem without excessive force.

Figure 5.2. A newly harvested crop hung to dry on wires.

5.4.1.8 Stripping

When sufficiently dry, all floral and leaf material was stripped from the stem by pulling the plant though the tightly clasped thumb and forefinger of a gloved hand. This floral and foliar mixture is referred to as Botanical Raw Material (BRM).

5.4.1.9 Garbling

Tyler *et al.,* 1998 defined garbling as ''the final step in the preparation of a crude drug and involving the removal of extraneous matter such as other parts of the plant, dirt and added adulterants. This is done to some extent during collection and should be carried out after the drug is dried and before it is baled and packaged''.

When grown in the glasshouse, minimal adulteration or contamination with dirt arose. The presence of low-potency stem material was undesirable and all material assessed as more than 2 mm in diameter would be manually removed.

5.4.1.10 Storage

As with other dried herbs, e.g. hops (Henderson, 1973) the aqueous content of stored dried herbs is greatly affected by the surrounding humidity. To minimise the possible effects of varying moisture content on microflora activity, secondary metabolite volatilisation and catabolism the herbal material was stored in a controlled environment

at 35 ± 5% RH. Low air moisture was achieved using industrial dehumidifiers. THC degrades more rapidly in the presence of light (Fairbairn, 1976). To discourage this, the material was stored in the dark prior to analysis or use.

5.4.1.11 Environmental Control System

Glasshouse temperatures and lighting conditions were controlled by a Priva Universal Computer (907) system. Warmed or chilled air was supplied via a rapidly responding air handling system to ensure that a target glasshouse temperature was achieved +/- 1.5°C. Supplementary lighting was programmed to be turned on at the desired times of the day, and only when natural daylight irradiance levels were below a selected minimum level. Similarly, glasshouse roof shades and or blinds were automatically opened or closed to control the ingress of natural light and to prevent light pollution from glasshouse lights after sunset.

5.4.2 Specific Methods

5.4.2.1 Uniformity of Plants Grown from Cuttings or Seed

THC variety G1 was selected for this test. Thirty plants raised from cuttings were compared to a similar number raised from seeds. The clones were all derived from one plant and code named clone M1. For the seed sown crop, seedlings were raised as described in paragraph 5.4.1.1. For the plants raised from cuttings, the propagation timings and conditions were as described in paragraph 5.4.1.2. Ten days after sowing, eighty individual seedlings were transplanted into pots using identical materials and methods employed for the propagation of plants from cuttings. The rooted cuttings were also transplanted on the same day.

Three weeks after transplanting, the plants were moved to a twelve hour light / twelve hour dark regime to induce flowering. All male plants and excess female plants were removed to leave thirty seed and thirty clone derived plants. These were maintained in neighbouring blocks at a density of ten plants per square metre. Eight weeks after the move to twelve hour days, the plants were harvested and dried. The total weight of foliar and floral material produced by each plant after removal of the stem was recorded. This mixture was then milled and the cannabinoid content studied using gas chromatography.

5.4.2.2 Effect of Duration of Flowering Period on Yield

Two clones used for the production of clinical trials raw material were selected viz. M1 and M7. Twelve commercially available varieties were also selected. All were commonly used for illicit recreational purposes and were described as being derived from tropical or subtropical areas. Plants truly derived from these contrasting locations would be expected to differ in the natural duration of their flowering period.

Seedlings of each of the twelve commercial varieties were raised in the glasshouse in a twenty-four hour daylength. When sufficiently developed, cuttings were taken from each plant and encouraged to root using the standard method (section 5.4.2). The original plants, from which the cuttings were taken, were moved to a twelve-hour daylength regime to encourage flowering. All plants consequently identified as male were disposed of along with all cuttings taken from them prior to gender identification. Up to three female clone lines of each variety were retained for further evaluation, each being regarded as the most potent or prolifically flowering examples of their variety. Just one clone line was chosen for evaluation from four of the varieties. Two or three clone lines were selected from the remainder. In the latter case, these varieties had shown a high level of phenotypic variability.

The propagation regime was based on that used for regular production of THC for clinical trial (Section 5.4.2 – 5.4.8). However, a temporary installation of supplementary glasshouse lights was in place for this test and this gave a slightly lower minimum irradiance level of 40 W m^{-2} PAR. Fifteen plants of each clone were propagated and five of each harvested after six, eight and ten weeks in flower. Harvested plants were dried in a dehumidified environment (final humidity < 35%) for seven days and the floral and foliar material stripped from the stem. The floral and foliar material of the five replicate plants of each clone line were thoroughly mixed. Five small samples of approximately 1 g were taken at random from the mixture, blended and analysed by High Performance Liquid Chromatography (Appendix 1).

5.4.2.3 Effect of Daylength on Cannabinoid Profile (Part 1) Comparison of 12 and 13 hour daylength

All plants were propagated in the glasshouse using the standard materials and methods. Eighty plants of each selected clone line were grown in five-litre pots and maintained in constant twenty-four hour daylength for the first three weeks after potting. Thereafter half were transferred to a glasshouse area with a twelve-hour daylength and the remainder relocated to a similar glasshouse area with a thirteen-hour daylength regime. In both areas the glasshouse target irradiance level was 75 Wm^{-2}. Within each regime, each clone line was divided into two batches of twenty plants. Single plant

batches of each clone line were placed alongside each other at a pot-density of 10 m^{-2}. Plants were watered by hand throughout the test. Eight weeks after the move to short daylength, one batch of each clone was harvested and hung to dry. The remaining batches were harvested and dried fourteen days later.

5.4.2.4 Effect of Daylength on Cannabinoid Profile (Part 2) Comparison of 11 and 12 hour daylength

The above method was duplicated. However, this test was located in a growth room with no natural lighting (Figure 5.3), and twenty plants of each clone (rather than five, as used in the previous test) were included in each replicate. High-pressure sodium lamps gave a uniform irradiance level of 70 W m^{-2}. Temperature was maintained at 25 ± 1°C. 'Ebb-and-flood' benches provided uniform levels of water to all plants. Vertical blinds between areas enabled plants to be divided into areas with equal environmental conditions but altered daylength.

Figure 5.3. An experiment to compare plant development and cannabinoid content when flowered in 11 and 12 hour daylengths. Plants are maintained on ebb-and-flood benches and lighting provided by high pressure sodium lamps. Duplicate batches of plants are maintained either side of the curtain with plants on the right receiving the longer daylength regime.

5.4.2.5 Plant height assessment

The height of each single plant (from the surface of the pot to the top of the tallest inflorescence) was measured by hand immediately before harvest.

5.4.2.6 Stigma senescence assessment

The stage of inflorescence development on each clone was recorded weekly, up to the final harvest date, by making a visual estimate of the relative proportions of still-viable white stigmas and senesced non-viable brown stigmas (Figure 5.4).

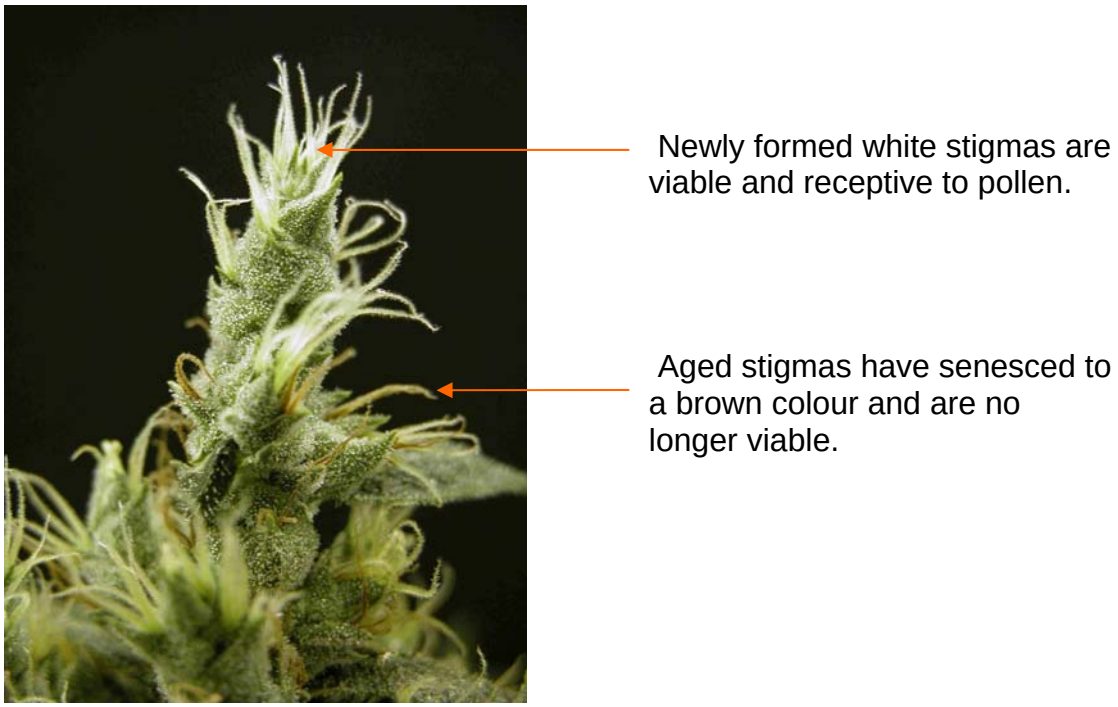

Figure 5.4. A close-up view of part of an unpolllinated cannabis inflorescence, showing viable and older non-viable stigmas.

5.4.2.7 Plant Weight Assessment

After being dried for seven days, the foliar and floral material from each individual plant was stripped from the stem and the latter discarded. The weight of combined foliar and floral material was weighed on a top pan balance.

5.4.2.8 Cannabinoid Content and Profile

The cannabinoid content and profile of the samples from the twelve versus thirteen hour assessment were analysed by HPLC. This facility was not available for the eleven versus twelve hour study. Cannabinoid yields and profiles were assessed using GC (Appendix 1).

5.4.2.9 Effect of Irradiance Level on Plant and Cannabinoid Yield

The mercury vapour (MBFU) lamps originally fitted in the glasshouse were only able to convert approximately 10% of the consumed electrical energy into photosynthetically active radiation. This compared to 30% with more modern High Pressure Sodium (HPS) and metal halide (MH) lamps. By replacing each 1000 watt MBF fitting with two 600 watt high pressure sodium (HPS) or metal halide (MH) units the electricity consumption within the glasshouse would have been raised by 20% to the maximum capacity of the existing power supply. However, the increase in irradiance achieved by new fittings would have theoretically been three-fold, taking levels from 16 to 53 W m^{-2}.

To measure the effect of introducing such improvements to irradiance levels, identical batches of plants were grown in the glasshouse and in a walk-in growth chamber. The latter was equipped with a combination of HPS and MH lamps to deliver an irradiance level of 70 W m^{-2} at the plant canopy. This equates to the light level typically existing in the glasshouse, as equipped at the time, during mid-afternoon on a bright summer day.

The experiment commenced in September and finished in December so as to recreate a harvest date when the previous year's findings suggested that glasshouse yields would be at or near their minimum. Records from the Priva Glasshouse Control computer system showed that on rare very clear days, noon irradiance levels inside the glasshouse peaked at approximately 50 W m^{-2}. However a maximum of approximately 25 W m^{-2} was more common, with morning and evening levels falling to a minimum of 17 W m^{-2}. Irradiance levels were measured using a hand-help Skye SKE 500 light metre at ten locations on the surface of the crop canopy.

One hundred rooted cuttings of each of the THC chemovars G1 and the CBD chemovar G5 were placed in pots of growth medium following the standard procedures for botanical raw material production. Fifty of each chemovar were propagated in the growth room and the remainder were propagated in the glasshouse along-side the crop being routinely grown for botanical raw material production. In both the glasshouse and growth room the temperature was maintained at 25.0 ± 1.5°C throughout the trial period. Plants were placed on a bench at maximum density (pot-to-pot) for the first three weeks and maintained in a twenty-four-hour-per-day lighting regime. The THC chemovar plants were then spaced out to ten plants per square metre or seventeen per sq metre (G5 CBD chemovar) for the remaining eight weeks before harvest. During these eight weeks the lighting regime was maintained at 12 hours light/12 hours dark per day. Plants in both areas were watered by hand. No additional plant nutrient was given to the plants in either growing regime.

Plants were harvested after the usual eight-week flowering period and dried and weighed. Mixed samples of stripped foliar and floral dry material of each chemovar from both regimes were analysed for cannabinoid content using HPLC.

In a subsequent larger scale test 250 m^{-2} of the glasshouse was converted to raise supplementary lighting levels from 16 to 53 W m^{-2}. Temperature and daylengths were kept identical in both areas. The monthly average crop yields in both areas were compared over the following year.

5.4..2.10 Effect of the length of flowering period on the cannabinoid profile of heterozygous plants of the mixed THC/CBD chemotype.

In addition to clones specifically bred to produce a mixed THC/CBD profile, one was discovered by chance (code name G159) while screening plants grown from commercially available seed. This was marketed for outdoor growing in the UK. Although most seedlings from this variety were found to be of the THC chemotype, five plants were of the B_TB_D genotype, as described by de Meijer *et al.* (2003). These produced plants containing THC and CBD in ratios varying between 1.5:1 and 1:1.5. These were adopted for detailed study in this thesis as they could be included in indoor and outdoor tests. Four rooted cuttings of each were grown in five litre pots of the standard growth media. Plants were kept in a walk-in growth room in continuous lighting for the first three weeks. A twelve hour light/ twelve hour dark regime was then implemented. Temperature was maintained at 25.0 ± 1.5ºC. Using high pressure sodium lamps, irradiance levels were kept at 75 W m^{-2} at foliage height. After four weeks in short day length a program of weekly sampling commenced, one entire inflorescence being removed from the side of each plant. These were analysed for their cannabinoid content by gas chromatography (Appendix 1).

5.4.2.11 Statistical Analysis

Analyses of variance (ANOVA), paired t-tests and F-tests were used as appropriate, utilising Microsoft Excel 2003 related software.

5.5 RESULTS AND DISCUSSION

5.5.1 Comparison of the Yield and Uniformity of Plants Grown from Cuttings or Seeds

The yield of raw material obtained from plants grown from seed (494 g m^{-2}) and cuttings (515 g m^{-2}) was very similar. An analysis of variance (n = 30) showed this small difference not to be significant ($p > 0.05$). However, the mean THC content of the cloned plants (14.6% THC w/w) was significantly higher (ANOVA, $p < 0.01$) than those grown from seed (11.1% THC). As a consequence, the cloned plants produced significantly more THC per unit area - 75.4 g m^{-2} ($p < 0.01$, ANOVA) than those from seed - 54.9 g m^{-2}. Clone M1 was one of five originally selected for further testing from approximately one hundred female plants of the variety G1. The selection was on the basis of its favourable ratings for vigour, yield, glandular trichome density, THC content and purity. The THC content of these one hundred plants had exhibited a normal distribution. It is not clear from this investigation whether the increased THC yield from the cloned material was entirely as a result of this selection process.

Only THC, CBG and CBC were found in detectable quantities in all samples. The mean CBG content seed sown plants was very similar to that collected from cloned plants, and no significant difference was found. Although the mean CBC content of cloned plants was 15% higher than that of seed derived plants, this increase was not significant (ANOVA, $p > 0.05$). F-tests showed the CBC potency of seed derived plants to be significantly more variable ($p < 0.01$). The difference in variability of CBG content of seed sown plants was much less pronounced. Seed derived plants were not significantly more variable in CBG potency than those raised from clones ($p > 0.05$). When the ratios of cannabinoids in the seed-sown and cloned plants were compared it was found that the cannabinoid profile of seeds-sown plants was more variable than that those raised from cuttings. F-tests showed that the CBC:THC ratio and CBG:THC ratios to be significantly more variable in plants grown from seed ($p < 0.01$).

To meet acceptable quality standards, phytomedicines have to undergo standardization process, this being -

the establishment of reproducible pharmaceutical quality by comparing a product with established reference substances and by defining minimum amounts of one or several compounds or groups of compounds….. (or in some cases) a maximum and minimum amount (Heinrich *et al.,* 2004).

The efficacy of cannabis can be attributed to more than one cannabinoid. Two of these, THC+CBD, have been shown to act together synergistically. (Williamson, 2001; Musty, 2004). The interaction of the other pharmacologically active cannabinoids is less well understood. Where active ingredients within a medicine act synergistically, alterations to the ratio of the synergists can have a greater effect than in a medicine where two active ingredients act additively. This investigation shows that the ratio of synergists is more strictly controlled in cloned plants. When pharmacologically active ingredients are extracted from botanical raw material, it could be argued that the overall secondary metabolite profile of the entire batch is important and that the profile of individual plants within that batch is of minimal significance. More work would be required to test this hypothesis.

5.5.2 Effect of Irradiance Level on Plant and Cannabinoid Yield

During the first year of regular propagation of cannabis chemovars the average monthly yield was recorded. A large seasonal yield variation was observed (Figure 5.5) with winter crops yielding less than those grown in summer.

Routine monthly analysis of cannabinoid content by GC showed that the potency of winter crops was as little as half that of summer crops. As a consequence of the combined drop in crop-yield and potency the cannabinoid yield of winter crops was found to be roughly a quarter of that achieved in summer (Figure 5.6).

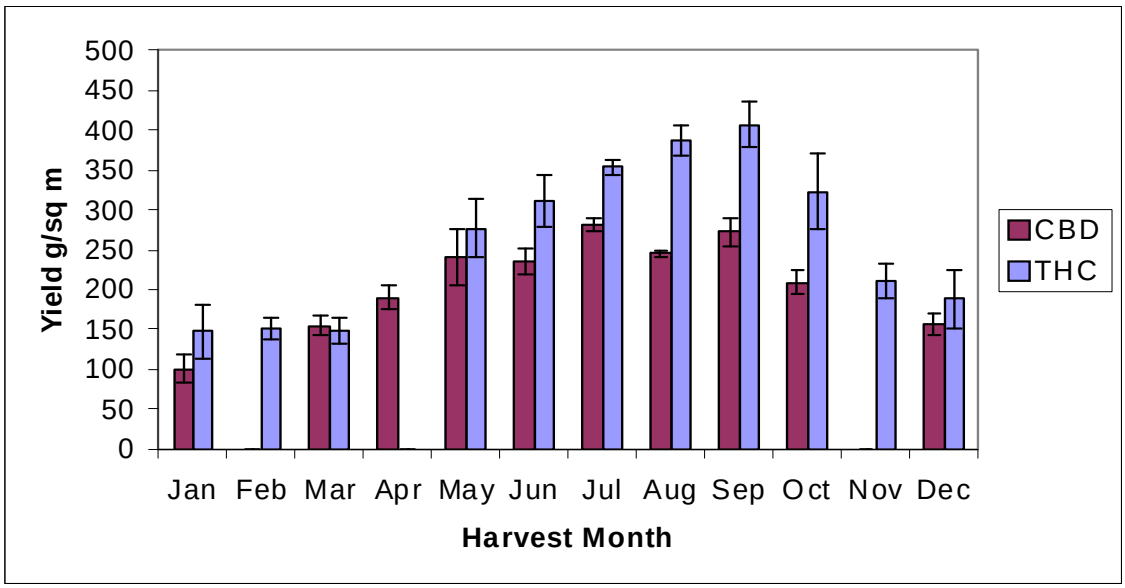

Figure 5.5. Average monthly BRM yield (two to four crops per month) (± SD) of THC and CBD chemovars during the first full year of propagation. (No THC chemovar was harvested in April and no CBD in February and November.)

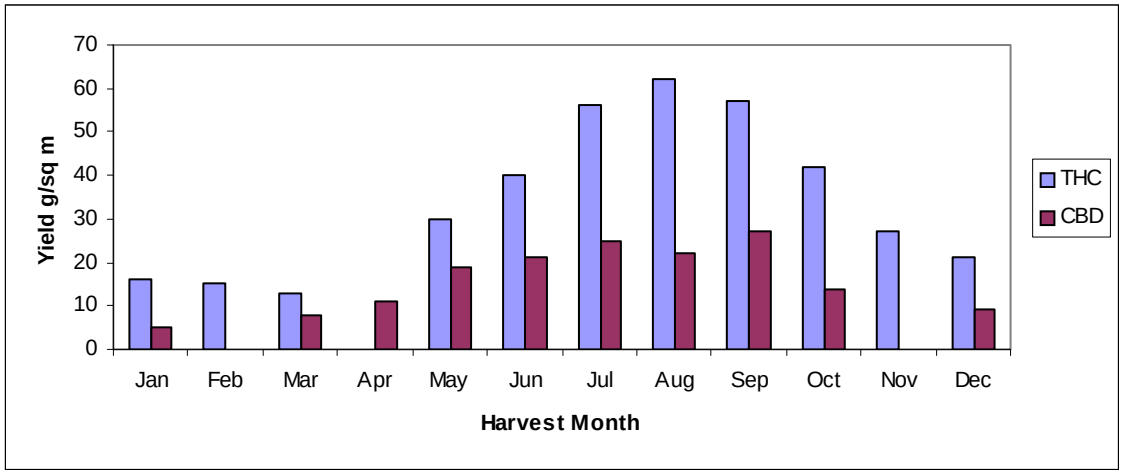

Figure 5.6. The seasonal variation in cannabinoid yield of THC and CBD chemovars during the first full year of propagation. Values shown were estimated by combining the average monthly Botanical Raw Material yield (two to four crops per month) and the average monthly THC or CBD content (w/w).

Table 5.4. shows the yield and potency of identical batches of crops propagated in the glasshouse and in the growth-chamber. The glasshouse light level varied according to time of day and outside lighting conditions, with mercury vapour lamps providing up to 17 W m^{-2}. A theoretical maximum irradiance level of 50 W m^{-2} was achievable in the glasshouse at noon on a bright winter day with supplementary lighting operating. An actual maximum of approximately 25 W m^{-2} was more typical. Growth room conditions were much brighter at 70 W m^{-2}. These crops were harvested during November and December when glasshouse grown yields were close to their minimum.

Chemovar	Location	Daytime Irradiance	BRM Yield	Cannabinoid Potency	Cannabinoid Yield
		W m^{-2} PAR	g m^{-2}	% w/w	g m^{-2}
THC	Glasshouse	17- 50	188	11	23
THC	Chamber	70	397	16	62
CBD	Glasshouse	17 - 50	157	6	9
CBD	Chamber	70	251	11	28

Table 5.4. A comparison of Botanical Raw Material yield and potency of a THC and a CBD chemovar when grown in two irradiance levels during winter.

Following this compelling observation that increased irradiance resulted in such a large increase in yield of plant material and cannabinoid, part of the glasshouse

supplementary lighting system was upgraded. Existing mercury vapour lamps were replaced with high pressure sodium lamps giving a greatly improved light output of a level similar to that achieved in the growth room test. During the following winter months, ten batches of each of the THC and CBD chemovar crops (> 50 plants per batch) were grown under both the mercury vapour lamps delivering 17 W m^{-2} PAR and the brighter high pressure sodium supplementary lighting system delivering 55 W m^{-2} PAR. Under the mercury vapour lamps the THC and CBD chemovar yields were 234 (± 25.6 SD) g m^{-2} and 162 (± 46.6 SD) g m^{-2} respectively. Under high pressure sodium lamps this increased to 478 (± 71.2 SD) g m^{-2} and 410 (± 82.6 SD) g m^{-2}. In a paired t-test the improvement in yield of both chemovars under sodium lamps was highly significant ($p < 0.01$).

