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Metabolic fingerprinting of *Cannabis sativa* L., cannabinoids and terpenoids for chemotaxonomic and drug standardization purposes

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ABSTRACT

Cannabis sativa L. is an important medicinal plant. In order to develop cannabis plant material as a medicinal product quality control and clear chemotaxonomic discrimination between varieties is a necessity. Therefore in this study 11 cannabis varieties were grown under the same environmental conditions. Chemical analysis of cannabis plant material used a gas chromatography flame ionization detection method that was validated for quantitative analysis of cannabis monoterpenoids, sesquiterpenoids, and cannabinoids. Quantitative data was analyzed using principal component analysis to determine which compounds are most important in discriminating cannabis varieties. In total 36 compounds were identified and quantified in the 11 varieties. Using principal component analysis each cannabis variety could be chemically discriminated. This methodology is useful for both chemotaxonomic discrimination of cannabis varieties and quality control of plant material.

1. Introduction

Cannabis sativa L. (cannabis) is an annual dioecious plant belonging to the family Cannabaceae. Cannabis has a long history of human use as a medicinal plant, intoxicant, and ritual drug (Russo, 2007). Today most nations' worldwide regard cannabis as an illegal drug of abuse. Despite the abuse potential of cannabis research into its chemistry and pharmacology has demonstrated that it also has medical properties. Chemical analysis of cannabis in the 1940s and 1960s led to the discovery of a unique group of terpenophenolic secondary metabolites, known as cannabinoids, of which *trans*-(−)-Δ^9-tetrahydrocannabinol (Δ^9-THC) was shown to be the primary psychoactive ingredient (Pertwee, 2006). At least 90 plant cannabinoids, also known as phytocannabinoids, have been isolated from cannabis (Ahmed et al., 2008; ElSohly and Slade, 2005; Radwan et al., 2009). In the early 1990s the G-protein coupled cannabinoid receptors (CB) were discovered. Two types of cannabinoid receptors CB_1 and CB_2 revealed a receptor based mechanism for the action of Δ^9-THC (Pertwee, 2009).

Clinical trials into cannabis, pure cannabinoids, and synthetic analogues have demonstrated some effectiveness as analgesics for chronic neuropathic pain, appetite stimulants' for cancer or AIDS patients, and multiple sclerosis. The increased medical interest in these substances has prompted the development of various cannabis based medicines such as the oral Δ^9-THC preparation Marinol® (Solvay Pharmaceuticals, Belgium), a synthetic analogue of Δ^9-THC Nabilone® (Valeant Pharmaceuticals International, USA), and Sativex® (GW Pharmaceuticals, UK) an oral mucousal spray containing 1:1 ratio of Δ^9-THC and cannabidiol (CBD) (Ben Amar, 2006; Hazekamp and Grotenhermen, 2010). Since 2003 The Netherlands has allowed the distribution of standardized herbal cannabis in pharmacies to patients with a prescription (Hazekamp, 2006). In the USA 14 states have legalized under state law the use of medical cannabis. In order to facilitate research into clinical safety and effectiveness the American Medical Association (AMA) has recently called for the rescheduling of cannabis's legal status from Schedule I to Schedule II (Hoffmann and Weber, 2010). These developments highlight the urgency to define the criteria necessary for the chemotaxonomic classification of medicinal cannabis for drug standardization and clinical research purposes.

There has been considerable debate over whether or not whole herbal cannabis has any additional therapeutic benefits when compared to pure cannabinoids (ElSohly et al., 2003; Llan et al., 2005; McPartland and Russo, 2001; Russo and McPartland, 2003; Wachtel et al., 2002). However, there is some evidence that certain cannabis preparations exhibit different effects when compared to pure cannabinoids (Fairbairn and Pickens, 1981; Johnson et al., 1984; Pickens, 1981; Ryan et al., 2006; Segelman et al., 1974; Whalley

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et al., 2004; Wilkinson et al., 2003). Both the terpenes and minor cannabinoids present in cannabis are known to have various biological activities (McPartland and Russo, 2001). A lack of detailed chemical characterization beyond Δ^9-THC, CBD or cannabinol (CBN) quantification is shown in the above mentioned preclinical as well as clinical research making it difficult to compare results across studies (Ben Amar, 2006; Hazekamp and Grotenhermen, 2010). It is not possible to draw any strong conclusions about what components other than Δ^9-THC and occasionally, depending on the study design CBD, present in cannabis preparations may have an influence on the drugs effects.

Cannabinoids are produced biosynthetically in cannabis as their carboxylic acid derivatives and are known as cannabinoid acids. Cannabinoid acids degrade into their neutral counterparts through the action of heat, sunlight, and storage (Taura et al., 2007). Cannabis is most commonly administered by smoking the dried flower buds due to the avoidance of first pass metabolism of orally administered Δ^9-THC as well as ease of self-titration by the user or patient (Williamson and Evans, 2000). In a recent study we demonstrated that cannabis ethanol extracts, smoke, and vapor produced by a vaporizing device are composed of a complex mixture of terpenoids and cannabinoids (Fischedick et al., 2010). Therefore quality control methods for the major volatile compounds in cannabis should be utilized prior to and during clinical studies of cannabis administered with a vaporizing device or by smoking.