The crop yields were monitored over twelve months, following the complete conversion of the glasshouse to the new lighting regime. The average monthly yields achieved before and after the lighting improvements are shown in Figure 5.7.

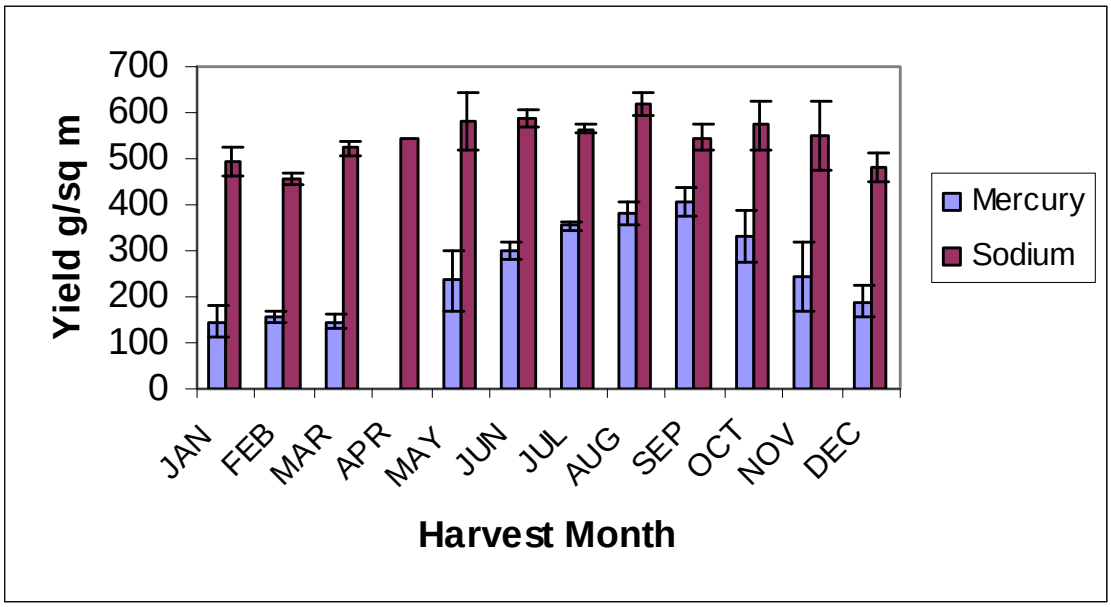

Figure 5.7. The average yield of the THC chemovar before and after the replacement of mercury vapour lamps (17 W m^{-2}) with high pressure sodium lamps (55 W m^{-2}) of improved supplementary lighting (± SD). The mean is typically for four crops per month. No crop was harvested in April of the first year.

This showed that the monthly average yields under the brighter HPS lamps were significantly much greater than those previously obtained under the mercury vapour fittings (t-test, $p < 0.001$). This was even the case in the late summer months when abundant natural light was available. Even on the brightest days, when outside light

levels reached 85 klux or more, the supplementary lighting was activated when outside light conditions fell below 60 klux - this always being the case in the early and late part of the day. The amount of total light energy to which plants were exposed daily was thus greatly increased.

The new lighting regime also significantly improved the uniformity of monthly average yields over the year (F-test, p = 0.013). However, despite this there was still some seasonal variation in yield. The average THC-chemotype summer yield (harvested May – October 573 g m^{-2}) was significantly greater (ANOVA, $p < 0.001$) than that of crops harvested over the rest of the year – 516 g m^{-2}. In theory, further increases in supplementary lighting would have increased winter yields. Alternatively uniformity could be improved by reducing the irradiance levels in summer, by less use of supplementary lighting or increased use of glasshouse shading.

The yields achieved in the first full year of crop growth in a solid building with no natural lighting were similarly monitored. Yields showed a downward trend, commensurate with the manufacturer's predicted age-related fall in irradiance from the lamps. A comparison of the variability in monthly yields of crops grown entirely indoors under lamps initially delivering 75 W m^{-2} , with those grown in the glasshouse under HPS supplementary lamps, revealed no significant difference (F-test, p = 0.05) between the two growing environments.

Having demonstrated such a clear correlation between irradiance levels and cannabis growth, the initial seasonal yield fluctuation prior to lighting improvements was reconsidered. With hindsight it was striking that crop yields were seen to peak in those crops harvested in August and September, a few weeks earlier than if grown outside at the same latitude. The peak in glasshouse average daily irradiance levels would exist around the summer solstice, five to nine weeks earlier, in late June. This suggests that the irradiance conditions at the very beginning of flowering have the greatest potential impact on the final yield. This supposition is supported when the clearly-similar seasonal pattern of glasshouse yields (prior to lighting improvements) is plotted alongside the seasonal variation of irradiance conditions existing at the commencement of flowering of each of these glasshouse crops (Figure 5.8). It would appear that *Cannabis sativa* has evolved to make maximum use of the light energy available, during the longest and brightest days of mid-summer, to develop as dense a foliar canopy as possible. In the following generative (flowering) phase the additional foliage formed would undoubtedly intercept more light energy. This would increase the photosynthetic ability of the plant, some of which would be diverted into secondary metabolite biosynthesis. The later senescence of the additional foliage would enable

proportionally more metabolites to be translocated via the phloem to the inflorescences for secondary metabolite biosynthesis.

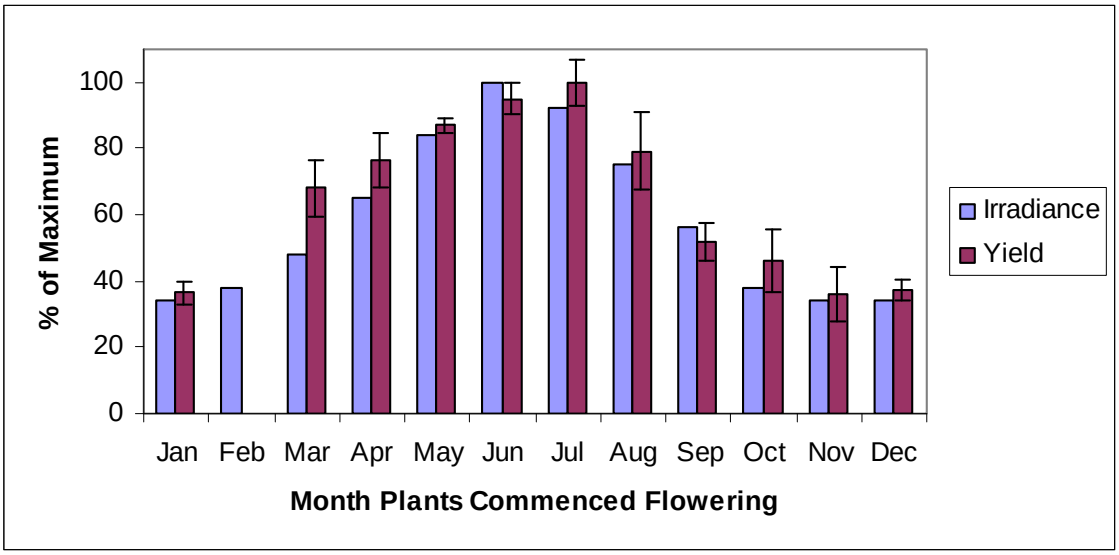

Figure 5.8. Pattern of irradiance level in the glasshouse between 7 am and 7 pm (prior to the improvements in supplementary lighting) and the pattern of average monthly yields of THC chemovar raw material ± SD (n = 4).

This data was alternatively viewed by plotting average monthly yield as a function of the glasshouse light level at the beginning of flowering (Figure 5.9). On both axes the data was expressed as a percentage of the maximum observed. A regression calculation confirmed the highly significant close-to-linear correlation (R^2 = 0.96, p < 0.001) between monthly irradiance level at the commencement of flowering and the subsequent final yield.

This clear linear correlation complemented the findings of growth room studies by Lydon *et al.* (1987) who showed that cannabis assimilated carbon dioxide linearly up to a photon flux density of approximately 500 µmol m^{-2} s^{-1}, 400 - 700 nm. This is equivalent to solar radiation levels of approximately 100 W m^{-2} and is well above the maximum daily-average irradiance encountered in this study. It is assumed that the linear assimilation of carbon dioxide observed by Lydon *et al.* (1987) corresponded to a linear increase in photosynthesis. A subsequent increased accumulation of both primary and secondary metabolites would be expected, although the relative proportions of these would possibly differ. The increased potency of plants grown in brighter conditions observed in this study supports the Carbon Nutrient Balance hypothesis proposed by Bryant *et al.* (1983). This predicts that the increased net

photosynthesis results in an increased Carbon/Nutrient ratio within the plant. This favours the development of carbon-based secondary metabolites.

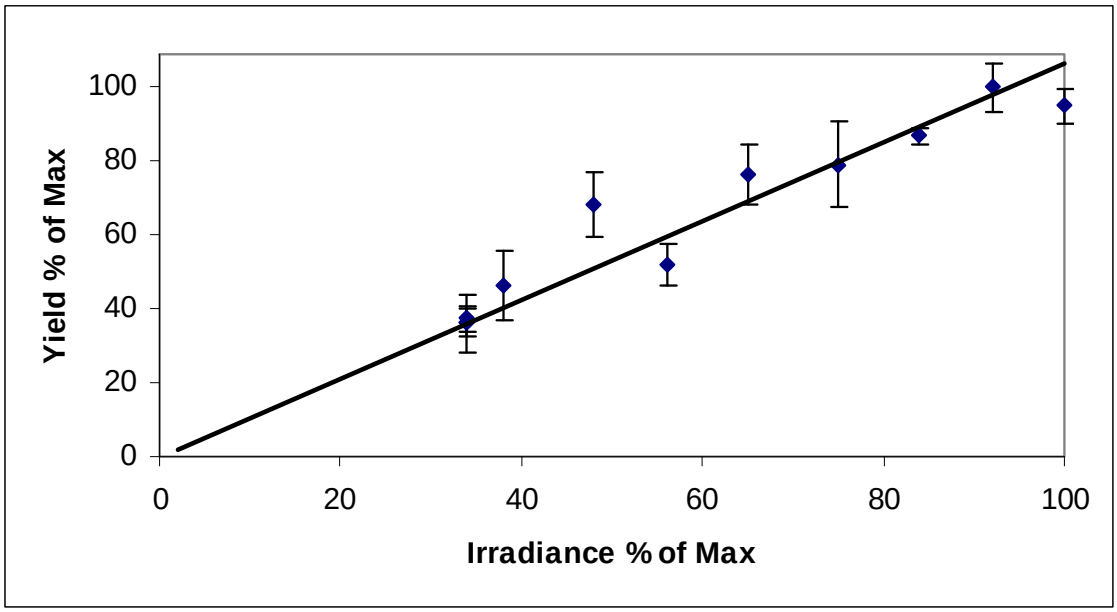

Figure 5.9. The average monthly yield as a function of the glasshouse light level at the beginning of flowering. On both axes the data was expressed as a percentage of the maximum observed ($r^2 = 0.92$, $p < 0.001$).

The requirement for such high levels of supplementary lighting is understandable. At latitude 50ºC, ignoring effects of cloud cover, average solar radiation levels at sea level are approximately half those encountered at 30ºC (Albuisson *et al.*, 2006). Although *Cannabis sativa* grown for fibre or seed is often planted at this latitude, the THC-chemotype grown outdoors for its cannabinoid content is more commonly found at latitudes of 30º or less (Small and Beckstead, 1973a,b). In addition to unfavourable latitude, light transmission through a typical glasshouse roof is further reduced by at least 30% (Heuvelink *et al.,* 1995). Supplementary lighting has been increasingly used in commercial glasshouses in the UK for food-crop production. Rarely do these deliver light levels more than 12 W m^{-2}, but recent installations of 25 W m^{-2} have been reported (Vale, 2008). The installation of a lighting system delivering 55 W m^{-2} over an area of 5000 m^{-2}, as used here, was highly unusual and possibly unique in the UK.

However this lighting level is less than that typically utilised by illicit growers. Evidence from indoor UK cannabis-growing scenes of crime (private communication) show much brighter lighting conditions. These appear to comply with the illumination levels recommended in cannabis growing guides (Green, 2003), which commonly suggest the use of one 600W high pressure sodium lamp per square metre of flowering crop.

Hough *et al.* (2003) quotes an experienced illicit UK cannabis grower as saying that "A decent grower can quite easily get one gram of dried flower head per watt of lighting used." This refers to electrical energy consumed per unit area. It implies that the use of one 600 Watt lamp per square metre could result in the production of 600 g m^{-2} of dry mature cannabis flower head. Such illicit yields are regularly claimed (Rosenthal, 2001) and appear credible. Six hundred watt HPS lamps typically convert about 30% of the electricity consumed into photosynthetically active radiation (Langton and Fuller, 2001). The irradiance in such a situation would therefore be 180 W m^{-2} PAR, i.e. approximately twice that encountered by the pharmaceutical crop studied for this thesis. This highlights the typical illicit growers' desire for yield over uniformity. This may be partly to meet consumer demand. However, it is extremely common for illicit growers to be using 'extracted' (stolen) electrical energy, and as such energy consumption and cost is often not a consideration.

5.5.3 Effect of Duration of Flowering Period on BRM and Cannabinoid Yield

Between the sixth and eigth week of flowering the 33% extension in flowering duration resulted in a mean cannabinoid yield increase of over 50% (Figure 5.10). In all clones this extra two weeks flowering was clearly advantageous. A further 25% increase in flowering period from eight to ten weeks resulted in a mean increase in THC yield of 31%, but for approximately half of the clones, including Sativex-related G1M1 and G2M7, the observed THC yield increase was less than 25% and the economic benefit of the extra two weeks flowering was doubtful. Paired t-tests showed that the mean increases in yield, from six to eight weeks and from eight to ten weeks, for the twenty five clones were highly significant (6-8 weeks $p < 0.001$, 8-10 weeks $p = 0.032$).

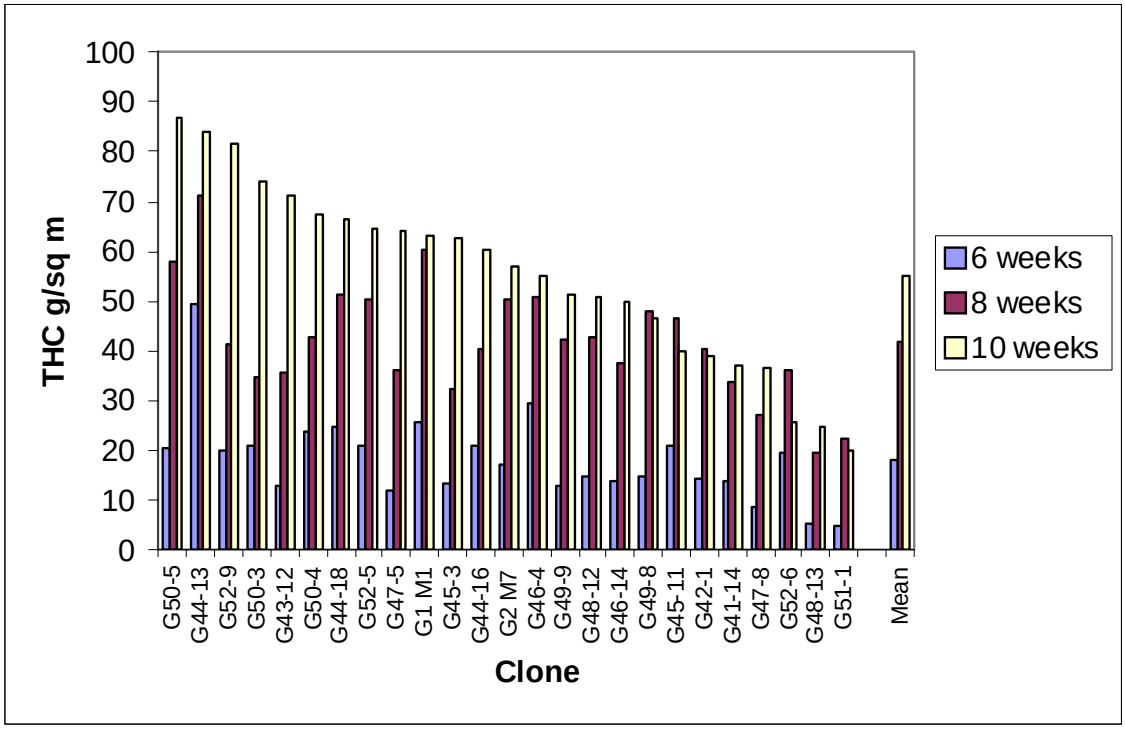

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Figure 5.10. The yield of THC achieved by each of the clones (n=5) after six, eight and ten weeks in flower. For clarity, the clone lines have been sorted in order of descending THC yields after ten weeks in flower. (Paired t-test 6-8 weeks $p < 0.001$ and 8-10 weeks $p < 0.01$).

The clones that clearly benefited from a longer ten week flowering period were generally those reported to have an equatorial and subequatorial areas provenance. In their natural habitat these would experience a longer growing season. However, as with all cannabis varieties that are used for illicit purposes, these variety names are not registered in the International Code of Nomenclature for Cultivated Plants, 1995 (Snoeijer, 2002). Thus true provenance cannot be authorised and caution is required when referring to the properties of such varieties.

5.5.3.1 Effect of Duration of Floweringt Period on Ratio of THC and CBG in THC Chemovars

Between the sixth and ten week of flowering, the mean average content of both THC and CBG increased in the twenty five clones. The mean proportion of CBG in the cannabinoid profile fell from 3.66% to 3.24% between the sixth and eighth week of flowering and fell further to 2.66% in the tenth week. Both decreases were highly significant (p = 0.0208 and p = 0.0003 respectively in two-tailed t-tests). This was probably due to the fact that as plant development slowed, CBG was being converted into THC faster by THC synthase than it was being renewed by

geranylpyrophosphate:olivetolate geranyltransferase. The relative proportions of CBG and THC in a pharmaceutical product must meet a tight specification to ensure its quality, safety and efficacy. This investigation shows that the ratio of these two cannabinoids is not genetically fixed and that harvest timing can have a significant effect.

Plant genetics were shown to have a greater impact on THC:CBG ratios than harvest timings. For example, the THC:CBG ratio of clone G44-13 was approximately 1:200 at all harvest timings whereas G48-12, G48-13 and G41-14 showed a ratio greater than 1:20. When the THC:CBG ratios from all three harvest date were combined (Figure 5.11) highly significant differences were observed between clones.

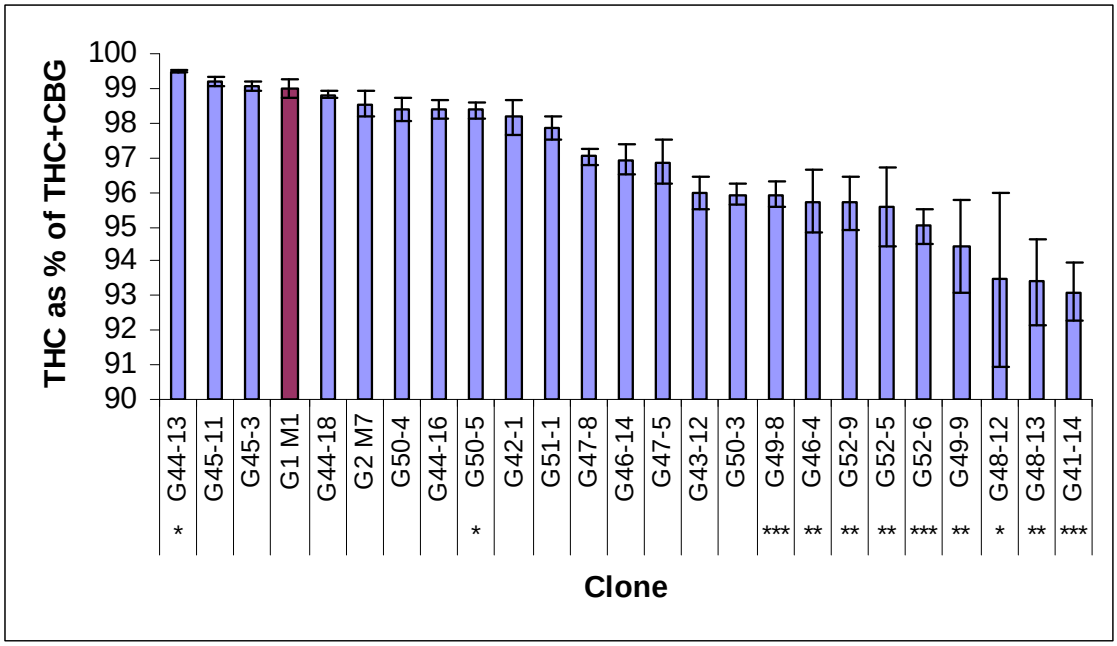

Figure 5.11. A comparison of the mean relative proportions (±SD) of THC and CBG in twenty five clones at three harvest dates. Analyses of variance (one-way) compared the proportion of THC in each clone to that in the Sativex-dependent clone G1 (shown in red). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

In some cases (G44-13 v G44-16 and G50-3 v G50-4 or G50-5), there were highly significant differences in the THC:CBG ratios of clones derived from single varieties (ANOVA, $p < 0.01$). This supports the data reported in Section 5.5.1 which indicates that uniform drug feedstock is more likely to be produced by plants grown from clones rather than seed. The especially high degree of variability in varieties G44 and G50 (provenance unknown) was possibly due to the parent-crosses having a higher degree of heterozygosity.

5.5.3.2 Effect of the length of flowering period on profile of heterozygous chemotypes with mixed THC/CBD profiles

Four weeks after being placed in twelve hour days, inflorescence development was rapid and capitate stalked trichomes were becoming abundant. This suggested that the plants were actively biosynthesising cannabinoids. Sampling commenced and was continued for the following six weeks. By the tenth week in short day length, no more viable stigmas were observed, indicating that inflorescence development and capitate stalked trichome formation had ceased. Table 5.5 shows that there were minimal increases in cannabinoid concentration in the last two weeks of the sampling period, but prior to that, cannabinoid contents had escalated rapidly.

In clone G159/1 the CBD/THC ratio remained constant over the entire flowering phase. However, in G159/9 and G159/16 there were significant proportional increases in CBD content (regression $p < 0.05$). Conversely, in G159/11 and G159/12 the proportion of CBD in the cannabinoid profile showed significant decreases with time (regression $p < 0.05$). The stable cannabinoid profile exhibited here by clone G159/1 suggests that single clones of the mixed CBD/THC chemotype can be used as phytopharmaceutical feedstocks. However, to produce such clones the plant breeder has the combined challenge of identifying genetics that not only exhibit a desired cannabinoid profile but also deliver this reliably and uniformly during the growing process. In the absence of such clones, desirable cannabinoid mixtures can be only be achieved by blending materials with differing profiles.

Weeks in 12 h days	Mean Cannabinoid Content % w/w (± SE)	Clone				
		G159-1	G159-9	G159-11	G159-12	G159-16
		CBD as % of CBD+THC (± SD)				
4	1.55 (± 0.08)	61.78 (± 0.14)	58.79 (± 0.70)	61.41 (±0.37)	42.60 (± 0.35)	59.49 (± 0.49)
5	3.12 (± 0.69)	61.43 (± 0.41)	58.55 (± 0.29)	62.21 (± 1.14)	43.14 (± 0.63)	60.02 (± 0.80)
6	5.69 (± 1.27)	62.03 (± 0.85)	59.65 (± 0.15)	61.20 (± 0.22)	42.95 (± 0.49)	61.19 (± 0.54)
7	9.22 (± 1.22)	61.84 (± 0.38)	62.05 (± 0.38)	59.23 (± 0.30)	43.06 (± 0.44)	61.49 (± 0.11)
8	11.15 (± 1.08)	61.53 (± 0.09)	62.79 (± 0.21)	58.91 (± 0.57)	41.86 (± 0.90)	62.21 (± 0.58)
9	11.65 (± 0.92)	61.32 (± 0.37)	63.00 (± 0.95)	59.11 (± 0.58)	42.06 (± 0.05)	62.17 (± 0.23)
10	11.95 (± 1.24)	61.99 (± 0.53)	64.16 (± 0.73)	59.60 (± 1.22)	41.08 (± 1.17)	62.29 (± 0.20)
	Regression	No significant change $p > 0.05$ r = 0.03	Significant Increase $p < 0.01$ r = 0.96	Significant Decrease $p < 0.05$ r = 0.81	Significant Decrease $p < 0.05$ r = 0.79	Significant Increase $p < 0.05$ r = 0.95

Table 5.5. The relative proportions of CBD and THC during plant development in five clones derived from variety G159. Results are shown as the proportion of CBD expressed as % of CBD+THC (± SD). The regression calculations test the significance of the changing proportion of CBD and THC in each clone, between the 4th and 10th week in 12 h daylength.

5.5.4 Effect of Daylength on Plant Development and Cannabinoid Profile

5.5.4.1 Comparison of Twelve and Thirteen Hour Daylength Regimes

Despite their varying provenance, all the varieties included in this investigation commenced to flower within ten days of being placed in a thirteen-hour day length. This suggested that all had a 'critical daylength' of over thirteen hours. As shown in Table 5.6 a daylength of thirteen hours occurs naturally at around August 18th, at a latitude of 26ºN, and progressively later at more northerly locations. Had a Columbian variety been included, perhaps originating for Santa Marta (latitude 11° north), it is

possible that it would not have flowered - as even at the summer solstice the day length is just 12 hour 45 minutes. The critical day length for plants from this latitude would be therefore expected to be substantially less than thirteen hours. It is possible that the plant would have eventually have flowered in response to plant age rather than daylength, as is common for tropical varieties.

All of the locations cited in Table 5.6 have been important in the breeding history of the varieties currently developed for pharmaceutical use. Skunk #1 and subsequently some of the other varieties within this test were originally bred using a mixture of Columbian, Mexican and Afghan genetics (de Meijer, 1999; Clarke, 2001). Despite the Columbian inclusion, all varieties appear to have inherited from their more northerly ancestors an ability to flower readily in a thirteen-hour daylength. The CBD-rich variety G5 was bred from landrace plants derived from the Black Sea coast in Northern Turkey, where thirteen hours day length occurs in early September, a few days earlier than in Southern England.

Latitude: -	11º	26º	36º	41º	51º
Climate: -	Tropical	Semi-tropical		Temperate	
Location: -	Santa Marta, Columbia	Monterrey, Mexico	Mazar al sharif, San Fransisco and Ketama*	Samsun, Turkey	London, UK
Jul-15	12.43	13.35	14.25	14.50	16.10
Aug-01	12.35	13.19	14.05	14.20	15.24
Aug-15	12.28	13.03	13.37	13.49	14.37
Sep-01	12.19	12.38	12.59	13.06	13.34
Sep-15	12.11	12.18	12.27	12.28	12.40
Oct-01	12.01	11.54	11.48	11.45	11.37
Oct-15	11.53	11.34	11.19	11.10	10.45

Table 5.6. The falling late-summer daylength (hours.minutes) in a range northern hemisphere cannabis growing areas. (* Contrasting locations at similar latitude in Afghanistan, USA and Morocco.)

Although floral development of all plants commenced equally promptly in a twelve or thirteen-hour daylength, the pattern of floral development differed greatly in the two

regimes. The visual assessments the degree of stigma senescence (Table 5.7) showed that the rate of flower formation slowed more rapidly in the twelve-hour regime. Assessed eight and ten weeks after being placed in short daylength, the mean proportion of senescence stigmas was significantly higher in the twelve hour daylength (paired t-test, $p < 0.01$, at both assessment dates). The difference was most dramatic in clone line M84, which after ten weeks in a twelve-hour daylength, had completely ceased to develop new flowers whereas the same clone in thirteen hours daylength continued to flower prolifically.

	Eight weeks in Short Days		Tenth Week in Short Days	
	12 hr Day length	13 hr Day length	12 hr Day length	13 hr Day length
Clone				
M1	60	33	50	45
M6	60	38	80	45
M57	80	35	80	55
M59	70	50	35	10
M60	95	35	100	98
M61	99	38	100	75
M79	70	15	60	50
M82	20	4	30	15
M84	50	3	100	10
M87	30	4	25	5
Mean	63.4 **	25.5 **	66 **	40.8 **

Table 5.7. A comparison of the proportion of senesced stigmas observed on ten clone when induced to flower in daylengths of twelve or thirteen hours. Assessments were made eight and ten weeks after the plants were placed in short daylength. ** Significant difference ($p < 0.01$, paired t-test).

A twelve-hour day length occurs at all northern hemisphere latitudes in the last week of September (Table 5.6), at around which time a cannabis crop grown for seed would typically be harvested (Bocsa and Karus, 1998). Any female flowers formed after early September would be unlikely to have sufficient light and warmth to produce viable seed before the plant died. Moreover, pollen formation typically ceases before the end of

August after which time male plants typically die thereby not competing with the female plants as they set seed. The formation of further female flowers would be a futile waste of plant resources.

After eight weeks in short daylength there was no significant difference in the mean height of the ten clones in twelve or thirteen hour days (paired t-test). After ten weeks significant differences were observed in the height of five clones (ANOVA), and this was most pronounced in vigorous F1 hybrids M82 and M87 (Figure 5.11). As a consequence there was a significant difference in the mean height for all ten clones in the two regimes (paired t-test $p < 0.05$). M82 and M87 were much taller than other clones. Excessive height is a disadvantage in a glasshouse grown crop and the longer daylength exacerbated this problem. Tall plants are difficult to support and to handle, and are less easily examined for pest and disease.

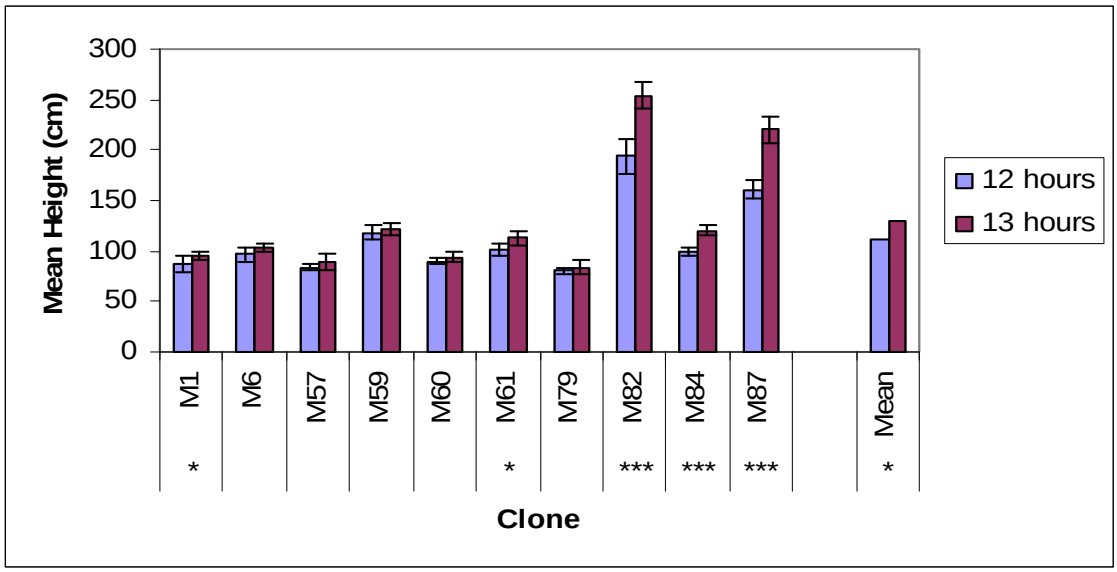

Figure 5.12. Effect of Daylength on Plant Height ± SD (n = 20) ten weeks after induction of flowering (* $p < 0.05$, *** $p < 0.001$, ANOVA for individual clones and paired t-test for the overall mean).

Eight weeks after flower initiation, minimal differences were observed between the botanical raw material yields of plants grown in twelve and thirteen hour daylengths. The two F1 hybrid clones M82 and M87, that had shown large differences in height, showed marked increases in yields at the longer daylength when harvested ten weeks after flower initiation (Figure 5.13). However, overall the longer daylength did not significantly increase the mean yield of the ten clones (paired t-test, $p > 0.05$).

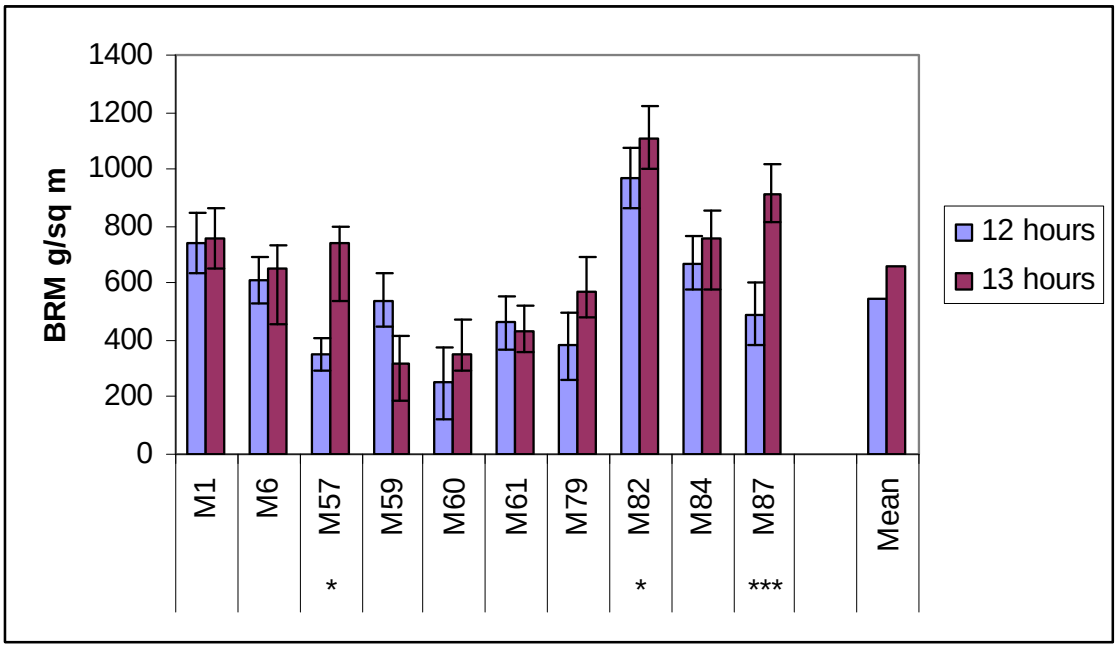

Figure 5.13. Effect of Daylength on Yield of Botanical Raw Material ± SD (n = 5 plants) ten weeks after induction of flowering (* $p < 0.05$, *** $p < 0.001$, ANOVA).

The cannabinoid content of the higher yielding clones M82 and M87 was greatly reduced in the longer daylength and this resulted in a marked decrease in mean cannabinoid yield of the ten clones at both harvest dates (Table 5.8). Conversely the clone lines M1 and M6, showed a possible yield benefit. When assessing the overall effect of daylength on the mean cannabinoid yield of the ten clones, the paired t-tests showed no significant advantage for either regime.

Clone	8 weeks in flower		10 weeks in flower	
	12 hr days	13 hr days	12 hr days	13 hr days
M1	57	79	70	73
M6	44	54	65	83
M57	64	64	51	87
M59	52	34	54	30
M60	41	45	36	39
M61	29	30	32	31
M79	50	53	43	49
M82	84	33	122	63
M84	34	35	55	53
M87	84	38	100	73
Mean	54	47	62	58

Table 5.8. Effect of Daylength on cannabinoid yield (g m^{-2}), eight and ten weeks after induction of flowering.

Daylength had highly significant effects on cannabinoid profile. Harvested eight or ten weeks after being placed in a twelve or thirteen hour daylength, all ten clones had a proportionally higher CBG content if grown in the thirteen hour regime (Table 5.9). The mean difference was significantly higher after eight weeks ($p < 0.01$) and ten weeks ($p < 0.05$, paired two-tailed t-tests).This was likely due to the fact that more new florets and accompanying glandular trichomes were being formed in the thirteen hour daylength, and these would be synthesising more CBG. Conversely plants in the thirteen hour regime possessed proportionally less THCV ($p < 0.001$ after eight weeks and $p < 0.01$ after ten weeks). This finding supports other results (not reported here) that propyl cannabinoid synthesis is more rapid than that of pentyl cannabinoids. As a consequence, in aging tissue the biosynthesis of THCV reaches completion before that of THC.

	CBG as % of CBG + THC					THCV as % of THCV + THC			
Daylength	12 h	13 h	12 h	13 h		12 h	13 h	12 h	13 h
Time in Short Days	8 weeks		10 weeks			8 weeks		10 weeks	
M1	1.00	1.20	0.65	2.00		0.52	0.48	0.55	0.46
M6	0.77	1.40	0.81	1.25		0.65	0.57	0.73	0.53
M54	3.96	4.38	3.30	3.97		0.39	0.34	0.33	0.29
M57	0.69	0.98	0.45	0.82		0.42	0.34	0.51	0.35
M59	1.27	2.63	1.11	0.96		0.47	0.45	0.57	0.42
M60	3.30	4.57	2.51	2.59		0.54	0.52	0.54	0.48
M61	1.53	3.43	0.98	1.44		0.29	0.20	0.32	0.18
M79	1.55	3.78	1.29	1.57		0.43	0.37	0.45	0.37
M82	5.77	7.15	4.41	6.11		0.32	0.16	0.31	0.17
M87	4.10	6.55	3.29	3.40		0.39	0.35	0.32	0.37
Mean	2.39	3.61	1.88	2.41		0.44	0.38	0.46	0.36
p, t-test (2 tailed)	0.0011		0.0176			0.0006		0.0015	

Table 5.9. The effect of day length during flowering on cannabinoid profile. Results shown are the proportion of CBG and THCV, expressed as a % of the CBG+THC or THCV+THC total, in ten clones after eight and ten weeks in short daylength.

5.5.4.2 Comparison of Eleven and Twelve Hour Daylength Regimes

Whereas reducing the daylength from thirteen to twelve hours had a dramatic effect on floral development, a further reduction to eleven hours only marginally accelerated the cessation of new flower formation, Table 5.10.

Clone	Eight weeks in Short Days		Tenth Week in Short Days	
	11 hr	12 hr	11 hr	12 hr
M1	30	30	60	55
M6	35	40	60	60
M11	80	70	100	98
M16	50	38	100	98
M57	15	8	65	55
M58	28	30	45	55
M59	30	40	85	70
M60	95	80	99	100
M61	20	25	98	70
M82	8	10	23	20
M84	15	10	50	45
M87	10	12	40	30
Mean	35	33	69	63

Table 5.10. A comparison of the proportion of senesced stigmas on ten clones in twelve and eleven hour daylength regimes when assessed eight and ten weeks after the induction of flowering. Just one overall visual assessment was made for each clone.

F1 hybrids M82 and M87 showed very large differences in height when grown in contrasting twelve and thirteen hour regimes. However, along with all other clones, these showed no significant difference in height when grown for eight or ten weeks (Figure 5.14) in eleven and twelve hour daylengths (ANOVA). Similarly, a paired t-test revealed no significant difference in the mean heights of the ten clones in either daylength.

The extra hour of day length resulted in significantly greater yields of most clones (ANOVA) at both assessment dates. For simplicity, just the result of the latter assessment is shown in Figure 5.15 (* $p < 0.05$ and *** $p < 0.001$). A paired t-test showed the mean weight of the twelve clones to be greater in the longer day length at both assessment dates.

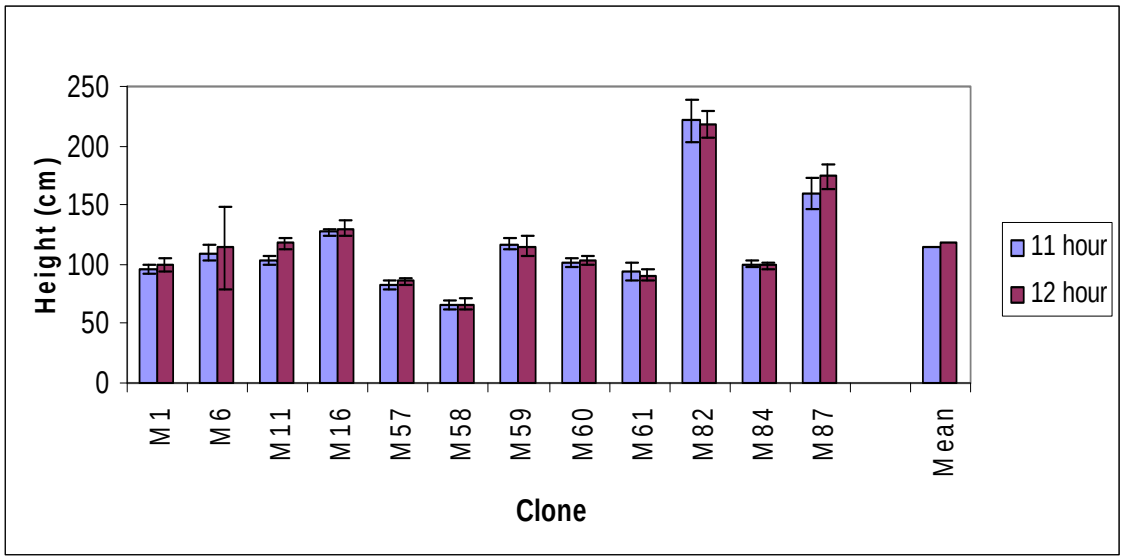

Figure 5.14. Effect of Daylength on Plant Height ± SD (n=20) ten weeks after induction of flowering. In a paired t-test there was no significant difference in the mean height of plants grown in 11 and 12 hour days.

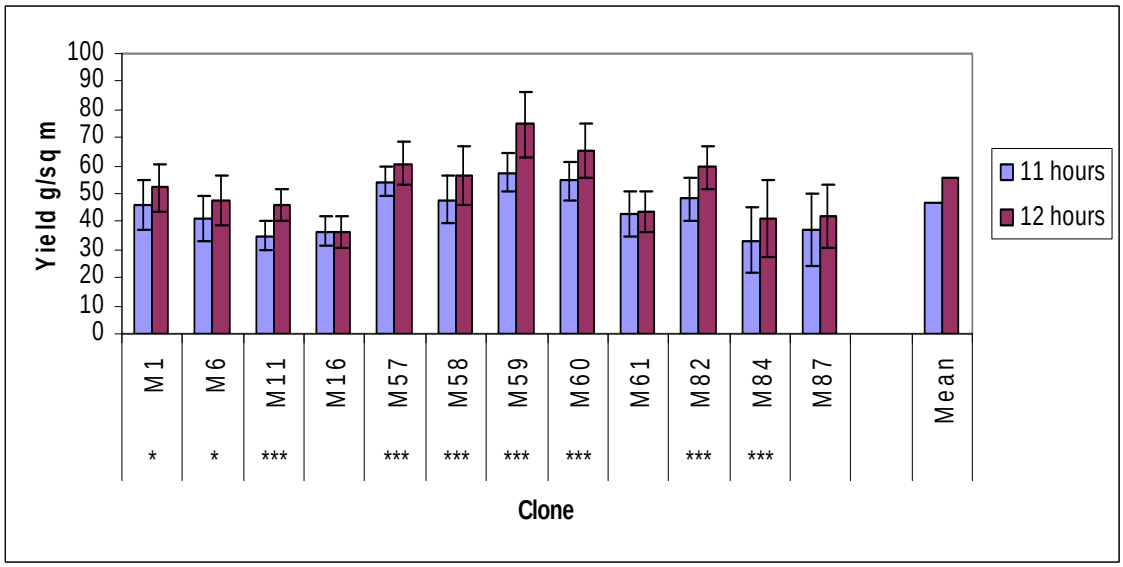

Figure 5.15. Effect of Daylength on Yield of Botanical Raw Material ± SD (n = 20 plants) ten weeks after induction of flowering. In the Analyses of Variance, the levels of significance were shown as * $p < 0.05$ and *** $p < 0.001$. In a paired t-test, the mean height of plants in the 12 hours days was significantly higher $p < 0.001$.

When harvested after ten weeks in the flowering regime, all clones showed a lower cannabinoid yield in the shorter daylength (Figure 5.16). The overall mean 22% reduction in cannabinoid yield was highly significant, ($p < 0.01$, two-tailed t-test). This reduction in yield could not be attributed to a prominent visible change in plant

morphology. Plants in the shorter eleven-hour daylength had received 9% less light energy per day. Conversely, these plants spent 9% longer in darkness and would be expected to have lost yet more energy as a result of a longer period of respiration. This would seem a major contributing cause of the yield reduction in the eleven hour daylength and is in marked contrast to the lack of yield difference observed when comparing the twelve and thirteen hour daylengths. In the thirteen hour regime the potential yield benefits of increased energy were not utilised by the plant.

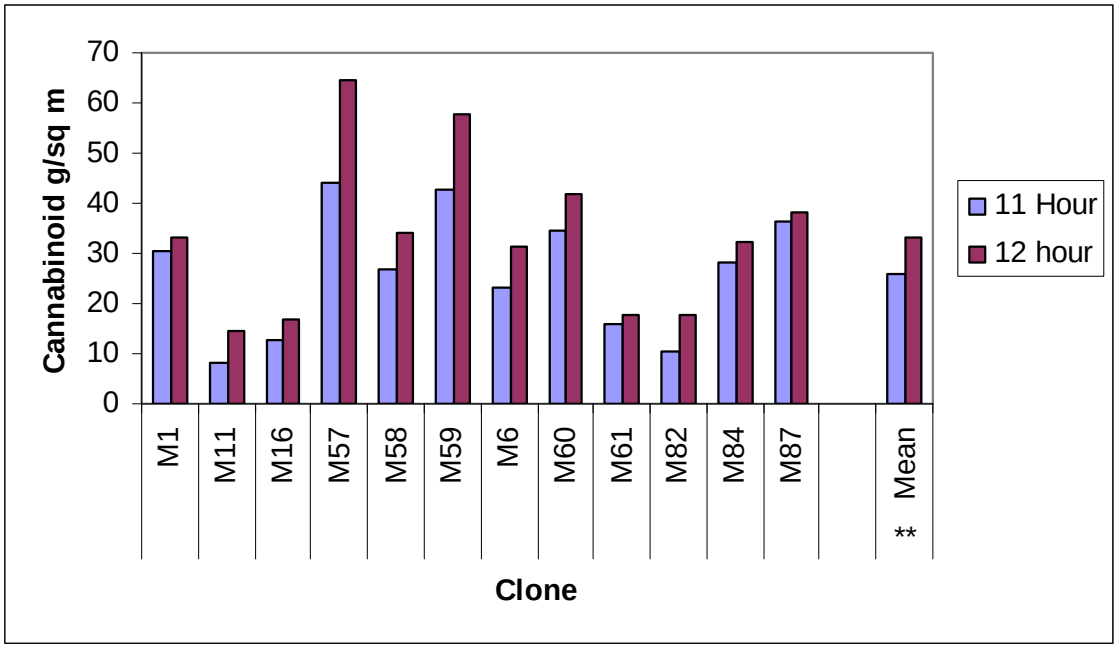

Figure 5.16. Effect of Daylength on cannabinoid yield ten weeks after induction of flowering. (Paired t-test, two tail ** $p < 0.01$).