Two morphological types of cannabis are commonly recognized, *C. sativa* being taller and more highly branched typically representing fiber type varieties and *Cannabis indica* being shorter with broader leaves typically representing strains used for recreational or medicinal purposes. Whether or not these two morphotypes are different species is still a matter of debate (Russo, 2007). A third subtype, *Cannabis ruderalis* has also been recognized, and is described as having low levels of cannabinoids with a bushy appearance (Hillig and Mahlberg, 2004). Today many cannabis varieties used recreationally and for medical purposes are hybrids of the various cannabis morphotypes mostly *C. sativa* and *C. indica*. Chemotaxonomic evaluation of cannabis has led to the recognition of three chemotypes, a drug type with higher levels of Δ^9-THC, a fiber type with higher CBD, and an intermediate type with similar levels of each (Fetterman et al., 1971; Small and Beckstead, 1973a,b). More recent studies using gas chromatography (GC) analyzing cannabinoids (Hillig and Mahlberg, 2004) or terpenoids (Hillig, 2004) have been performed for chemotaxonomic purposes. ^{1}H NMR has been used to fingerprint cannabis aqueous extracts and tinctures (Politi et al., 2008) as well as to chemically differentiate cannabis cultivars (Choi et al., 2004). However, none of these methods offer validated quantitative methods for the analysis of cannabis terpenoids and cannabinoids simultaneously. Furthermore the sample preparation used by Hillig (2004) for terpenoid analysis utilized extensive sample drying (2 months at room temperature) and heating at 30 °C prior to analysis. This would have resulted in a higher rate of volatilization for the monoterpenoids thus biasing the chemotaxonomic evaluation towards the less volatile sesquiterpenoids.

Metabolic fingerprinting, also known as metabolic profiling, is a targeted analytical approach which aims to quantify a group or groups of compounds found in an organism or group of organisms. Metabolic fingerprinting with GC, HPLC, coupled with mass spectrometry, or ^{1}H NMR is useful for studying plant biochemistry, chemotaxonomy, ecology, pharmacology, and quality control of medicinal plants (Van der Kooy et al., 2009). To metabolically fingerprint cannabis we validated a GC–flame ionization detection (GC–FID) method for monoterpenoids, sesquiterpenes, and cannabinoids. The analytical method was used to study the chemical composition and variability of terpenoids and cannabinoids in 11 cannabis varieties grown under standardized environmental conditions. Principal component analysis (PCA) was used to identify the compounds most important in distinguishing cannabis varieties. We also studied the variation on cannabis chemical profiles as a result of growing plants in different batches and with deviations in growth time. This study establishes useful criteria for quality control and standardization of cannabis varieties for clinical studies as well as chemotaxonomy.

2. Results and discussion

2.1. Plant material

Bedrocan BV (Groningen, The Netherlands) is a company licensed and contracted by the Dutch government to produce standardized cannabis plant material under Good Agricultural Practice (GAP) conditions to be supplied to patients on prescription, through pharmacies (OMC, 2010). All plant material in these experiments was grown by Bedrocan BV. The varieties Bedrocan® (Bedrocan), Bedropuur® (Bedropuur), and Bediol® (Bediol), have been breed by Bedrocan BV for use in medicine or research. All other varieties grown in this study are currently used for research purposes only. In total 11 cannabis varieties were grown (Table 1). Standard growth conditions are defined as the optimum vegetative and flowering growth times for each variety. The morphological type classification for each variety is based on morphological traits as well as knowledge Bedrocan BV has of the varieties origin and breeding history. Hybrids are described as having either equal morphological traits from *C. indica* or *C. sativa* (i.e. hybrid indica/sativa) or having traits of both but mostly having traits representative of one of the morphotypes (i.e. hybrid mostly sativa). The letter codes have no meaning other than to distinguish between varieties. All plants were grown from clones of a 'motherplant'. A motherplant is defined as a female cannabis plant from one distinct variety used for cloning (vegetative propagation) only.

Two female cannabis plants were grown for each batch and each growth treatment. Five random samples of dried flower material were selected for the analysis of each batch and each growth treatment. The purpose of growing plants in different batches and with deviations from standard growth conditions was to test the robustness of our chemical classification as well as determine the reproducibility of a cannabis varieties chemical profile. The AO variety was grown in five batches at the same time. Each batch originated from a different seed from the same cannabis variety. Seeds were grown and female plants were selected for cloning. Each number for the AO variety thus denotes a different original seed and its subsequent female clones. Therefore each AO batch was not genetically identical. For all other varieties the plants grown were genetically identical. The AO7 batch was grown for an extra week in the flowering state. The varieties AG, AE, Ai94 were each grown in three separate batches (1, 2, and 3). Batches 1 and 2 were grown about a month apart while batch 3 was grown at the same time as 2 except with an extra week of vegetative growth and an extra week of flowering (Table 1). Bedrocan was grown in two batches at the same time. One batch had its lower branches clipped (c) while the other batch was grown under standard conditions (Table 1). Bedropuur was grown in four batches at the same time with one batch grown under standard conditions and the other three batches grown with deviations from standard conditions (Table 1).

2.2. Method validation

Results of GC method validation are summarized in Table 2. For precision the percent relative standard deviation (RSD) of the peak