The HPLC analysis method that was used to analyse the cannabinoid profile showed limited sensitivity to CBG and THCV, and these proved to be below the detectable level in some clones. Nevertheless, sufficient data was available to assess the proportion of CBG, as a percentage of the CBG+THC+CBD total, in six clones (Table 5.11). In a paired test, there was no significant difference in the mean proportion of CBG present.

Daylength	CBG as % of Cannabinoid Total	
	11 h	12 h
M57	0.45	0.90
M58	4.23	4.52
M59	1.31	1.33
M60	2.56	2.29
M84	1.73	1.67
M87	3.13	2.82
Mean	2.24	2.26
Paired t-test	$p = 0.98$	

Table 5.11 The effect of day length during flowering on cannabinoid profile. Results shown are the proportion of CBG expressed as a % of the CBG+THC+CBD total, in six clones after eight weeks in short daylength.

5.5.4.3 Review of the comparisons of plants induced to flower on daylengths 11, 12 and 13 hours.

Amongst the major findings, from the studies comparing the effect of daylength, it was found that increasing the daylength from twelve to thirteen hours increased energy consumption by at least 8%, but resulted in no beneficial increase in botanical raw material yield. There was sometimes also an unwelcome increase on plant height. Effects of increased day length on cannabinoid yield were variable, with some clones showing a large decrease in cannabinoid production at the longer day length. Conversely, reducing the day length to eleven hours saved energy by approximately 8% but resulted in an economically unacceptable and statistically significant 15 – 20% mean reduction in plant yield ($p < 0.01$). There was also a significant reduction in the mean cannabinoid yield ($p < 0.01$ after 10 wks in short days).

Placing indoor-grown cannabis in a short daylength to induce the plants to flower is widely practised. The concept of flower initiation in response the arrival of the critical daylength, and the part that phytochromes play in this are widely reported (Halliday and Fankhauser, 2003). The effect of short daylength on inflorescence development is less well studied. In addition to a critical daylength, at which point flowering is induced, this

study suggests that there is a previously unreported second critical daylength at which development of further female flowers is inhibited.

In a glasshouse setting, where crops in various stages of floral development grow side-by-side, it is not practicable to recreate the steadily shortening day length experienced by outdoor grown cannabis. A standardised daylength has to be maintained once flowering has commenced. The flowering daylength adopted can dramatically affect the appearance of the inflorescence, with plants in thirteen hours day length continuing to produce significantly more fertile female flowers for much longer than those in twelve or eleven hour regimes ($p < 0.05$). An assessment of the proportion of senesced stigmas in an inflorescence is often regarded as useful when deciding upon harvest date of illicit crops. However, this investigation concluded that stigma senescence is not useful for judging the cannabinoid content of crops grown for pharmaceutical purposes.

Eleven or thirteen hour daylength regimes are less economical in producing cannabis for the production of cannabinoid-based medicines than the twelve hour regime initially adopted. Future research to explore small adjustments to the adopted twelve-hour regime may be beneficial. Research with ornamental pot-grown chrysanthemums (short day plants) has shown that adjustments of just ten minutes to the daylength can have a dramatic effect on the economic value of the plants produced (Langton and Fuller, 2001).

Rather than simply altering the lighting regime to save costs, much greater savings could be made by growing the crop outdoors. Whereas the glasshouse environment provided uniform temperature, irradiance levels and daylength the outdoor environment exposes the crop to variable conditions. If these variabilities were only found to affect secondary metabolite yield this would be tolerable. The production of plants with a significantly different secondary metabolite profile or poor quality would raise serious concerns. The next chapter evaluates this possibility.

5.6 CONCLUSIONS

This chapter examined the development, and more especially its secondary metabolite content, when grown indoors. The overall aim was to determine ways of identifying growing methods and harvest timings that improved yield and crop uniformity when growing medicinal cannabis crops indoors in the UK.

Specific tests with the Sativex® dependent variety G1 showed that cannabinoid profile uniformity was significantly improved by growing plants from clones (cuttings) rather than seeds.

The duration of the flowering period prior to harvest was seen to have a statistically significant effect on cannabinoid profile when tested on a wide population of genotypes. However, limited data suggested that the proportion of CBG in the cannabinoid profile of Sativex-dependent varieties G1 and G2 had stabilized after eight weeks in flower. The ratio of THC and CBD in heterozygous plants did not remain stable during floral development, and this instability is one supportive reason for producing THC and CBD for Sativex® from separate homozygous chemotypes.

When inducing and maintaining floral growth, a twelve hour daylength was shown to be more energy efficient than eleven and thirteen hour regimes.

The most pronounced findings, from the research for this chapter, resulted from studies with glasshouse supplementary lighting. Significant improvements were achieved in the year round uniformity and yield of botanical raw material. Cannabinoid uniformity and yield were similarly improved. It was shown that the irradiance levels at the commencement of flowering had the greatest effect on yield. However, these improvements were only achieved by the consumption of lighting energy levels well above those typically used in the UK horticultural industry. One way of avoiding this energy use would be to grow the crop outdoors. Rather than simply altering the lighting regime to save costs, much greater savings could be made by growing the crop outdoors. Whereas the glasshouse environment provided uniform temperature, irradiance levels and daylength the outdoor environment exposes the crop to variable conditions. If these variabilities were only found to affect secondary metabolite yield this would be tolerable. The production of plants with a significantly different secondary metabolite profile or poor quality would raise serious concerns. The next chapter evaluates this possibility.

Chapter 6 . The Outdoor Propagation of Phytopharmaceutical Cannabis

6.1 INTRODUCTION

The concept of growing phytopharmaceutical cannabis outdoors was initially rejected due to plant quality and security concerns. However, prior to this thesis, an experimental outdoor trial was planted. This demonstrated that potentially high yielding cannabis crops could be grown in southern England (Potter, 2004), the Sativex-dependent clone G5 M11 producing 500 g m^{-2} of dry BRM. This was approximately the same yield as that achieved in a glasshouse crop. The trial did not aim to explore the maximum yield that could be achieved and with improved knowledge higher yields may have been obtained. No attempt was made to analyse the effect of outdoor growing on secondary metabolite profile. In the following two seasons further batches of the clone G5 M11 were planted and crop establishment was good. However, both crops encountered fungal spoilage by *Botrytis* in the last week before planned harvest. Subsequent attempts to grow clones of the Sativex-dependent THC chemovar G1 failed, as the plants commenced flowering far too late in the season to produce a worthwhile crop. However, another high-THC genotype did achieve high yields and produced a potential phytopharmaceutical feedstock containing 11.8 ± 1.5% (w/w) THC. This was close to the average potency of illicit sinsemilla cannabis circulating in England in 2005 (Potter *et al*., 2008), most of which was thought to have been secretly grown indoors. The first field trial also compared the establishment of plants grown from rooted cuttings and from seed. Both methods proved satisfactory but establishment from seeds was markedly less labour intensive.

From an agricultural perspective it therefore appeared that outdoor growing of cannabis was feasible in England, although there was little data to demonstrate the potential quality of this material. Concerns remained about the security of outdoor cannabis crops as the medicinal cannabis crop has a potentially high cash value, and an unusual cachet that appeals to the curious and mischievous. Hemp-fibre and seed crops of *Cannabis sativa* grown in the UK have frequently been damaged by the public, who have mistaken the crop for recreational cannabis. An all-female CBD chemovar crop bears a closer similarity to recreational cannabis, both in appearance and odour, and would be more likely to be tampered with. Any pharmaceutical crop is also possibly

vulnerable to the attention of those protesting against the animal testing associated with the industry. An outdoor crop would require a very discrete and secure location.

In marked contrast to the field environment the glasshouse conditions were well controlled, and harvest timings could be routinely anticipated and labour timetabled accordingly. If the timing of harvest of field-grown crops could be predicted with similar confidence there would be clear benefits. When commencing this research it was not known how predictable the date of outdoor harvest would be, and how long crops could be regarded as being at the appropriate stage. As only one crop had been successfully harvested prior to this thesis, the predictability of harvest date was still little understood.

It was anticipated that a major difficulty of outdoor growing would be the requirement to dry large quantities of material at the exact point when crops were deemed ready for harvest. An early attempt to dry bulk quantities of outdoor grown crop resulted in failure. The crops were dried using the same method employed for the glasshouse crop, and much of the material was lost due to fungal spoilage. Exceeding the microbial count threshold, as stated in the European Pharmacopoeia, is the most common cause of rejection of plant material (Baier and Bonne, 1996). Prior to this thesis a crop was successfully dried in the oast house, using a diesel fuelled oast furnace, in a similar fashion to that traditionally used for hops. The lower humidity and rapid drying conditions prevented fungal proliferation. This type of drying appeared to warrant further evaluation. However, although the diesel used here is an acceptable fuel source for the drying of food-grade hops, this would not be acceptable for a medicinal crop. GAP Guideline 6.6 dictated that medicinal herb crops had to be dried naturally or using methane, propane or butane (EMEA, 2006). For future drying experiments, alternative GAP-compliant methods would be necessary.

A major advantage of growing outdoors is that it avoided the large energy cost incurred in the glasshouse (> 500 kW hr per kilo of dry botanical raw material) and consequently created a much smaller carbon footprint. At the start of this study it appeared that outdoor growing was feasible and, if disease and crop drying difficulties could be overcome, there were clear cost and environmental advantages. However, the effect of outdoor growing on cannabinoid and terpene profile remained untested.

6.2 AIM and OBJECTIVES

Trials were performed annually over four consecutive years. The overall aim was to enable the growth and harvest of good quality plants, with the same cannabinoid and terpene profile as glasshouse crops. This necessitated an improvement in the understanding of how outdoor growing affected plant development, secondary

metabolite production and susceptibility to pests and disease. Specific tests would then be needed with the objective of overcoming these problems and improving harvest and drying techniques. These objectives are described in specific details as follows.

6.2.1 The effect of growing environment on female plant development

Unlike the glasshouse crop, which routinely took eleven weeks to grow from a rooted cutting to fully mature plant, the outdoor grown plant took up to twice as long. As such, specific observations were performed to ascertain how the development of outdoor grown cloned all-female plants compared to that of a glasshouse crop and how optimum time for harvest could be visually judged.

6.2.2 Comparison of the secondary metabolite yield and profile of fresh plant material and enriched trichome preparations made from them

As shown in Chapter 4, enriched trichome preparations could be produced from glasshouse grown crops which captured most of the cannabinoid content of the raw plant material. These were less bulky materials from which essential oil samples could be steam-distilled to allow detailed terpene analysis. Trials were performed to compare the secondary metabolite profile of freshly harvested plant material and enriched trichome preparations. If the results indicated that glandular trichomes collected from fresh material had the same secondary metabolite profile as the intact plant material, this collection method might prove more efficient than harvesting, drying and then processing whole plants.

6.2.3 The effects of harvest timing on secondary metabolite yield and profile

One detailed trial aimed to assess how the cannabinoid and terpene yield and profile changed during the latter phases of inflorescence maturation. This would help ascertain the growth stage at which plants were most appropriately harvested to meet the preset specification.

6.2.4 Comparison of the secondary metabolite profiles of glasshouse and outdoor grown plants

In 2006 studies were performed to compare the terpenoid content and profile of glasshouse and field grown plants, during inflorescence maturation. The objective was to ascertain if and how secondary metabolite content was altered by growing conditions.

6.2.5 Evaluation of outdoor pest and disease issues

The trial program would aim to find out what major pest and disease problems might arise and assess their potential effect on plant quality and yield.

6.2.6 Evaluation of Crop Drying Methods

A range of drying methods was evaluated with two objectives. The first was to find a way of speeding the processing of a single seasonal harvest. The second was to assess how the drying method might be improved, to reduce fungal spoilage.

6.3 MATERIALS

Materials	Source
Seed for cannabis varieties G1, G5	HortaPharm BV Schimkelhavemkade 1075 VS Amsterdam Netherlands
Seed for cannabis variety G159	Undisclosed commercial source.
Propane gas heater and accompanying bottled gas.	Local hire. Location withheld for security reasons.
Diplex portable handheld thermometer/ hygrometer (calibrated in-house)	Diplex Ltd., PO Box 172, Watford England, WD17 1BX
Carbolyte drying oven	Carbolite - Parsons Lane, Hope, Hope Valley. S33 6RB UK

Table 6.1 Propagation Materials and Equipment used in the field trials program to evaluate the outdoor propagation of *Cannabis sativa* L.

The sieves, microscopes and analytical equipment were the same as those described in Chapters Two to Four.

6.4 GENERAL AGRONOMIC METHODS

6.4.1 Seedbed Location, Preparation and Crop Establishment

Being mindful of security concerns, and to satisfy Home Office license conditions, the crop was grown in a discrete high-walled garden. Seedbeds were prepared manually using a fork and rake and metaldehyde-based molluscide pellets applied to the soil to provide slug control.

Cuttings were raised as described in Chapter Five. These were placed outside the glasshouse for seven days to acclimatise to outdoor conditions. They were then planted in rows one metre apart at a density of four to six cuttings per square metre, as

found adequate in first year's trial. Predictably, variable weather and soil moisture conditions were encountered in the first weeks of growth and plants were watered by hand when insufficient soil moisture was naturally available.

6.4.2 Field Trial Design

In the first three years single 100 m^{-2} plots of clone M16 were established, and specific tests performed on this single clone. The 2006 trial compared the performance of the G5 CBD chemovar at five harvest timings. Seven plots of each regime were laid in a randomised block. A small area outside of the main trial was dedicated to some heterozygous cultivars producing approximately equal quantities of THC and CBD. All were derived from the single variety G159.

6.4.3 Soil Nutrition

All trials were given a light dressing of approximately 40kg/ha of fertilizer, containing 34.5% w/w nitrogen, before or soon after planting. However, no account was taken of residual fertilizer already existing in the soil. In all years lush growth was observed and no deficiency symptoms were observed.

6.4.4 Pest and Disease Monitoring and Management

In each season the trials were examined at least once per week throughout the season. Pests and disease were identified and their severity noted. At no stage were any chemical treatments applied. Rabbits caused extensive damage to a crop planted in 2001 and subsequent trials were surrounded with protective fencing. Weeds were a constant problem and were manually removed on a regular basis each year.

6.4.5 Harvest

In all cases plants were cut at the base below the lowest side-branch upon which any floral material was observed. Plants were placed on clean sheeting prior to relocation to an oast house for drying.

6.4.6 Crop Drying and Stripping

Crops were hung to dry on suspended wires in an oast house drying chamber. Propane gas burners provided hot air and fans ensured that this moved freely within the drying environment. Excess heat was allowed to escape through controlled ventilation ducts in the ceiling above the drying chamber. Thermostats within the drying chamber maintained drying conditions at the selected temperature, and this was monitored (in compliance with GAP Guideline 4.6) using the oast house environmental management recording system. Humidity was read with a hand-held meter.

In each of the three years 2003-2005, separate batches were dried in temperatures of 30º, 40º and 50ºC (± 2ºC). At the end of the drying period the relative humidity in these respective regimes was recorded as being approximately 30%, 20% and 10% respectively. Ten samples (approximately 1 g each) were taken at three-hourly intervals during the drying process. Their moisture content of these was determined by weighing the moisture loss when dried for 24 hours at 105ºC. Once judged by touch to be sufficiently dry, the leaf and floral material in the oast was stripped from the stems by hand. This material was relocated to a dehumidified store at 30-35% RH to equilibrate. It was expected that in this environment the samples would settle to a uniform predicted moisture content of 15 ± 2%, based on experience with glasshouse-grown crops.

6.4.7 Assessment of Crop Development

The 2005 field trial was visited weekly and the height of thirty randomly selected plants was measured. A typical plant would produce a dominant main inflorescence, and many shorter side branches. Plant height was taken as the height of the top of this central inflorescence as measured from the soil surface. In the following year's trial, subjective weekly assessments were made of the state of development of the inflorescence, from the appearance of the first stigmas through to complete stigma senescence. An assessment was also made of the overall proportion of senesced stigmas in each of the seven replicates. To compare the pattern of development of the field and glasshouse-grown plants, the same observations were made on the inflorescences of three consecutive crops of the same clone.

6.5 Secondary Metabolite Purification and Analytical Methods

6.5.1 Production and Collection of Enriched Trichome Preparations

Ten litres of crushed ice were placed in a fifty litre bucket and twenty litres of tap water added. Seven plants were collected at random from the field trial. Approximately ten litres of foliar and floral tissue were stripped from the plants. These were promptly plunged into the iced water and the temperature allowed to stabilize at below 1ºC. The mixture was agitated with a food mixer at maximum speed for ten minutes. The resultant slurry was then poured through 220 µm and 25 µm 'Bubblebag' sieves. The material settling on the 25µm sieve was placed in a 100ml Duran bottle and moved to a refrigerator at 4ºC. The procedure was repeated until all the material from the seven plants had been processed. A subsample was examined, using a Brunel MX3 binocular microscope, to check the efficiency of the process. Over 90% of the solid material present was judged to be glandular trichome resin heads, the remaining proportion

consisting of capitate trichome stalks, cystolythic trichomes and fragments of mesophyl tissue. The ETP was then relocated to a deepfreeze at -20ºC prior to analysis.

6.5.2 Steam distillation of trichome rich preparations

Samples were distilled at Botanix Limited, Hop Pocket Lane, Paddock Wood, Kent, UK according to the company's Standard Operating Procedure for the Determination of Volatile Hop Oil (Institute of Brewing Method). In compliance with this method samples were sufficiently thawed within their sealed Duran 200ml bottles to enable the contents to be decanted into individual one-litre round bottom flasks with a B55 neck. A few anti-bumping granules were added to the flasks before connecting each one to a British Pharmacopoeia still, using a B55/34 adaptor. The flasks were then heated using a heating mantle, and the contents distilled for three hours. Using this method Howard (1970) showed that the extraction of essential oils from hop lupulin (glandular trichomes) was reliably complete in this time. During this period the flow of condensate was controlled to cause minimum disturbance to the oil in the trap. At the end of three hours the volume of oil was recorded and then decanted for analysis by GC (Appendix 2).

6.5.3 Steam distillation fresh foliar and floral material

Two kilograms of each sample of fresh material and five litres of water were placed in ten litre round bottomed flasks and a few anti-bumping added. The flasks were then connected to the still using a B55/34 adaptor. The mixture was boiled for two hours, after which time no further volatiles were seen to be condensing within the stills. During this period the flow of condensate was controlled to cause minimum disturbance to the oil in the trap. At the end of two hours the volume of oil was recorded and decanted. These oil fractions were analysed for qualitative terpene content by GC at Botanix Ltd. The quantitative content of a small range of terpenes within this mixture was determined by GC at GW Pharmaceuticals Ltd (Appendix 2).

6.6 Statistical methods

Standard deviation, analyses of variance, and regression calculations were performed using Microsoft Excel 2003 software.

6.7 RESULTS and DISCUSSION

6.7.1 Observations on Crop Establishment and Plant Development

Crops were successfully harvested over five seasons. A summary of planting dates, flowering dates and yields are shown in Table 6.2. Planting dates ranged from 12th

May to 25th June and yields varied between 451 and 728 gm-2. No clear correlation was observed between planting date and final yield. However, yields data needed to be made with caution as the trials differed in their location, planting density, soil fertility and harvest dates and as expected weather conditions varied between seasons (Appendix 4). To put the observed yields into perspective, clinical trials with Sativex® indicated that when patients were able to self-titrate, for the symptoms of MS and neuropathic pain, the mean daily consumption of CBD was 25 – 40 mg day-1 (9 - 14 g year -1) in combination with a similar dose of THC (Wade *et al*., 2004, Barnes, 2006). This suggests that one square metre of the CBD-chemovar could have provided sufficient feedstock for one patient for one year (not allowing for losses during extraction and formulation). If approved as a prescription-only medicine, the entire UK demand of CBD for this medicine could theoretically have been met by a crop area of just a few hectares.

Year	2000*	2003	2004	2005	2006
Cultivar(s)	M11	M16	M16	M16	M16
Planting Date	12th May	11th Jun	25th Jun	23rd Jun	23rdJun
First Flowers	No record	1st Sep	22nd Aug	25th Aug	22nd Aug
Harvest	6th Oct	8th Oct	9th Oct	10th Oct	17th Sep to 15th Oct*
BRM g/sq m	502	598	451	728	609*
CBD % w/w	6.0	8.8	_	7.0	7.0*
CBD g/sq m	30	53	_	50	33

Table 6.2 Summary of Agronomic and Yield Data from field trials performed between 2000 and 2006. *The 2000 trial was performed before commencement of this thesis, and the data is included for comparison.

In all the field trials, when grown from cuttings, the CBD crop gained little in height in the first four weeks after transplanting. Mid-June planting appeared to be sufficiently early for good crop establishment and no obvious benefits were observed in planting in May. In each year, irrespective of weather conditions and planting date, the maximum rate of vegetative growth was observed in the period mid-July to end August. This is

demonstrated in Figure 6.1, which shows the results of the weekly monitoring plant development in the 2006 trial. The sigmoidal growth curve is similar to that reported in a monecious hemp crop grown from seed (Bócsa and Karus, 1998), although flowering occurred much earlier in hemp as the variety had been bred to mature earlier in the season.

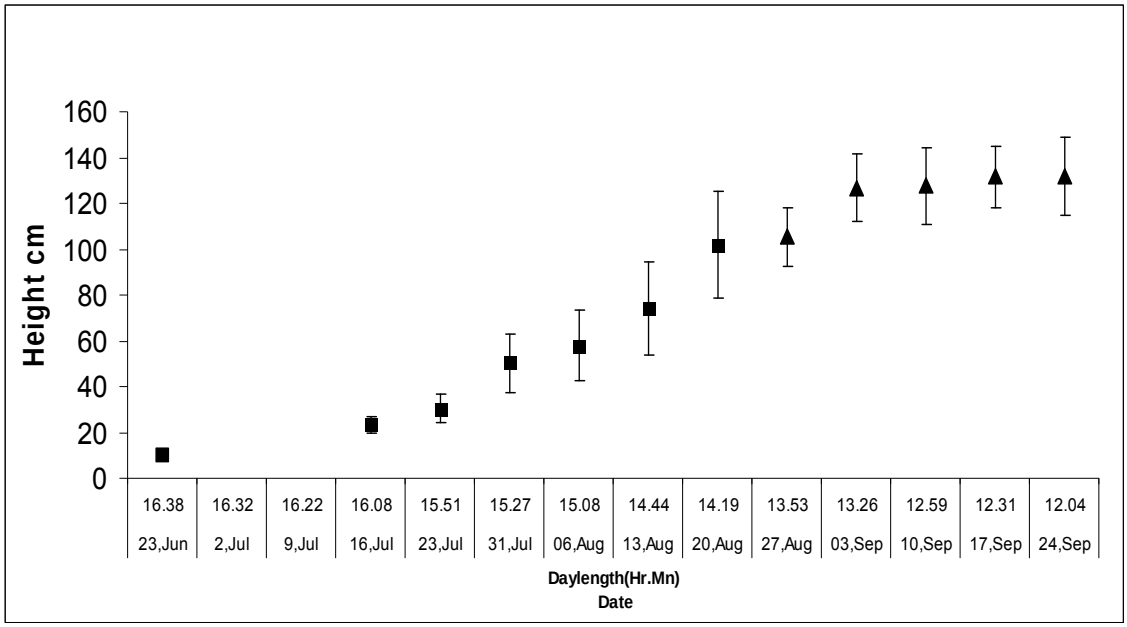

Figure 6.1 The mean height (± SD) of G5 M16 crop as observed at weekly intervals in 2005. Thirty plants were measured on each occasion. Data points are shown as square symbols during the establishment and vegetative phase. Data points are shown as triangle during the flowering (generative) phase.

Observed during 2003-2006, flowering always commenced between 22nd August and 1st September (Table 6.2). As with the glasshouse crop, the increase in height almost totally ceased seven to fourteen days after the appearance of the first stigmas. The final height of the crop varied from year to year, being clearly influenced by the growing conditions and soil nutrition. In 2005 the final crop height was 1.3 metres but the same clones grown in the same location in 2006 reached 2.1 metres. This was likely due to marked difference in mid-summer temperatures and sunshine-hours (Appendix 3) experienced, the conditions in Jun and July being markedly brighter and warmer. In four trials CBD yield was measured. Yields varied from 30 to 53 gm^{-2}. However, none of the trials were designed with the aim of achieving the maximum possible yield.

In 2006 floral development was observed in detail. Table 6.3 describes the stage of inflorescence development and stigma senescence during the flowering period, as recorded weekly between August 14th and October 8th. The table also compares the

pattern of development with three consecutive crops of the same cultivar when grown in the glasshouse. As reported in Chapter 5, the proportion of senesced stigmas within the inflorescence gives the most useful measure of the state of maturity. Figure 6.2 compares the pattern, speed and duration of inflorescence development and maturation and showed that outdoor and indoor crops of variety G5 exhibited a very similar pattern. This was despite the very different day lengths and growing conditions. In each year's trial, floral development was rapid from mid-September into the first days of October, when development would cease. This cessation of growth was recorded in some detail in the 2006 trial. Stigma senescence was observed to have commenced just before 23rd September and to have been almost complete fourteen days later.

The 2006 outdoor crop yielded well, despite the fact that mean temperatures were approximately 8°C lower (16.9°C – 17.3°C, Appendix 3) than the temperatures in the glasshouse (25°C) during the August – September flowering period. Measured ten kilometres away, at the glasshouse site, daily total radiation levels in autumn 2006 were close to those predicted for this latitude using a UK global radiation algorithm (Hamer, 1999). This showed total daily radiation falling from 6.8 to 3.5 MJ m^{-2} d^{-1} over the flowering period. This was slightly above that encountered by a glasshouse production crop, which encountered a winter minimum 3.4 MJ m^{-2} d^{-1} rising to a summer maximum 5.8 MJ m^{-2} d^{-1}. Even though the indoor crop received supplementary lighting, the glasshouse roof structure reduced the light transmission levels by approximately a third. The glasshouse crop also experienced a shorter period of daylength (12 hours) throughout the flowering period, whereas the field crop experience longer daylengths during the first weeks of flowering. The higher radiation level at the commencement of outdoor flowering may be especially pertinent. Glasshouse studies (Chapter 5) showed that the greatest cannabinoid yields were achieved in crops harvested in August. These had encountered the brightest light conditions of the summer solstice during the very early phase of floral development.

Observation Timing and Pertaining Daylength				Development stage of both indoor and outdoor crops.	
Indoor Crop		Outdoor crop			
Week in short days	Day length (Hr.mn)	Date observed	Day Length (Hr.mn)	Inflorescence Development	Stigma Development
0	12.00	13th Aug	14.40	Florets absent	Stigmas absent
1	12.00	20th Aug	14.16	First florets formed	First stigmas visible
2	12.00	27th Aug	13.46	Rapid formation of new florets	Fertile stigmas common
3	12.00	3rd Sep	13.23	Rapid formation of new florets	Prolific stigma formation
4	12.00	10th Sep	12.56	Rapid formation of new florets	Prolific stigma formation
5	12.00	17th Sep	12.28	Floret formation slows	Fewer fertile stigmas observed. Older stigmas senesced.
6	12.00	24th Sep	12.01	Development almost ceased	Rapid stigma senescence
7	12.00	1st Oct	11.34	Development ceased	Senescence near complete
8	12.00	8th Oct	11.11	Development ceased	Senescence complete

Table 6.3 A comparison of the pattern of inflorescence development and stigma senescence in indoor and outdoor crops of cannabis chemovar G5. The stages of development of the glasshouse crop are shown from the point at which the plants are moved into a 12hour light/12hour dark until they are routinely harvested eight weeks later. The field crop development is shown from mid-August, just before stigma formation commenced.

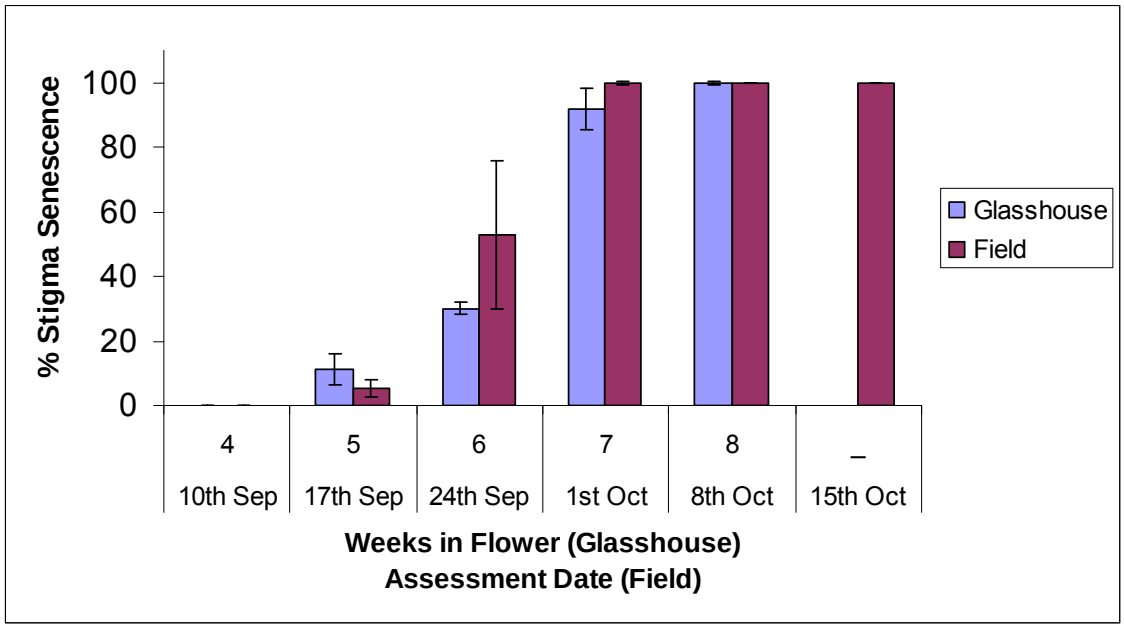

Figure 6.2 A comparison of the pattern of stigma senescence (%, ± SD, n = 7) in 2006 field trial plants between 10th September and 15th October with that observed in five consecutively grown routine indoor crops of the same variety (± SD, n = 5).

6.7.2 Comparison of the secondary metabolite yield and profile of fresh plant materials and enriched trichome preparations made from them

The 2005 trial incorporated a study to compare the essential oil profile of steam distilled fresh mature cannabis and steam distilled ETP made from the same material. An essential oil fraction, produced by steam distilling foliar and floral material from ten plants taken at random from the crop on October 4th, yielded 7.7 ml m^{-2} (7.0 g m^{-2}) The approximate CBD potency of plants analysed from the same crop was 7% w/w, indicating a CBD yield of 50 g m^{-2} (500 kg ha^{-1}). It is useful to note that the essential oil:CBD ratio was therefore approximately 1:7 (w/w).

The previous day (Oct 3rd) ETP was made from fresh G5 M16 plant material collected from the same field trial. A similar batch was made from glasshouse plants at the same state of maturity. These were immediately frozen and steam distilled alongside the fresh plant material. The essential oils produced were analysed by GC at Botanix Ltd. The results are shown in Table 6.4. The very similar terpene profiles of the fresh plant material, and the ETP made from it, suggested that the complete range of monoterpenes and sesquiterpenes had been equally well captured during the making of the enriched trichome preparation. If the results of this single test were to be routinely replicated, this would indicate that the terpene profile of a fresh cannabis sample could be fairly assessed by studying an ETP made from that material. ETPs

made in this way are much more conveniently stored pending analysis. Being more concentrated they enable more minor constituents to be quantified, as demonstrated by trans-nerolidol in this test. Steam distillation further concentrates these terpenes.

	Field		Glasshouse
	Fresh Leaf and Flower	Enriched Trichome Preparation	Enriched Trichome Preparation
α-pinene	7.7	7.5	9.9
β-pinene	4.6	4.4	4.2
Myrcene	53.2	41.9	38.4
Limonene	6.9	7.9	8.3
β-ocimene	13.4	13.0	4.7
Linalol	1.5	1.1	2.6
α caryophyllene	2.6	4.3	5.4
β caryophyllene	10.3	17.0	22.7
Trans-Nerolidol	<1.0	3.1	1.4

Table 6.4 A comparison of the terpene profile of freshly harvested fully mature field grown cannabis leaf and flower material (cultivar G5 M16) and ETP made from the same fresh material (2005 Field Trial). Also included is ETP made from similar mature glasshouse grown material. The data show the relative peak areas when assessed by GC at Botanix Ltd. In each case one batch of material was analysed.

The terpene profiles were seen to be dominated by the monoterpene myrcene and the sesquiterpene trans-caryophyllene. Together they accounted for at least 60% of the total peak area. The limited data looking at the cannabinoid, myrcene and trans-caryophyllene ratios of cannabis inflorescences, and the enriched trichome preparations made from them, indicated that the ratios were maintained during the making of these preparations (Table 6.4). If enriched trichome preparations could be made in bulk from freshly harvested field grown crops, they would avoid the requirement for crop drying. They would also provide a greatly enriched feedstock for drug production. A similar process is sometimes adopted in the hop processing industry. Hop glandular trichomes (known as lupulin) are collected by agitating and sieving deep-frozen material.

It is interesting to note that the essential oil yield of 7.7 ml m^{-2} achieved here is much higher than the 1.8 ml m^{-2} previously recorded from unpollinated *Cannabis sativa* (Meier and Mediavilla, 1998).

6.7.3 The effects of harvest timing and growth stage on yield and cannabinoid profile

6.7.3.1 Botanical Raw Material Yield

G5 was believed to be a uniquely high-yielding high-CBD chemovar, derived from Turkish parents naturalised at 41º N. When grown in the UK at 51º N this commenced flowering in the fourth week of August, in response to a phytochrome-mediated hormone switch, when the day-length was about 14 hrs:30 mns. At 41º N, this day-length would have occurred in the last week of July. This suggests that, if grown in Turkey, flowering would have commenced three weeks earlier. However, by the last week of September, the day-length would have been 12 h in both locations. Research findings described in Chapter 5 indicated that phytochrome-controlled systems also appear to induce the cessation of floral development. Floral development would therefore have probably ceased in both locations around the time of the autumn equinox at the end of September. Outdoor growing of this variety might be expected to be more successful if G5 production was moved closer to 41º N. The flowering period would be extended and conditions would likely be less conducive to the proliferation of *Botrytis*. However, the logistics of importing the crop would add to licensing difficulties and costs.

In the 2006 trial, where five harvesting dates were compared (Figure 6.3), there was no significant difference in the yields of raw material harvested weekly between 17th September and 15th October (ANOVA). This appeared to be at least in part due to early-harvested crops having a higher proportion of foliar material. Later-harvested crops had a higher proportion of floral material but crop weight did not show a corresponding increase. Much of the foliage in the older crops had senesced and abscised by the time of harvest. It is likely that many of the primary metabolites within the older foliage had been translocated to the developing inflorescences in the last weeks of growth.

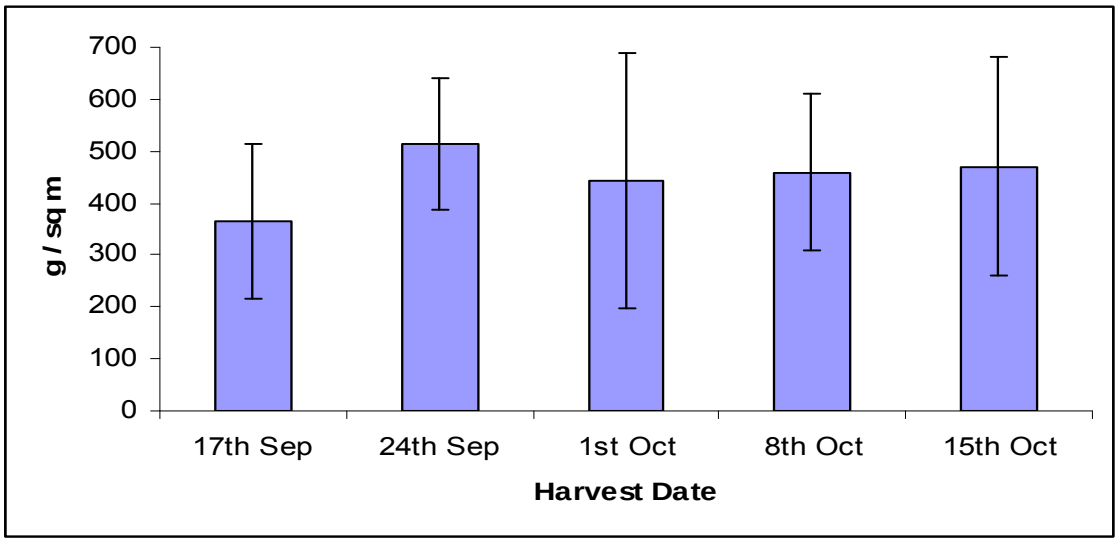

Figure 6.3 Yield of Botanical Raw Material in the 2006 trial showing the effect of planting date and harvest date. The results are the mean dry weights (gm^{-2} ± SD) harvested from seven replicates.

6.7.3.2 CBD Concentration

Data comparing the CBD content of crops harvested between 2000 and 2005 showed a range of approximately 6-9% w/w. The 2006 trial studied the effect of harvest date on potency, and there was a clear linear upward trend from an initial content of 2.3% to 7.0% w/w (Figure 6.4). Analyses of variance showed highly significant weekly increases in potency between 24th September and 8th October ($p < 0.01$). A regression calculation, examining the correlation between days in flower and % CBD content throughout the five assessments gave a highly significant upward linear trend, $p = 0.0028$, $R^2 = 0.983$.

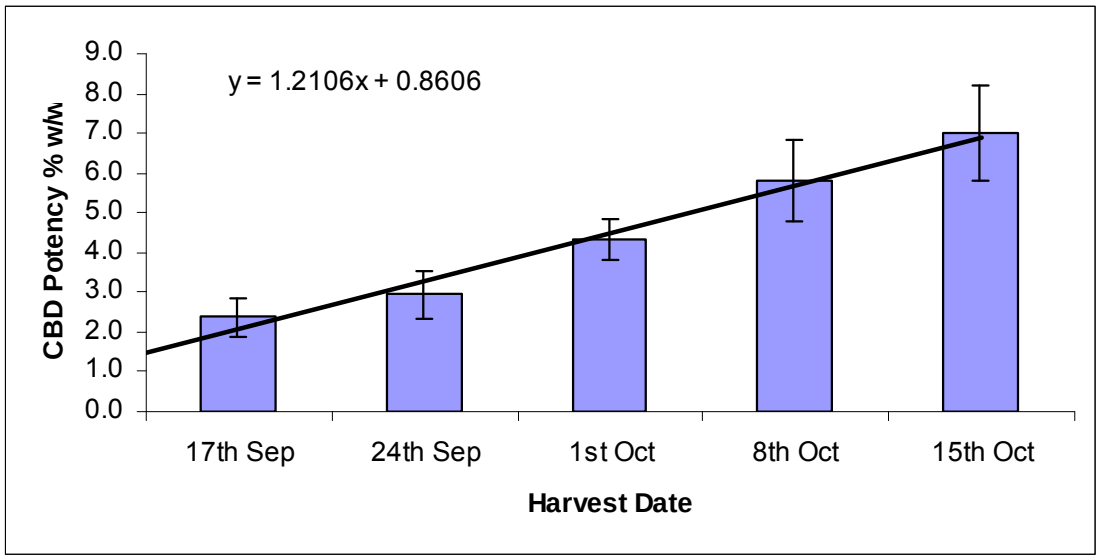

Figure 6.4 The effect of harvest date on cannabidiol concentration in Botanical Raw Material in the 2006 trial. The results are the mean % CBD (± SD) content of samples from each of the seven replicates, as measured by GC. Regression model, n = 7, $p = 0.0028$, $R^2 = 0.983$.

6.7.3.3 CBD Yield

The yield of CBD, expressed as gm^{-2}, is shown in Figure 6.5. A highly significant linear upward trend is observed from $8gm^{-2}$ on 17th September to $33g\ m^{-2}$ on October 15th. (Regression p = 0.0011, $R^2 = 0.994$).

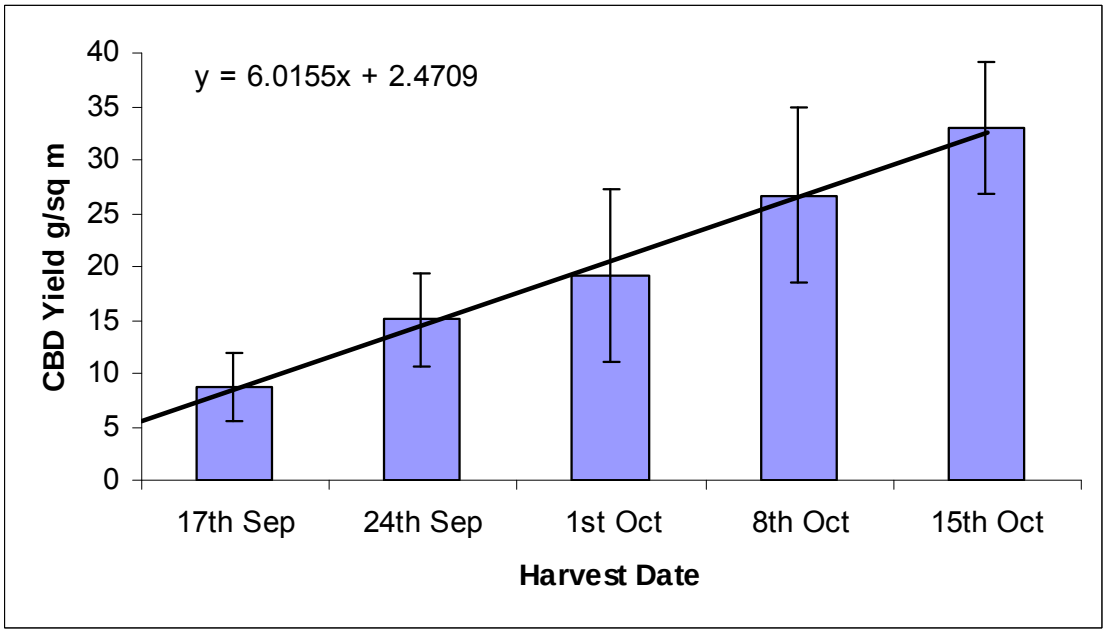

Figure 6.5 The yield of CBD in the 2006 trial, showing the effect of harvest date. The results are the mean CBD yields (gm^{-2} ± SD) produced in each of the seven replicates. Regression model, $p = 0.0011$, $R^2 = 0.994$

Despite there being no clear increase in the dry matter yield from the mid-flowering phase onwards, the 2006 trial showed cannabinoid yield increasing significantly in the first two weeks of October (ANOVA, $p < 0.01$). Of the acetyl CoA, NADPH and ATP generated by photosynthesis during this phase of growth, the plant appeared to be diverting a greater proportion to secondary metabolite biosynthesis. Part of the slowing in biomass increase during the flowering period is possibly due to the higher energy requirement for the production of floral tissue. Hemp crops were calculated to have a radiation efficiency of between 2.0 and 2.3 g/MJ PAR for vegetative growth, reducing to between 0.6–1.2 g/MJ PAR after flowering (Struik *et al*., 2000). The biosynthesis of the cannabinoids and terpenes would have a high energy requirement per unit weight. The energy required to biosynthesise the monoterpenes myrcene, pinene and limonene ($C_{10}H_{16}$) has been calculated to be a factor of 3.5 greater than that required for the same weight of glucose (Gershenzon, 1994). Although the energy requirement for CBDA and THCA biosynthesis is not known, metabolites with a similar C:H:O ratio have an slightly lower energy cost than the monoterpenes, due to the lower state of reduction. Additional energy would be utilized in the formation of the complex trichome structure, which contains cellulose, cutin and a variety of enzyme proteins. Secondary metabolites formed in the last days before harvest may have been increasingly synthesised using metabolites released and translocated from older rapidly senescing tissue.

Although the cannabinoid yield showed a continuous and significant increase up until the final harvest date in mid-October ($p = 0.0011$), the quality of the material deteriorated markedly as a result of fungal infection. Removal of all substandard material would have negated much of this yield increase.

6.7.3.4. Effect of Harvest Date and Growth Stage at Harvest on Cannabinoid Profile

Analysis of the BRM by GC from the earliest harvest timings showed CBD to be the only cannabinoid present in detectable quantities in plants of the G5 chemovar. Meaningful comparison of cannabinoid profiles was only achievable by studying the content of the much more potent enriched trichome preparations made at each harvest-timing. CBD, THC and CBG were present in measurable quantities in all samples. The ratios of these are shown in Table 6.5.

Harvest Date 2006	17th Sep	24th Sep	1st Oct	8th Oct	15th Oct
CBG as % of CBG+CBD (± SD)	5.61 ± 0.11	5.31 ± 0.12	3.30 ± 0.18	2.31 ± 0.12	1.59 ± 0.01
THC as % of THC+CBD (± SD)	4.35 ± 0.20	4.27 ± 0.13	4.08 ± 0.11	3.93 ± 0.04	3.85 ± 0.01

Table 6.5 The changing proportions of CBG and THC with respect to CBD (Mean ± sd) in enriched trichome preparations produced from plants harvested at weekly intervals between 17th September and 15th October. Just one ETP sample was made on each date after bulking together one plant from each of the seven replicates. Three subsamples of each preparation were analysed.

Results showed the importance of harvesting the crop at the correct time to achieve the desired cannabinoid profile. Table 6.5 showed that the CBG:CBD ratio fell rapidly during the last weeks of growth, the CBG proportion of the CBG + CBD total decreasing steadily from 5.6% on September 17th to 1.6% on October 15th. The final value was close to that typically found in glasshouse crops at harvest. Forty seven consecutively processed batches of glasshouse-grown high-CBD chemovar were analysed as part of the routine growing operation. The mean proportion of CBG in the CBG + CBD total was 1.78% ± 0.21% SD. CBG is a cannabinoid with known pharmacological activity (Formukong *et al.,* 1988). Excessive quantities of this minor cannabinoid in a CBD-based medicine would result in batches of feedstock being rejected as having an unacceptable specification. To achieve the same low CBG:CBD ratio as the glasshouse crop, the results of the 2006 trial suggested that the outdoor harvest would have had to have been delayed until mid-October. However, *Botrytis* rendered the quality of this crop unacceptable before this date. The THC:CBD ratio also showed a downward but less pronounced trend over the harvest period. In the above-mentioned batches of glasshouse-grown CBD chemovar the mean proportion of THC, expressed as a percentage of the THC+CBD total, was 3.89 ± 0.16% SD (n = 47). This is very close to the ratio found in the field grown plants in the later harvests (3.85 ± 0.01, Table 6.5). Homozygous high-CBD chemovar plants like G5 produce only CBD synthase and no THC synthase (de Meijer *et al*., 2003). CBD synthase however always appears to produce a small amount of THC, as well as CBD. It is postulated that CBD synthase is present within CBD chemotypes in more than one isoform, and these variably affect the final THC:CBD ratio. The ratio of these two cannabinoids in commercial hemp varieties is reported to be typically around 1:20 (Mechtler *et al.,* 2004, Hillig and Mahlberg, 2004). The parental lines used in the breeding of G5 were selected on the basis of several characteristics including a

THC:CBD ratio as low as possible. As a consequence, the ratio in G5 plants was lower than most commercial hemp varieties (deMeijer, pers comm), decreasing from 1:23 in the early harvest to 1:26 over subsequent weeks (tentative n = 1 data only). This apparent decrease in later harvested plants was possibly due to isoforms of CBDA synthase being present which differed in their ability to function in the increasingly autumnal growing conditions. However despite this, as the CBD content rose to 5.8 % and 7.0 % w/w in the final weeks (Figure 6.4), the corresponding THC content rose to 0.23 % and 0.27 %.

As designated under the Misuse of Drugs Act *Cannabis sativa* can be licensed only for research or "other special purposes" providing the Secretary of State is of the opinion that it is in the public interest to do so. The only "special purpose" currently recognised is the cultivation of EU-approved varieties of hemp for commercial and industrial purposes. All such EU-approved varieties have THC contents below 0.2%. When this program of field trials commenced, the maximum THC content permitted in floral samples of licensed hemp crops was 0.3% w/w. This was subsequently reduced by the European Union (EU regulations (VO (EG) Nr. 1420/98)) to 0.2%. Although a minimum content of 0.3% was readily achievable by chemovar G5, the lower limit of 0.2% may be too low for the G5 chemovar to qualify for an EU subsidy payment, and this would have a small effect on the cost of outdoor growing. Growing a variety containing more than 0.2% THC is not illegal, but does necessitate a dispensation from the Home Office Licensing. Enquiries to the Home Office (Drugs Licensing) indicate that such an application would be met favourably (Evans, 2008).

6.7.4 Effect of Growth Stage and Harvest Date on Essential Oil Profile

The 2006 trial compared of the terpene profiles of field grown cannabis plants harvested at weekly intervals between 17th September and 15th October. This was achieved by first steam distilling an enriched trichome preparation made from fresh botanical raw material collected on each date. Between 30 ml and 100ml of enriched trichome preparation was produced from each, but this contained a large unmeasured volume of residual water. It is not possible to quantify the essential oil concentration of each sample on the basis of the non-aqueous fraction only. When distilled these produced between 0.25 ml and 2.1 ml of essential oil and each was analysed by GW Pharmaceuticals using GC. The relative peak areas for the twenty most abundant terpenes detected are shown in Table 6.6. These twenty accounted for more than 97% of the total peak areas. The monoterpene:sesquiterpene ratio of each of the essential oil samples showed a weekly increase. A regression calculation showed the weekly linear increase in the proportion of monoterpenes to be strongly correlated with time (p

= 0.0052, R^2 = 0.973, y = 0.844x + 0.6219). All individual sesquiterpenes, of which trans-caryophylene was the most dominant, showed a decreasing presence in the overall terpenoids mixture.

The data show that of the monoterpenes, myrcene was by far the most dominant. It was notable that when expressed as a % of the total peak area, the proportion of myrcene more than doubled over the sampling period. Limonene and beta-ocimene also showed clear though less dramatic increases. The pinenes conversely showed a possible downward trend.

	Harvest Date 2006				
	17th Sep	24th Sep	1st Oct	8th Oct	15th Oct
	% of Peak Area				
Monoterpenes					
Alpha-pinene R	3.49	5.27	4.25	3.13	3.24
Alpha-pinene S	4.30	2.35	3.63	3.78	3.00
Beta-pinene	3.32	3.50	3.33	3.18	3.00
Beta-Myrcene	22.63	36.30	40.86	46.02	51.48
Limonene	2.75	3.91	4.31	4.68	5.07
Beta-ocimene	2.64	4.13	4.04	4.63	4.76
Sesquiterpenes					
t-Caryophyllene	37.44	29.10	26.57	23.97	20.17
Bergotamene	0.34	0.18	0.16	0.12	0.08
Humulene	11.31	8.57	7.60	6.71	5.67
Aromadendrene	0.67	0.34	0.31	0.19	0.14
Selinene	1.17	0.63	0.52	0.36	0.29
Anon	0.84	0.46	0.41	0.26	0.21
Z,E Farnesene	0.18	0.09	0.08	0.06	0.07
alpha farnesene	1.70	1.77	1.44	1.25	1.11
alpha Gurjunene	0.17	0.15	0.11	0.09	0.07
Bisabolene	0.78	0.42	0.37	0.22	0.16
Nerolidol	0.66	0.62	0.38	0.26	0.23
Caryophyllene Oxide	4.13	1.70	1.29	0.91	1.05
Diepicedrene-1-oxide	0.89	0.38	0.28	0.17	0.19
Alpha-Bisabolol	0.59	0.12	0.07	0.02	0.02
Total Monoterpenes	39.12	55.46	60.41	65.42	70.55
Total Sesquiterpenes	60.88	44.55	39.59	34.58	29.45

Table 6.6 A comparison of the terpene profile of steam-distillates of enriched trichome preparations made from freshly harvested plants on five dates between 17th September and 15th October 2006. One bulked sample was analysed on each date.

Although the relative peak area data in Table 6.6 show very clear changing ratios between individual terpenes, and between the overall ratios of monoterpenes to sesquiterpenes, the true content of each of the twenty terpenes could not be accurately gained from this data. Quantitive data was generated for eight terpenes for which analytical standards were readily available. The results are shown in Table 6.7.

Harvest Date	17th Sep	24th Sep	1st Oct	8th Oct	15th Oct
α-Pinene	9.18	8.44	7.82	7.09	6.18
β-Myrcene	40.45	58.16	61.00	70.01	74.30
Limonene	2.89	2.61	3.84	2.82	2.84
Linalool	0.40	0.60	0.35	0.13	0.26
trans-Caryophyllene	32.36	21.60	19.52	15.37	12.68
α-caryophyllene	9.18	5.93	5.27	4.02	3.30
Caryophyllene Oxide	4.14	1.52	1.46	0.02	0.03
t-Nerolidol	1.39	1.13	0.74	0.53	0.41
Ratio Myrcene/ t-caryophyllene	1.25	2.69	3.13	4.55	5.86

Table 6.7 Ratio of eight terpenes in steam distilled enriched trichome preparations made from freshly harvested field grown plants of cultivar G5 M16. The result for each terpene is expressed as a weight percentage (% w/w) of the total within each column. The table also shows the ratio of myrcene (the dominant monoterpene) and trans-caryophyllene (the dominant sesquiterpene).

The qualitative peak area data in Table 6.6 gives a valuable though not accurate measure of the ratios between the individual terpenes. Comparing quantitative data, as in Table 6.7, is more accurate but limited by the availability of reference standards. The two analytical methods are compared in Table 6.8, which shows the myrcene/trans-caryophyllene ratios when calculated from Relative Peak Area and quantitative w/w data. Each is expressed as a % of the October 15th value. Whether determined from the peak area data or the w/w data, the 'normalised' values give very similar results with the myrcene/trans-caryophyllene ratio doubling between September 24th and October 15th.

Harvest Date	17th Sep	24th Sep	1st Oct	8th Oct	15th Oct
	Myrcene/t-caryophylene				
	Actual Value				
Peak Area	0.60	1.25	1.54	1.92	2.55
w/w	1.25	2.69	3.13	4.55	5.86
	Normalized Value Shown as % of 15th Oct Value				
Peak Area	24	49	60	75	100
w/w	21	46	53	78	100

Table 6.8 Comparison of myrcene/trans-caryophyllene ratios when calculated from Relative Peak Areavalues and w/w data.

6.7.5 Comparison of the secondary metabolite content of glasshouse and outdoor grown plants

As reported above in Section 6.7.6, a single field trial suggested that the THC:CBD ratio of late harvested G5 CBD-chemovar plants was similar if plants were grown in either environment. A more dramatic effect of outdoor growing was demonstrated by five heterozygous B_TB_D genotypes derived from variety G159. These produced a significantly lower mean THC:CBD ratio when grown outdoors (two-tailed t-test, $p < 0.001$, Table 6.9).

	THC as % of THC+CBD (n = 4)					
Clone	G159/9	G159/11	G159/12	G159/13	G159/16	Mean
Field	33.1 ± 2.4	33.2 ± 0.2	48.9 ± 1.9	33.9 ± 0.0	33.3 ± 0.1	36.5
Glasshouse	38.8 ± 1.5	39.9 ± 0.6	57.4 ± 0.9	40.6 ± 0.6	38.6 ± 2.0	43.1

Table 6.9 The relative proportions of THC and CBD synthesised in heterozygous B_TB_D clones derived from variety G159. The proportion of THC produced is shown as a % of the THC+CBD total. Just one sample of dry inflorescence material was analysed from each plant.

In a separate study not reported here, where these clones were grown in a growth room in identical conditions apart from contrasting growth temperature of 15ºC and 25ºC, the cooler temperature significantly increased the proportion of CBD within the cannabinoid profile. A possible explanation is that in cooler conditions the CBD synthase within these plants is able to compete more favourably than THC synthase for the common precursor CBGA. No previous reports could be found describing this temperature effect. As THC synthase and CBD synthase have been suggested to exist

in more than one isoform (de Meijer, 2003), other heterozygous mixed THC/CBD chemotypes might exist which synthesise different ratios of one or both of these synthases. These in turn may synthesise differently to temperature. It is also possible that the increased proportion of CBD observed here is specifically induced by the activation of an unidentified gene. In either case, the ability to produce proportionally more CBD in cooler conditions may be an inherited trait which has at some point improved the survival ability of *Cannabis sativa*.

Changing temperatures have been found to alter the ratios of terpenoids in glandular trichomes of other species e.g. *Pelargonium xhortorum*. Walters and Harman (1991) showed that growing temperature affected the ratio of C_{22} and C_{24} unsaturated anacardic acids, both of which have similar molecular weights to the cannabinoids. It was postulated that plants may be exhibiting a genetically controlled response to temperature by altering the proportion of less viscous secondary metabolites within the trichome, to maintain uniform viscosity. A potential benefit of doing so would be the retention of the trichome's ability to ensnare insects. Although the effects of cannabinoid profile on trichome viscosity have not been tested, this explanation seemed unlikely in cannabis. At room temperatures THCA is an oil and CBDA is a crystalline solid. To retain stable viscosity as temperatures fell, it seems probable that the trichome contents would require proportionally more of the oil. Observations in this study demonstrated that the opposite had occurred. The cannabinoid profile may have been affected by the higher proportion of UV light encountered outdoors. This part of the spectrum is less able to penetrate a glasshouse and the amount produced by HPS lamps is negligible. However, contrary to the proportional decrease in THC synthesis observed in this outdoor crop, Lydon *et al*. (1987) showed that UV_B radiation significantly increased THC biosynthesis, whereas CBD biosynthesis did not show a significant increase. However, Lydon *et al.* (1987) did not show the actual CBD data and did not include a test of the statistical significance of the difference in biosynthesis of THC and CBD when exposed to increasing UV_B radiation.

To facilitate the comparison of the terpene profile of indoor and outdoor grown plants of cultivar G5 M16, the mean peak area for each of the terpenes was calculated with each of the five sampling dates being regarded as a single replicate. The results are shown in Figure 6.6. To allow a clearer comparison between the contents of both major and minor terpenes, the results are expressed as % of Total Peak Area using a logarithmic scale. The terpene profile of these indoor and outdoor grown plants was seen to be very similar, with no significant difference in mean % peak area in eight of the fourteen terpenes ($p > 0.05$). Being based upon one indoor and one outdoor crop the relevance

of these differences has to be viewed with caution. The terpenes showing the greatest significant difference in content were ocimene and nerilidol, which had proportionally lower contents in outdoor-grown plants. This may have been a result of higher UV light conditions outdoors. UV_B radiation has been found to alter terpene profiles in other species e.g. basil *Ocimum basilicum* (Johnson *et al.,* 1999), thereby affecting the flavour and commercial value of this culinary herb, but no obvious survival advantage was attributed to this terpene profile change. The reason for the altered terpene profile in outdoor grown cannabis plants may alternatively be a phytochrome-mediated response, as observed in outdoor grown thyme, *Thymus vulgaris* (Tanaka *et al.,* 1989). Many of the terpenes found in glandular trichomes have been found to be repellant to specific insects, and there may be an inherited benefit in only biosynthesizing certain secondary metabolites to coincide with the life-cycle of a major predator.

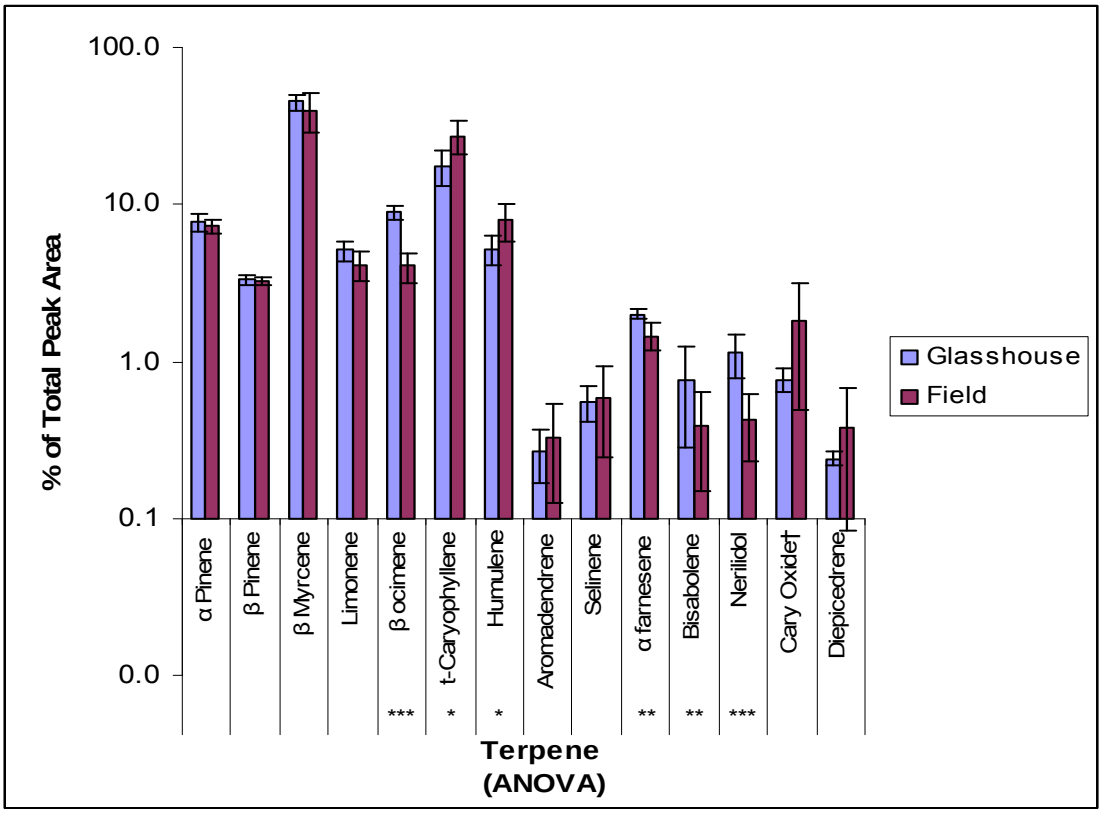

Figure 6.6 A comparison of the terpene profiles, as a proportion of the total peak area, of enriched trichome preparations prepared from glasshouse and outdoor crops († = Caryophyllene Oxide). The results are the mean of five samples produced at weekly intervals towards the end of flowering (± SD). Glasshouse plants had been in a 12 hour day length for 6 to 10 weeks. Field grown crops were sampled between September 17th and October 15th. (ANOVA, Glasshouse v Field, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

6.7.6 Summary of Pest and Disease Problems in the Field Trials

The main pest and disease problems incurred are summarised in decreasing order of severity in Table 6.10.

	Severity Score 1-5 (1-absent, 2-minor, 3-moderate, 4-severe, 5-fatal)				
Year: - / Pest	2000	2003	2004	2005	2006
Grey Mould *Botrytis cinerea*	1	3	3	3	1-5*
Caterpillars (Various spp)	2	3	3	3	2
Stemphylium Leaf Spot	1	1	1	1	3
Common Nettle Capsid † *Liocoris tripustulatus*	-	-	-	2	2
Leaf Miner *Liriomyza strigata*	1	1	1	1	2
Powdery Mildew *Sphaerotheca macularis*	1	1	1	1	2
Black Bean Aphid *Aphis fabae*	1	1	1	1	1‡

Table 6.10 Summary of pest problems experienced, in decreasing order of magnitude using a subjective 1-5 score. * Botrytis initially minor but extremely severe in late harvested plots. † Symptoms not recognized as pest damage until 2005. ‡ Aphids absent in trial plots but moderate infestation of black bean aphid *Aphis fabae* observed on neighbouring cannabis seed-crop with lower secondary metabolite content.

Grey Mould *Botrytis cinerea* was observed in all but the first year's trial. The disease regularly attacked the inflorescence tissue of mature plants (Fig 6.7) but was never observed on the floral tissue of G5 plants before 1st October. Botrytis is reported to be the most common disease of cannabis, proliferating in high humidity which encourages conidia germination (McPartland *at al*., 2000). The disease would typically establish deep within the inflorescence, where the humidity was especially high. In many cases the disease was only discovered by manually parting the bracts. In the 2006 field trial the level of *B. cinerea was* assessed on plants as they were harvested on five harvest dates between 18th September and 17th October. On each occasion the number of plants visibly infected in each plot was recorded. The results are shown in Table 6.11. The first infected plants were observed on 2nd October. Although 12% of plants were

visibly spoiled, only a small proportion of each plant was affected. To comply with Paragraph 3.1 of the GAP Guidelines the harvest should take place when the plants are of the best possible quality according to the different utilizations. If harvested at this stage, the crop would have to be laboriously graded to remove the poor quality diseased material. By the 8th October a large proportion of the crop was affected and grading the crop to only retain high quality material would have been especially difficult. A week later on 17th October almost all of the plants were affected and the crop was regarded totally unacceptable as a phytopharmaceutical feedstock.

Figure 6.7 Fungal damage of a cannabis inflorescence due to *Botrytis cinerea*.

Harvest Date	17th Sep	24th Sep	1st Oct	8th Oct	15th Oct
% of Plants Infected	0	0	12.2	30.6	98.0

Table 6.11 The level of infection with *Botyrytis cinerea* observed on plants harvested on five dates between 17th September and 15th October 2006. Scores are the mean % infection rates in the seven plots of each treatment

Other diseases experienced were Stemphylium Leaf and Stem Spot (*Pleospora tarda* E.G. Simmons) and powdery mildew (*Sphaerotheca macularis*). *Stemphyllium* has been recorded as a significant problem in hemp crops in Canada, Holland and Eastern Europe (McPartland *et al.,* 2000). In the field trial program the disease was only observed in 2006. The identification was made by CABI Bioscience, Bakeham Lane, Egham, Surrey, TW20 9TY, UK. The fungus was reported to be in its anamorphic state

- *Stemphylium botryosum* Wallr. Symptoms became widespread on foliage in August. The discoloured leaf tissue within the spot necrosed and frequently disintegrated to leave a characteristic 'shot hole' in the leaf. Towards late September these spots would coalesce leading to a significant loss of leaf area. Throughout this latter phase however, the bract tissue within the inflorescences was totally unaffected by the disease.

Powdery mildew appeared in a field trial for the first time in 2006 as a minor disease. No plants could be regarded as being of insufficient quality for harvest. Of the insect pests to attack the crop caterpillars (various species) were present each year and caused greatest damage, especially in late summer. However, damage was generally restricted to the large leaves that subtended the top inflorescence. In each case the capitate stalked trichomes on the bracts and bracteoles proved an excellent deterrent to predation, as shown in Figure 6.9. Active caterpillars were often discovered within the inflorescences but were observed not to be eating the resinous bracts and bracteoles.

Figure 6.8 A cannabis plant at the late flowering stage. Resinous bracts are unaffected but leaves below the inflorescence are heavily grazed. In some cases little more than the midrib of the leaf remains.

Other insects pests observed included leaf miner *Liriomyza (Agromyza) strigata* (Meigen), black bean aphid *Aphis fabae* and common nettle aphid (*Liocoris tripustulatus).* In no year was more than an estimated 2% of the foliage lost to leaf

miner, the problem being most severe in 2006 when prolonged higher-than-average temperatures favoured attack. Aphids were notable for their total absence in all seven year's crops of the G5 CBD chemovar. However, the pest was noted in 2006 on a susceptible cannabis variety planted from seed alongside the G5 CBD trial. An analysis of variance showed that the males were significantly more susceptible than the females (males 11.9%, females 1.3%, $p < 0.001$). Cannabis appears therefore to be typical of so many dioecious crops where the male is more susceptible to predators – a clear example of sex-biased herbivory (Herms and Matson, 1992). The all-female G5 crop appeared to be benefitting from both a varietal and sex-based resistance to *Aphis fabae.*

Common nettle capsid is reported to feed on common nettle (*Urtica* spp.) and to occasionally cause economic damage in a number of glasshouse crops (Malais and Ravensberg, 1992). This was observed on regular occasions in outdoor crops during August when larvae from the summer generation have reached the adult stage. Commercial fibre and seed-hemp crops would typically be approaching their natural harvest date in August and be beyond a stage at which serious damage could occur. However, the all-female cannabis drug-crop would be producing ample succulent foliage at this time and clearly more susceptible to attack. Also encountered but not quantified were rabbits and arable weeds. After excessive grazing damage in 2001/2002 all subsequent trials were protected with rabbit proof fence. Weeds were manually removed at intervals through the season.

6.7.7 Effect of Raised Temperatures on Crop Drying Time

The initial attempt to dry cannabis plants on the floor of an oast house drying chamber was only partially successful. To maintain a uniform rate of drying through a hop crop, the material needs to be spread evenly on the floor in sufficient depth to substantially restrict air flow. Such a crop is typically dried within a few hours. The depth of the cannabis (approximately 15 cm) was much less than the one metre depth of hops traditionally processed in the oast, and the inlet temperature of 40ºC was at the lower end of the typical range (Neve, 1991). To successfully dry the crop this way required twenty four hours.

The crops in 2004-2006 were hung to dry on wires suspended within the oast house. Cannabis crops typically feel sufficiently dry to be stripped when below 15% moisture. Figure 6.10 suggests that in 30, 40 and 50ºC, the mean times taken to achieve 15% moisture were approximately 36, 18 and 11 hours respectively. Plants of the same cultivar, dried in the glasshouse-crop drying facility at 25ºC typically took 4.5 – 5.0 days to reach this same moisture level. However, ventilation levels differed between the two

locations and the longer drying time cannot be entirely attributed to a lower drying temperature. This prolonged drying time was highly favourable to the proliferation of fungi and bacteria, and plants already showing low levels of disease were seen to deteriorate in these conditions. Increased drying temperatures greatly reduced the time within which such fungal spoilage could occur. It was accepted that proportionally more of the volatile monoterpenes would be lost compared to the sesquiterpenes at the higher drying temperatures. However, it was considered likely that the increased monoterpene losses at 40ºC and 50ºC were minor compared to those that would have been lost during the decarboxylation process, when milled BRM was heated at much higher temperatures to convert the cannabinoid acids into the neutral forms.

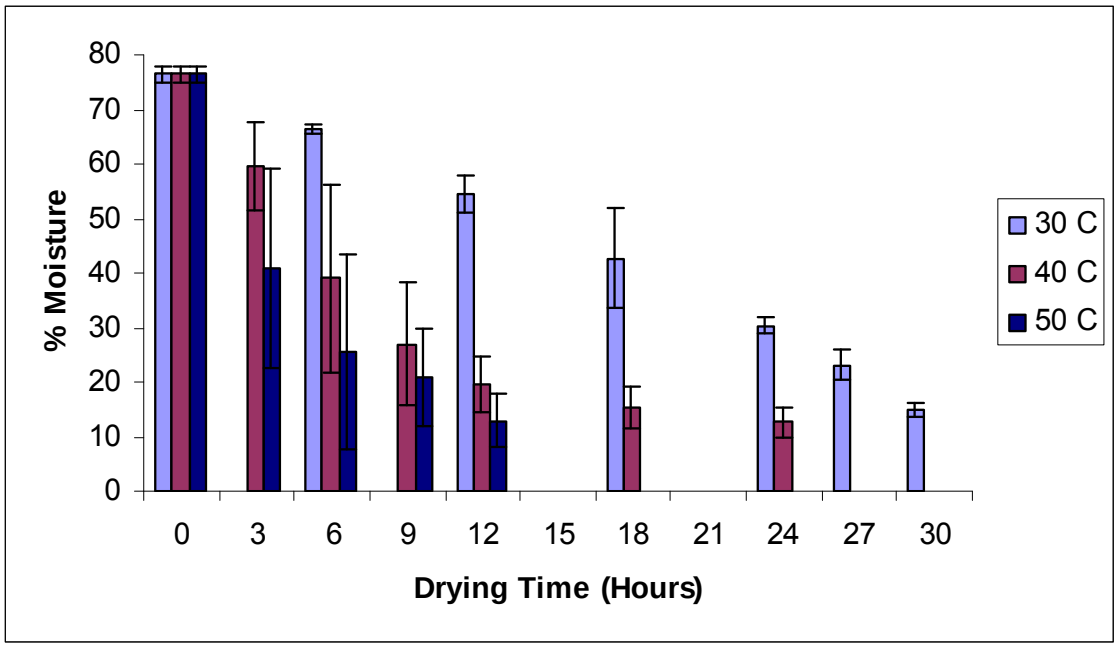

Figure 6.9 The rate of moisture loss of field grown cannabis, when dried at three temperatures (30, 40 and 50ºC). The results are the mean of three crops dried in 2004-2006 (± SD) and show the pattern of moisture loss until mean moisture content was <15%.

6.8 CONCLUSIONS

The research reported here indicated for the first time that the outdoor propagation of *Cannabis* as a phytopharmaceutical in the UK was possible. However, the largest problems to overcome were disease control and the logistical difficulties encountered in harvesting a large volume of crop in a limited period of time. Cannabinoid and terpene profiles of indoor and outdoor gown crops were shown to be very similar if both crops were harvested at the end of floral development, as denoted by complete stigma

senescence. However, the desired cannabinoid profile was only achieved in mid-October, when levels of *Botrytis* infection were high. The reliable outdoor production of this crop appears impossible therefore without the use of fungicides. This would necessitate harvested crops being tested for fungicide residues.

The cannabinoid profile of the outdoor crop appeared less stable than that of crops grown in the glasshouse. As a result, the harvest would need to take place within a very short period when cannabinoid levels were assessed as being within the agreed specification. This could coincide with unfavourable weather conditions, which would hamper harvest operations. However, the ability to rapidly dry the crop was demonstrated. An alternative way of exploiting outdoor grown *Cannabis* may be to collect the glandular trichomes from this material. The crop could conceivably be harvested and promptly fresh frozen for processing at a later date. Similar harvest operations have been used in the frozen pea industry for decades. As stated in the previous chapter, the bulk removal of *Humulus* trichomes is also practised industrially. The concept may warrant evaluation in forthcoming seasons.

Chapter 7 General Discussion

GW Pharmaceuticals plc, the sponsoring company, has been resolute in developing cannabinoid-based phytopharmaceuticals from *Cannabis sativa* L. At the commencement of this thesis in autumn 2003 the company was propagating two chemotypes to produce the medicine Sativex®. This was undergoing Stage III clinical trials for the treatment of symptoms of multiple sclerosis (MS) and for various forms of pain relief. In Canada in 2005 Sativex® received provisional approval with conditions for the treatment of central neuropathic pain in MS, and in 2007 for intractable cancer pain. In 2008 GW Pharmaceuticals commenced Stage III clinical trials in the US to evaluate the efficacy of this medicine for the control of pain in terminal cancer patients. Since 2005 Sativex® has been available in the UK on a 'named-patient' basis, with the Home Office being notified of each recipient. Many other countries have since allowed prescription of the medicine on the same basis. The medicine contains two major cannabinoids, Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), which both had reported pharmacological properties. The choice of these two cannabinoids was based upon evidence that, in addition to them individually being pharmacologically active, the two acted together synergistically, with CBD abrogating the side effects of anxiety and intoxication associated with THC alone (Fowler and Law 2006). Sativex® was a botanical drug, i.e. a well characterised, multi-component standardised drug extracted from plant sources. In addition to THC and CBD, the medicine also contained lesser amounts of other cannabinoids, as well as monoterpenes and sesquiterpenes. *In vivo* rodent studies showed cannabis extracts to have significantly increased pharmacological activity over cannabinoids alone, (Williamson, 2001) and the monoterpenes and sesquiterpenes were suspected of being at least partly responsible for this increase. It was envisaged that these terpenes might also prove to be advantageous ingredients in Sativex®.

To meet the quality and safety standards demanded of a medicine by the Regulatory Authorities, the Botanical Raw Material used as the starter material for Sativex®, and possible future botanical medicines, had to contain a minimum and maximum level of each of several secondary metabolites. To increase the probability of each batch of botanical raw material meeting this specification, the growing conditions initially adopted were kept as uniform as possible and plants routinely harvested when of a fixed age. Initial attempts to grow year-round uniform material were thwarted by a seasonal fluctuation in yields. The cause of this was suspected to be seasonal variation in irradiance levels within the glasshouse. Relatively little was known of how alterations

to the growing protocol would affect the secondary metabolite profile of the harvested plants. It was possible therefore that any attempts to improve the growing conditions might produce feedstock that failed the product specification. A series of tests was performed to analyse the effect on plant development and secondary metabolite content of altering growing conditions. As part of this, seed-sown plants were tested alongside those derived from cuttings (clones). Plants were compared when grown in varying daylengths and under differing irradiance conditions. A range of harvest timings was also compared.

Most if not all other phytopharmaceuticals are produced from outdoor grown crops. Growing cannabis indoors provided better growing conditions and higher level of security. However, the process was costly and produced a large carbon footprint. The energy consumed for lighting alone exceeded 0.5 kW hr per gram of dry feedstock for glasshouse crops, and reached approximately 1 kW hr per gram in a totally enclosed environment. Applying equally high levels of security to the growing of CBD chemovar crops was arguably excessive. It was recognised that outdoor growing of cannabis would reduce energy costs, but the effects on secondary metabolite profile and plant quality might be unacceptable. The vagaries of the weather would be expected to influence yields but how this would affect secondary metabolite profile was not known. Pest and disease problems would likely be different to those encountered by a glasshouse crop and this might unacceptably affect quality. Drying the crop would also present logistical difficulties. Prior to commencing the work of this thesis, one CBD crop had been grown successfully outdoors and the cannabinoid yields achieved were similar to those achieved with a single glasshouse crop. For this thesis a sequence of CBD-chemotype crops was grown outdoors and plant development studied. Pest and disease levels were monitored. Crops were harvested over a range of dates from mid-September to mid-October and the effect of harvest date on cannabinoid and terpene profile compared to that of glasshouse crops. In an attempt to reduce the degree of crop spoilage during the drying process, trials were performed over three seasons to compare crop drying regimes at a range of temperatures.

When characterising any phytopharmaceutical feedstock, a systematic and illustrated report of the microscopical details is generally required. In *Cannabis sativa* L, the trichomes were perceived to be the most important parts of the plant, as the cannabinoids and terpenes are biosynthesised and sequestered within these structures. A review of the form, function and distribution of these constitutes Chapter Three of this thesis. As the chapter shows, there are differing trichome forms. The cannabinoid and terpene profile of the plant changes through its life and this can often

be at least partly explained by the presence of changing proportions of so-called sessile and capitate stalked trichomes. Most notable examples are the higher proportions of CBC and sesquiterpenes within the secondary metabolite profile of foliage compared to floral material, this being attributable to the absence of capitate stalked trichomes on foliage. In potent female floral material the total volume of secondary metabolites in sessile trichomes is almost negligible compared to that in the larger capitate stalked trichomes. Consequently, although they have a different profile, the sessile trichomes have little influence on the overall secondary metabolite profile of the plant. So-called 'manicuring' of inflorescence, to remove bract tissue lacking capitate stalked trichomes, is time consuming. Although favoured by producers of recreational cannabis, seeking to improve potency and flavour, the process has little effect on the profile of the remaining feedstock.

The removal of glandular trichomes from cannabis floral material produces an 'enriched trichome preparation' that has a very similar cannabinoid and terpene profile to that of the plant material from which it came, but is much more potent. One major exception is the diterpene phytol, which is associated with the biosynthesis and catabolism of chlorophyll and Vitamin E, and this is predominantly found outside the trichome. Due to the naturally small volume of the sessile trichome population, the collection of these from aerial tissues produces very small quantities of material. However, the secondary metabolite profile of the collected material can be of great potential interest. The removal and sieving of sessile trichomes from aerial parts of a CBC-rich chemovar containing 1.4% w/w CBC produced a preparation that contained 44% w/w CBC. The stage of development of these sessile trichomes can greatly affect their cannabinoid profile. In the example just quoted CBC accounted for 61% of the total cannabinoids present in the raw material. However, the agitating and sieving process predominantly captured the older more mature sessile trichomes and the CBC purity level of the resultant filtrate was 94% w/w. This method of producing an enriched source of CBC, by selectively capturing mature sessile trichomes, was submitted for patent protection. Enriched trichome preparations, predominantly containing capitate stalked trichome resin heads, were produced which contained up to 67% w/w THC. This material has the clear advantage of being less bulky than feedstocks containing botanical raw material. It also lacks the chlorophyll-based pigments and other substances found in foliage, which may be regarded as undesirable in some botanical medicines. When considering possible ways of mechanising the removal and collection of glandular trichomes, it was recognised that harvesting of cannabis glandular trichomes is also the basis of illicit cannabis resin manufacture. The efficiency of this was assessed in

studies to characterise the illicit cannabis materials typically used in England in 2005/2005.

Resin is just one form of illicit cannabis circulating in the UK. Also commonly found are imported outdoor-grown 'herbal cannabis' and a more potent intensively indoor-grown all-female cannabis product called sinsemilla - or more colloquially 'skunk'. Illicit cannabis is used for recreational and medicinal purposes. Indeed, it was partly on the basis of increasing evidence of the efficacy of this material that GW Pharmaceuticals was given Home Office permission to evaluate cannabis as a phytopharmaceutical. However, little was known of the cannabinoid profile of this material and the impact that this would have on its efficacy and safety. For this thesis, the first survey of UK cannabis cannabinoid content was performed.

The study showed that herbal, sinsemilla and resin contained very variable quantities of THC and herbal and sinsemilla cannabis were almost devoid of CBD. Resin contained extremely variable quantities of CBD, and along with herbal cannabis often contained high levels of CBN. The ratio of these cannabinoids in resin varied greatly, much of this due to the fact that THC catabolised much faster than CBD. In theory, a variable content of one cannabinoid could be overcome by patients if they self-titrated to achieve the desired dose level. When using resin, the only significant source of CBD, self-titration did not offer the ability to correctly dose both THC and CBD. Although the only significant source of CBD, previously unseen survey data analysed for this thesis showed that resin was unfavoured amongst the majority of medicinal cannabis users. Whereas the appearance of resin gave no indication of its likely cannabinoid content, simple visual organoleptic assessments did give a significant clue as to the THC content of sinsemilla. Part of the preference for sinsemilla amongst some medicinal users is the ability (albeit illegal) to grow the material at home. Although seed is commercially available, it is almost always of the high-THC genotype. The study showed that the resin's share of the illicit cannabis market was in decline, with sinsemilla becoming more dominant. The THC content of sinsemilla in the UK was showing a significant upward trend. The psychoactive potential of the average cannabis sample was thus increasing. This was due to the increasing THC content, combined with a decline in the presence of the antipsychotic cannabinoid CBD. This data was used by the Advisory Council on the Misuse of Drugs before making recommendations to the UK Government on the possible reclassification of cannabis (ACMD, 2008).

Chemical characterisation involved the analysis of whole cannabis plant tissues and glandular trichomes collected from them. Unpollinated female floral tissues were known

to be the greatest source of cannabinoids and terpenes and studies were focused here. Most cannabis varieties showed a strong photoperiodic response and, after an initial few weeks of vegetative growth in continuous lighting, plants generally commenced flowering within ten days of being switched to a daylength of 12 hours. Inflorescences produced increasing numbers of florets over subsequent weeks but after eight weeks in short days, growth was slowing rapidly and few additional fertile stigmas were formed. The cessation in floral development was mirrored by a slowing in cannabinoid biosynthesis. Over this period the cannabinoid profile changed, with the proportion of CBG decreasing significantly. Outdoor grown CBD chemovars showed a very similar pattern of floral development and cessation of flowering was matched by the same decrease in the proportion of CBG in the cannabinoid profile. The terpene profile of the plant also changed during plant development and, whether grown in stable glasshouse conditions or outside, the pattern of change was similar with the profile stabilising as the formation of new florets ceased.

The cannabinoid profile of other chemovars also showed a marked change during the flowering process. This was most pronounced in heterozygous chemovars producing more evenly mixed cannabinoid profiles. One of these (variety G159) was the source of several clones that synthesised THC and CBD in approximately equal quantities, and the ratio was observed to change significantly during the flowering process. These clones all produced a significantly higher proportion of CBD when grown outdoors in cooler average temperatures. Growth room tests confirmed that cooler propagation temperatures significantly increased the proportion of CBD within the cannabinoid profile. This could be simply due to the CBD synthase enzyme having a proportionally greater efficiency than THC synthase at lower temperatures, or to the plant switching on some mechanism to enhance CBD synthesis at the expense of THC. This finding has a wider implication for the understanding of how THC and CBD chemovars perhaps evolved and were exploited by man. THC-dominant chemovars are typically associated with latitudes of 30º or less (Small, 1976) where the plant is most capable of producing potent material and the local culture enables its production. CBD-dominant chemovars are associated with temperate latitudes, and this is heavily influenced by licensing restrictions that only allow the exploitation of this chemotype for fibre or seed production. This observation with heterozygous cannabis suggests that in a landrace population, which would include homozygous THC and CBD chemovars and heterozygous mixed THC+CBD chemovars (de Meijer, 2003), a higher proportion of THC would be synthesised in the warmer climates irrespective of the involvement of man.

Although the glasshouse used for this research had sophisticated environmental management and supplementary lighting systems, as stated earlier there was a large seasonal fluctuation in light conditions within the building. This was the presumed explanation for large seasonal variations in crop yield. Although the existing supplementary lighting system was capable of adding 16 W m^{-2} of photosynthetically active radiation (PAR) to the natural light, and originally considered adequate to support the healthy growth of many plant species, this proved inadequate for uniform year-round growth of *Cannabis*. Winter yields of botanical raw material were significantly reduced and there was an even greater reduction in the yield of cannabinoids. Following growth room tests, this was overcome by the provision of an improved supplementary lighting system capable of adding photosynthetically active radiation levels of 53 W m^{-2} to that provided naturally by daylight. This level of lighting is more than twice that typically used in the production of UK glasshouse-grown food-crops. It was discovered that high irradiance levels appear to be most essential at the beginning of the flowering process and greater efficiency and reduced costs could perhaps be achieved by apportioning more of the light energy to crops at this growth stage. It was also shown that by using lamps to provide irradiance levels of 75 W m^{-2}, year-round crops of cannabis could be grown in a totally enclosed environment with no natural lighting. These showed no significant difference in yield compared to those grown in the glasshouse under supplementary lighting.

It was found that the yield of botanical raw material produced per unit area was linearly proportional to the average irradiance level in that growing environment. Raising the irradiance level also caused the plant to divert a higher proportion of energy to the biosynthesis of cannabinoids. The amount of cannabinoid produced was therefore strongly related to the consumption of electrical lighting energy. In a drive to produce flowering cannabis crops with a uniform secondary metabolite content throughout the year, irradiance levels were kept as uniform as practicable throughout the twelve hour day. In mid-summer, this could mean that supplementary lighting would be used to achieve target irradiance levels during dull periods of the day, but on clear days glasshouse roof shades would be closed to reduce the ingress of free sunlight around noon. This was not necessarily the most energy efficient policy. It is possible that the allocation of carbohydrate to primary or secondary metabolite biosynthesis is less dependent on the irradiance level (i.e the light energy per unit area per unit time) and more closely related to the total quantity of light energy falling on the crop during any one twelve hour day - or possible an even longer period. As observed in the study here with variegated cannabis, cannabinoid biosynthesis continues in tissue that is not

photosynthesising. Indeed Crombie (1977) reported that cannabinoid biosynthesis continues during the night, the carbohydrate used therefore having been formed many hours earlier. Had uniformity of crop been less important, the glasshouse supplementary lighting system could have been switched on continuously on summer days and this would have boosted yields of raw material and cannabinoids even further, but possibly altered the secondary metabolite profile. The most likely changes within the secondary metabolite profile would have been alterations to the ratio of monoterpenes and sesquiterpenes, as a consequence of a predicted altering in the ratio of foliar and floral material.

Field trials over several seasons showed that the Sativex-dependent CBD crop could also be propagated outdoors in the UK. The costs were greatly reduced but a range of problems were encountered, of which fungal infection with *Botrytis cinerea* caused the greatest crop losses. Initially low levels of infection would frequently ruin crops within the first 48 hours of the drying process, if plants were dried at 30ºC or below. Increasing crop drying temperatures up to 50ºC considerably reduced crop drying times and offered some reduction in the level of fungal spoilage. Over a five year period, the crop regularly commenced floral growth in the last ten days of August and it was judged ready for harvest in the second week of October, when environmental conditions favoured fungal attack. Future outdoor growth appears unlikely without the use of fungicides to control disease. Sativex-dependant THC varieties commenced flowering too late in September to produce a satisfactory crop, but it was shown that other high yielding THC genotypes could produce healthy high-yielding crops outdoors in Southern England.

A major first step in the optimisation of cannabis as a phytopharmaceutical was to check that botanical raw material produced from clones was more uniform than that in plants grown directly from seeds, even in a highly inbred variety exhibiting pronounced phenotypic uniformity. It is common practise amongst licensed and illicit growers to grow plants from seed and to make clones from the highest yielding or most potent progeny. The plants grown to maturity from these clones typically maintain the desirable trait. Research here showed that cloned plants exhibited a significantly more uniform cannabinoid profile as well as a higher cannabinoid yield.

Grown indoors or out, the cannabinoid and terpene profiles were shown to change throughout the female floral development stages, with both tending to stabilise once the formation of new florets within the inflorescence ceased. The near or complete absence of newly formed stigmas was a visible conformation of the readiness of plants for harvest. Grown in tightly controlled indoor conditions, this growth stage could be

routinely achieved after a fixed time and harvest times could be planned in advance. In outdoor growing conditions plant development was under strict photoperiodic control. Over five consecutive seasons crops of the CBD chemovar G5 were seen to commence flowering within a nine day period between the 22nd August and 1st September, when daylength was approximately 14 hours. In all trials the crop finished flowering in the second week of October, just two weeks after the autumnal equinox, and this date could be predicted as optimum for future harvests of this variety at this latitude. Studies of the cannabinoid profile and terpene profile in both environments showed that by the end of the flowering period the cannabinoid and terpene profiles were most stable and the proportion of CBG within the cannabinoid profile was at a minimum. This was the ideal time to harvest the plant for economic and quality reasons. The proportion of propyl (THCV, CBDV etc.) and pentyl side-chained cannabinoids (THC, CBD etc.) were seen to change through the flowering period and hence the ratio of these would be affected by growth stage at harvest.

As recommended in many growing guides, illicit cannabis is typically induced to flower by exposing it to a steady twelve hour daylength. In an outdoor setting this is the daylength naturally occurring at the autumn equinox when outdoor grown crops were seen to finish flowering. The CBD chemovar, grown outdoors, was seen to commence flowering when daylength fell to approximately fourteen hours per day, and this would be defined as its 'critical daylength'. At this point a phytochrome-controlled hormone release mechanism induces flowering. The use of a twelve hour day in the glasshouse is thus shorter than that required to induce flowering in this chemovar and denies a flowering crop access to an additional two hours light exposure and associated photosynthetic activity. This in turn might reasonably be expected to affect raw material and cannabinoid yields. However, glasshouse studies showed this prediction to be incorrect. The studies in question compared the development of plants grown in eleven, twelve or thirteen hour daylengths. In all regimes the plants were observed to commence flowering equally quickly. However, those maintained in thirteen hour days were slower to cease vegetative growth. This resulted in significant changes in the cannabinoid profile, a significant increase in height and no increase in the feedstock yield, despite a proportional increase in electrical lighting energy costs. This finding suggests that there is more than one critical daylength in cannabis, one of which induces flowering and a shorter daylength at which vegetative growth is hormonally inhibited. Further reducing the daylength below twelve hours resulted in significant decreases in raw material and cannabinoid yield. Consequently, it is recommended

that a twelve hour daylength should continue to be used as the standard daylength in which to maintain the cannabis crops through the flowering stage.

Looking ahead, the survey of the cannabinoid content of illicit cannabis in the UK in 2005 was clearly 'a snapshot in time'. The results showed that the potency and market share of cannabis resin, herbal cannabis and sinsemilla were changing and this warranted a future study to monitor these changes. Indeed, such a survey was performed by the Home Office Scientific and Development Branch in 2008, and acknowledged assistance was given (Hardwick and King 2008). A further Home Office study is planned to monitor on-going changes in the potency of street cannabis in late 2009.

Within the GW Pharmaceuticals glasshouse a number of additional studies are foreseen. More research is recommended to investigate ways of further improving the winter and summer yield of glasshouse grown crops by alterations to light energy levels. In addition to placing greater emphasis on total the light energy received by the plant, rather than the irradiance conditions prevailing, future studies should be performed to research the correlation between the total quantity of light energy received and the relative allocation of carbohydrate to primary or secondary metabolite biosynthesis. The observation that high irradiance levels appear to have greatest impact on cannabinoid yields when applied at the beginning of flowering requires further investigation. If confirmed, supplementary lighting may be selectively concentrated on plants at this growth stage. The analytical studies, in which terpene profiles were monitored during plant development, should be repeated in view of the small number of crops assessed to date.

To enable CBD chemovar crops to be cultivated outdoors, the application of fungicides seems increasingly likely. A range of fungicides should be tested and residues within the crop assessed. Alternatively, a more southerly growing location should be evaluated. Plant breeding of earlier flowering varieties may overcome disease problems, but it may be impossible to breed such varieties with sufficiently similar cannabinoid and terpene profiles to be regarded as being of the same chemotype. Rather than relying on a mixed feedstock of dried foliar and floral material, the collection and storage of enriched trichome preparations offers distinct advantages. Further evaluation of trichome collection methods is highly recommended. The technique is already performed on an industrial scale with the closest relative genus - *Humulus* (the hop) and further work is recommended to see if the available machinery could be adapted for use on cannabis.

As the site of the biosynthesis of cannabinoids, monoterpenes and sesquiterpenes, the glandular trichomes are arguably the most important part of the plant. A greater understanding of the activities within these trichomes would possibly enable the grower to identify when these structures were operating at their optimum. The grower would then be able to assess how difference in growing conditions altered the level of activities within the trichome. As part of a general study of these structures, the capitate stalked form was seen to be the most important type, producing the bulk of the cannabinoids on female floral material. Using relatively simple light microscopes, the study showed that the terpenoids are sequestered in a resin head. Within a mature resin head a disk of secretory cells is visible, occupying about 20% of the volume. Vital stains were perceived as a tool to locate and highlight those tissues where respiratory activity was taking place within the trichome, and to indicate when this was most active. One such stain 'tetrazolium red' suggested that almost all the metabolic activity took place within the secretory cells, even though the site of cannabinoid biosynthesis is purported to be within the storage space above the cells (Sirikantaramas *et al.,* 2005, Taura *et al.,* 2007). The secretory cells would indeed consume much energy in the biosynthesis of cannabinoid precursors and cannabinoid synthase enzyme. However, the lack of apparent activity within the storage space may be purely an anomaly, due to the inability of these water based stains to penetrate this area. The use of lypophylic alternatives may improve the assessment of metabolic activity within the secretory head.

Enriched trichome preparations, almost entirely containing capitate stalked trichome resin heads, were found to have a wide range of cannabinoid contents from approximately 30% up to more than 67% % w/w (dry). This may be due to varying quantities of membrane tissue, enzymes and terpenes. If enriched trichome preparations were to be used as phytopharmaceutical feedstock, rather than floral and foliar material, it would be important to gain further understanding of the cause of this variability.

In September 2008, GW Pharmaceuticals plc reported positive results from a placebo-controlled randomized withdrawal study of Sativex in patients with neuropathic pain due to MS. In February 2009 the company reported positive results in a similar trial with patients experiencing spasticity due to MS. These studies showed that the efficacy of Sativex in the treatment of both neuropathic pain and spasticity due to MS is maintained in long-term use. A few days before completion of thesis GW Pharmaceuticals plc announced positive results from a pivotal Phase III double-blind randomised placebo-controlled study of Sativex® in patients with spasticity due to MS,

who have achieved inadequate spasticity relief with existing therapies. This Phase III study used an enriched design whereby 573 patients initially received Sativex for four weeks in a single blind manner (Phase A), following which Sativex responders (n = 241) were randomized to continue on Sativex or switch to placebo for a further twelve weeks in a double-blinded manner (Phase B). The prospectively defined primary efficacy endpoint of the study - the difference between the mean change in spasticity severity of Sativex vs Placebo in Phase B - was highly statistically significantly in favour of Sativex (p = 0.0002). The difference between Sativex and placebo was also significant for a number of secondary endpoints. 74% of Sativex patients achieved an improvement of greater than 30% in their spasticity score over the entire study versus 51% on placebo (p = 0.0003). These three studies was performed following regulatory guidance from the UK regulatory authority (MHRA) and provided evidence of long term efficacy to be included as part of a forthcoming European regulatory submission planned for mid 2009. If successful this would require a large increase in the quantity of crop grown. The research performed for this thesis aids the reliable propagation of that crop. In the UK alone, MS is the most common disabling neurological disease of young adults, affecting approximately 85000 people. Approximately 2% of the entire UK population experiences neuropathic pain. Regulatory approval of Sativex® would lead to the medicine being widely available on prescription. For many of these patients, illicit cannabis would have been the only form of medicinal cannabis available hitherto.

In early 2009, the company was performing Phase I trials to evaluate the cannabinoid THCV for appetite control in treating obesity. Other cannabinoids were also undergoing a range of in-vitro and in-vivo studies. A range of different *Cannabis* chemovars had been specifically bred to be dominant in cannabinoids other than THC and CBD. The parent plants used to breed these chemovars differed in provenance, and the optimum growing conditions for these had not been determined as fully as those used to produce THC and CBD. More horticultural research would be required to optimise their propagation.

This thesis has emphasised many times the fundamental importance of the glandular trichome on *Cannabis sativa* L. It is here, within the vesicles of the glandular trichome that the enzymes and precursors of cannabinoid biosynthesis are secreted and the phytocannabinoid story starts. Further research will aim to improve the understanding of the biosynthetic activities within these trichomes and take this story forward.

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Appendix 8.1 Analysis of cannabinoid content using HPLC and GC

8.1.1 HPLC Analysis

8.1.1.1 HPLC and GC Equipment

Apparatus	Source
Sonicator Model UR-324T	Gemini B.V., Elsweg 57, 7311 GV, Apeldoorn, Netherlands
Hewlett Packard/Agilent 1100 HPLC incorporating: - Autosampler DE594901133 Diode Ray Detector DE91605830 Column Oven DE91609412 Mobile Phase Degaser Unit DE9605830 Discovery C8 150 x 4.6 mm column and 30 x 4.6 mm precolumn packed with 5µm Kingsorb ODS packing material. Personal computer with HP Chemstation software.	Agilent Technologies UK Ltd. South Queensferry, West Lothian.
Sonicator Model UR-324T	Gemini B.V., Elsweg 57, 7311 GV, Apeldoorn, Netherlands
Hewlett Packard 6890 GC with FID incorporating an HP-5 320 µm x 30 m column with a 0.25 µm film and an autoanalyser	Pre-owned item

Table 8.1.1 Analytical Equipment

8.1.1.2 Analytical Materials

Materials	Source
11-Nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid 50 µg/mL in methanol.	Sigma Aldrich Ltd., Fancy Road, Poole, Dorset
CBGA CBDA CBCA	Applied Analysis Ltd. Rowley House, Tokenspire Business Park, Beverley, Yorkshire.
Chloroform HPLC Grade	Ultrafine Ltd. Marlborough House, 298 Regents Park Road, London
Methanol HPLC Grade	Fisher Scientific UK Ltd. Bishops Meadow Lane, Loughborough, Leics.

Table 8.1.2 Analytical Materials

8.1.2 HPLC determination of cannabinoid content

Previous forensic analysts have reported that investigations into cannabis potency are made difficult by the inhomogeneous nature of herbal cannabis. Within a well-mixed single large batch of crude material, and following removal of unwanted matter, different aliquots could lead to quite different analytical results (King 2004). Batches of the 'naturally inhomogeneous material' were well mixed before sampling. A minimum of three subsamples was analysed if sufficient material was available.

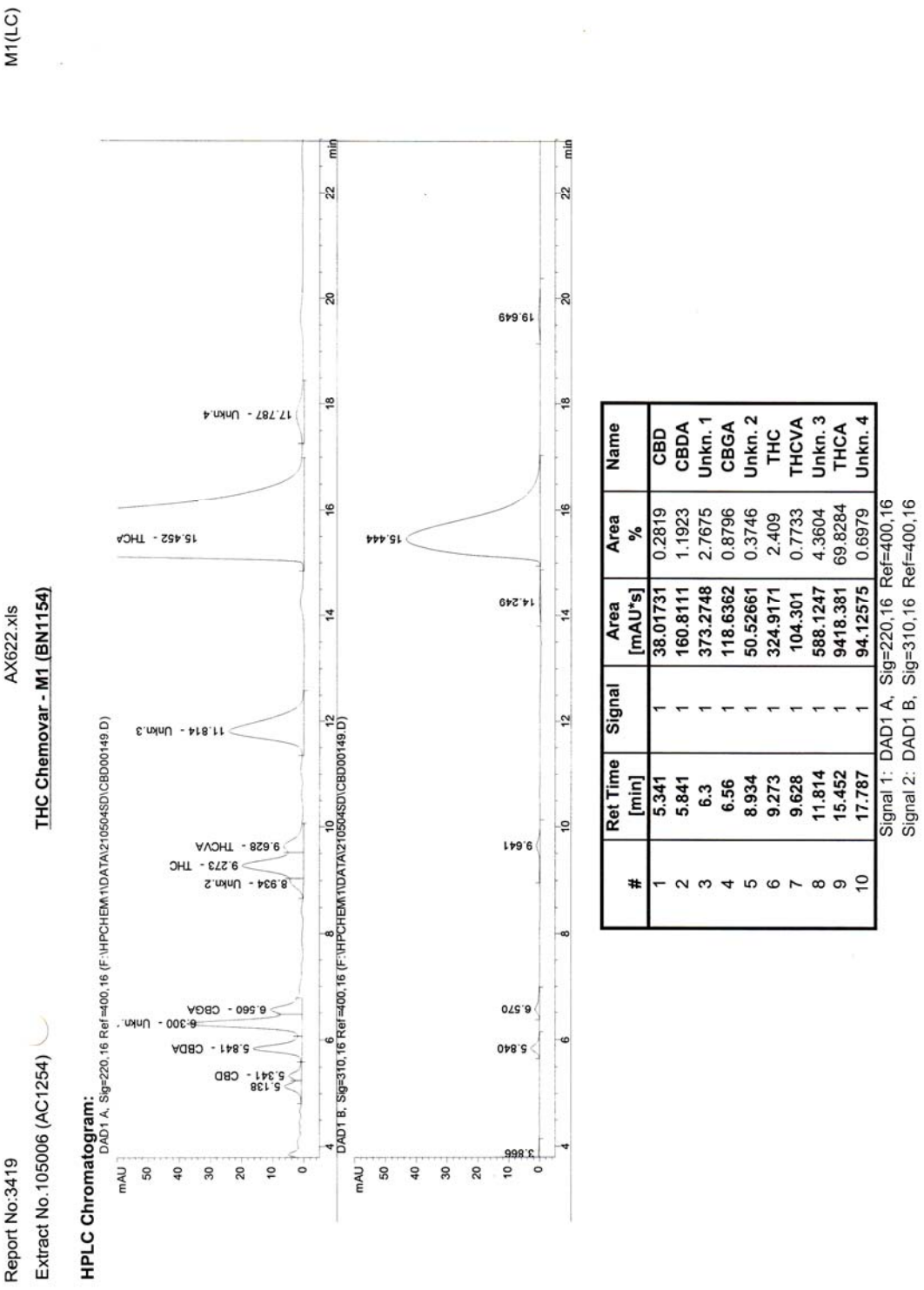
The botanical raw material is thoroughly mixed. Five small samples of approximately 1 g are taken at random from the mixture and blended. From this, a single sub-sample of 100 mg is extracted with 1.0 ml methanol-chloroform (9:1 v/v) by sonication for fifteen minutes. 100 µl of the filtered extract is diluted with 300 µl of methanol and aliquots of 1 µl used for HPLC.

Using a Discovery C8 150 x 4.6 mm column and a 30 x 4.6 mm precolumn containing 5µm Kingsorb ODS packing material, an operating temperature of 25ºC and a UV wavelength of 220 nm were adopted. The run time was 23 minutes and THC the internal standard.

Apart from THC, which was purchased from Sigma-Aldrich, most cannabinoid analytical standards were not available at the commencement of this study. Cannabinoids such as CBGA were identified and quantified in the HPLC trace by

comparing chromatograms with those produced by Lehmann and Brenneisen (1995). CBGA, CBDA and CBCA were later purified and identified at Applied Analysis Ltd (Flockhart pers comm.) and the original identification of their HPLC peaks confirmed.

A chromatogram produced when analysing an extract of Clone Line G1 M1 is shown in Figure 8.1.1. The CBGA and THCA peaks are observed at 6.56 and 15.45 minutes respectively. Following the later acquisition of a CBGA standard it was ascertained that when equimolar preparations of THCA and CBGA are evaluated by HPLC using this method, CBGA produces a peak area greater than THCA by a factor of 1.2. Concentrations of the two in this thesis have been calculated accordingly. Corrections for the peak characteristics of other cannabinoids have been similarly implemented.



#	Ret Time [min]	Signal	Area [mAU*s]	Area %	Name
1	5.341	1	38.01731	0.2819	CBD
2	5.841	1	160.8111	1.1923	CBDA
3	6.3	1	373.2748	2.7675	Unkn. 1
4	6.56	1	118.6362	0.8796	CBGA
5	8.934	1	50.52661	0.3746	Unkn. 2
6	9.273	1	324.9171	2.409	THC
7	9.628	1	104.301	0.7733	THCVA
8	11.814	1	588.1247	4.3604	Unkn. 3
9	15.452	1	9418.381	69.8284	THCA
10	17.787	1	94.12575	0.6979	Unkn. 4

Signal 1: DAD1 A, Sig=220,16 Ref=400,16
Signal 2: DAD1 B, Sig=310,16 Ref=400,16

Figure 8.3.1 Chromatogram of a preparation from Clone G1 M1.

8.1.3 GC determination of cannabinoid content

The botanical raw material was thoroughly mixed. Five small samples are normally taken at random from the mixture and blended. From this, a single sample 50 mg was taken and prepared for analysis.

The samples were then prepared and analysed as developed by de Meijer et al 2003. I ml of ethanol (>99.7%) was added to the filtration tube and the sample sonicated for 15 minutes and the extract then centrifuged at 4000 rpm for 10 minutes. This procedure was then repeated a further three times and the resultant 4 ml of ethanol containing the cannabinoid extracts then transferred to a five ml volumetric flask. 0.25 ml of a phenanthrene stock solution (10mg/ml) in ethanol was added as an internal standard and adjusted to 5 ml with ethanol. Extracts were homogenised and transferred to GC vials.

Gas-chromatographic analyses were performed on a Hewlett Packard 6890 GC equipped with an autoanalyser, a flame ionization detector and an HP-5 320 µm x 30 m column with a 0.25 µm film.

Appendix 8.2 Analysis of terpene content using GC

8.2.1 Analysis Equipment

Apparatus	Source
Gas Chromatograph with split/splitless capillary injector (Agilent Technologies 5890 or 6890) Flame ionisation detector (FID) and/or Electron Ionisation Mass Spectrometer (EI-MS) Autosampler DE594901133 Diode Ray Detector DE91605830 Column Oven DE91609412 Mobile Phase Degaser Unit DE9605830 Discovery C8 150 x 4.6 mm column and 30 x 4.6 mm precolumn packed with 5µm Kingsorb ODS packing material. Personal computer with HP Chemstation software.	Agilent Technologies UK Ltd. South Queensferry, West Lothian.

Table 8.2.1 Analytical equipment

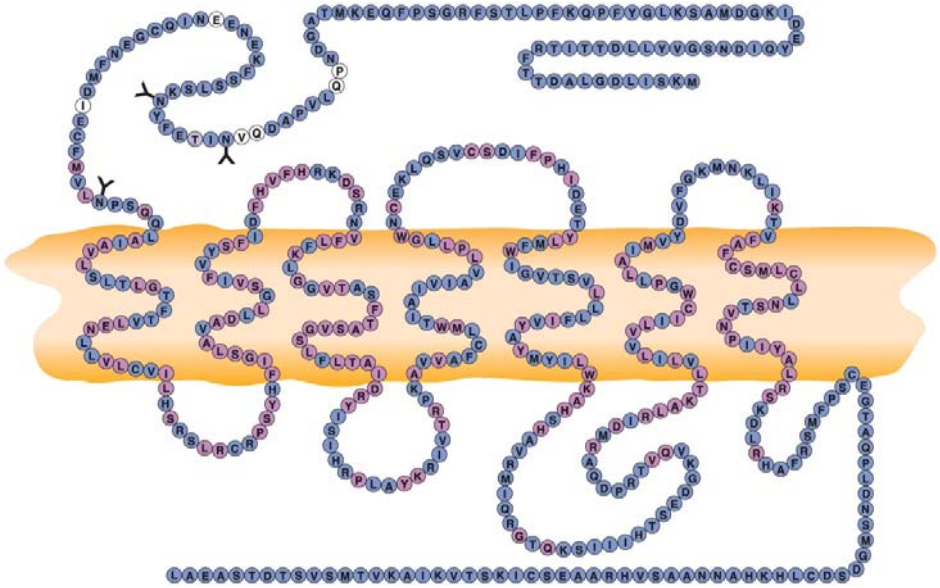
8.2.2 GC Analysis Method

The botanical raw material was thoroughly mixed. 1 g samples are taken at random and extracted with approximately 40 ml of methanol:chloroform (9:1 v/v) in a 50 ml volumetric flask by sonication for thirty minutes at 25ºC. The solution is allowed to cool and the volume made up to 50ml with methanol:chloroform (9:1 v/v). An aliquot of each extract is centrifuged at 3000 rpm for 2 minutes. 1.5ml of the supernatant is added to a 2ml auto-sampler vial, capped and securely crimped prior to analysis. Injector and Detector temperatures are 250ºC and 325ºC respectively. The injection volume is 1 µl with a split ration of 5:1. The FID fuel gases were Hydrogen 40 ml min-1, Air 50ml min^{-1}, Helium 45 ml min^{-1}.

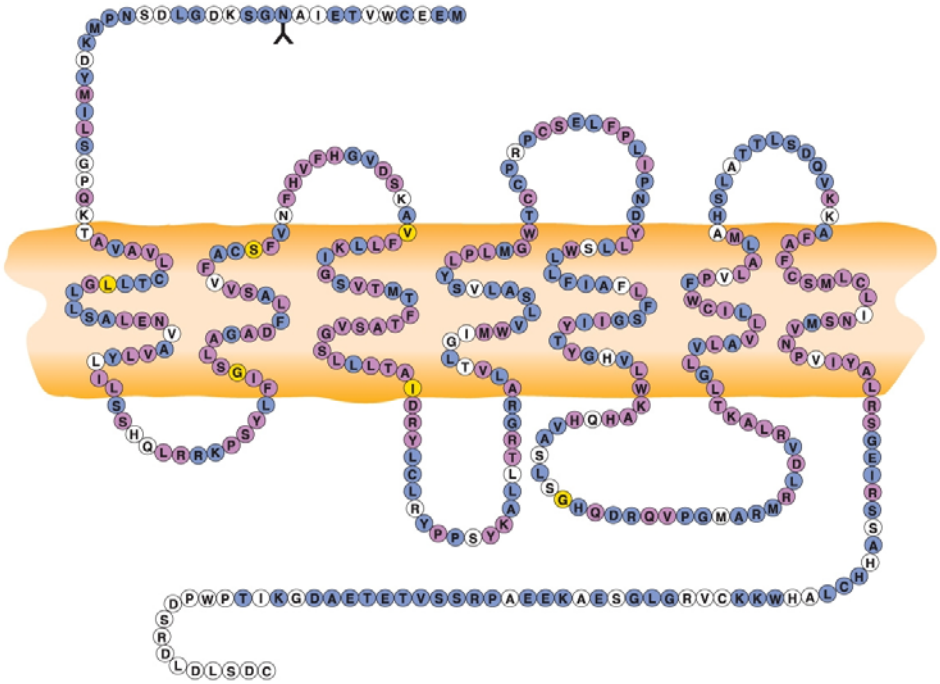
Groups of terpenes may be identified at retention times of approximately 4-5 minutes for monoterpenes and 14-20 minutes for sesquiterpenes. Peak identifications may be made by retention time comparisons with certified standards and by using gathered mass spectra. For generation of accurate quantitative data the losses and uncertainties of the injection and volatilisation process can be reduced by using an internal standard - Phenanthrene at 1 mg $ml^{-1.}$

The assay methods described in the above two appendices have been validated in accordance with ICH Guidelines and have subsequently been included in product license applications. The exact details of this validation remain the intellectual property of GW Pharmaceuticals Ltd.

Appendix 8.3 A Comparison of the Structure of CB_1 and CB_2 Receptors



a)



b)

The structure of CB1 (a) and CB2 receptors (b) in human tissue. Above the cell membrane is the extra-cellular N-terminal and below the intracellular C-terminal.

Appendix 8.4 Meteorological Data pertaining to UK Field Trials

	2000	2003	2004	2005	2006	Mean
May	12.7	12.2	12.5	11.8	8.4	11.5
Jun	15.3	16.3	15.9	16.0	16.2	15.9
Jul	15.7	17.9	16.5	17.3	20.3	17.5
Aug	17.2	19.2	18.0	16.7	16.9	17.6
Sep	15.3	14.7	15.4	16.0	17.3	15.7

8.4.1 Mean daily temperatures in the field trial region as recorded by the Meteorological Service.

	2000	2003	2004	2005	2006	Mean
May	196	202	206	224	168	192
Jun	174	224	232	215	274	224
Jul	174	206	189	202	311	216
Aug	215	240	202	242	181	216
Sep	128	194	173	158	164	163

8.4.2 Total number of sunshine hours each month in the field trial region as reported by the Meteorological Service.

The above data was downloaded from the Meteorological Office web page: -

http://www.metoffice.gov.uk/climate/uk [accessed on 6th February 2009].

Appendix 8.5 Definitions of Social Grade as defined by National Readership Survey

The Social Grade classification used by NRS was developed for the Survey over 50 years ago. It remains a highly effective way of classifying readers of different publications, and is widely used in planning advertising, not just in newspapers and magazines, but in other media too. The grades were also used by the Government when compiling the UK Population Census.

Social Grade is determined by the occupation of the Chief Income Earner (CIE) in each household. Additional criteria such the size of the organisation, and the number of people for which the CIE is responsible, are used to refine the process.

A brief description of the six grades is as follows:

	Social Status	Occupation
A	Upper Middle Class	Higher managerial, administrative or professional
B	Middle Class	Intermediate managerial, administrative or professional
C1	Lower Middle Class	Supervisory or clerical and junior managerial, administrative or professional
C2	Skilled Working Class	Skilled manual workers
D	Working Class	Semi and unskilled manual workers
E	Those at the lowest levels of subsistence	Casual or lowest grade workers, pensioners and others who depend on the state for their income