Terpenoids for medicine

Fischedick, J.

Chapter 4

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.

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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 1940's and 60's 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 1990's 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 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 (Fairbarin and Pickens, 1981; Johnson et al., 1984; Pickens, 1981; Ryan et al., 2006; Segelman et al., 1974; Whalley et al., 2004; Wilkinson, 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 drug's 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 3 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; Small and Beckstead, 1973b). 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.

Materials and Methods

Chemicals

Reference terpenoids of caryophyllene-oxide, camphor, α-bisabolol, β-pinene, myrcene, α-pinene, γ-terpineol, (*R*)-limonene (limonene), (*S*)-limonene, 1-8-cineol, carvacrol, and β-caryophyllene were purchased from Sigma-Aldrich (Steinheim, Germany). Terpineol mixture of isomers, α-humulene, and linalool were purchased from Fluka (Steinheim, Germany). Geraniol was purchased from Chromadex (Irvine, California, U.S.A). Camphene, α-thujene, sabinene, terpinene-4-ol, 1-4-cineol, Δ^3-carene, *p*-cymene, terpinolene, citronellal, geranyl acetate, pulegone, citral, α-terpinene, α-fenchyl alcohol, calamanene, γ-cadinene, bornyl acetate, a mixture of *cis/trans*-ocimene, α-cedrene, α-phellandrene, nerol, β-phellandrene, nerolidol, and piperitone-oxide were from a chemical bank of the authors. The cannabinoid references for Δ^9-THC, Δ^8-THC, CBD, cannabigerol (CBG), cannabichromene (CBC), *trans*-(-)-Δ^9-tetrahydrocannabivarin (THCV), and cannabinol (CBN) were purified and quantified by PRISNA BV as previously described (Leiden, The Netherlands) (Hazekamp et al., 2004a; Hazekamp et al., 2004b). All cannabinoids references were > 98% pure, except THCV. Absolute ethanol (EtOH) used for extraction and sample preparation was of analytical reagent (AR) grade (Biosolve BV, Valkenswaard, The Netherlands). 1-Octanol (HPLC grade) was purchased from Sigma Aldrich (Steinheim, Germany).

Plant material

Cannabis plant material was grown indoors. The plant material was produced by taking cuttings from standardized plants (mother plants) kept under vegetative conditions. Cannabis plants were grown in two growth cycles. First a vegetative period in which plants are grown under 18 h of uninterrupted light per day producing only roots, stems, and leaves. After an optimized vegetative period plants are switched to 12 h of uninterrupted light per day which induces flowering. The period for which each variety exists in each phase can differ and has been optimized by Bedrocan BV for efficient growth.

Environmental conditions for all varieties were the same. Plants were harvested after a standardized amount of days when the pistils faded from white to brown and the branches started to hang. The plants were then dried under the same environmental conditions. After one week drying the plant material lost 73% of its

weight. The plants were then processed by removing leaves from the buds and clipping buds from the main stems. Remaining plant material (buds) was packaged into 50 ml Falcon® tubes and stored at -20 °C until extraction.

Sample preparation

Cannabis plant material was weighed to the nearest mg with a typical weight range of 0.9-1.1 g. The plant material was crushed with a metal spoon within a falcon tube and the spoon was rinsed with a few ml of EtOH into the falcon tube. The volume was then brought up to 45 ml with ethanol. Falcon tubes were placed on a Yellow Line Orbital Shaker OS 2 Basic (IKA GmbH, Staufen, Germany) at 400 revolutions per minute (rpm) for 15 min. Samples were centrifuged briefly for 30 s at 2000 rpm. Supernatant was collected in a 100 ml glass volumetric flask. Samples were extracted two more times with 25 ml ethanol. As an internal standard 1 ml of an EtOH soln. containing 1-octanol (1%) was added to the volumetric flasks. Samples were finally brought to a volume of 100 ml with ethanol. Samples were filtered into 20 ml glass vials with a PTFE syringe filter (0.45 µM, 25 mm diameter). Samples were stored air tight in the dark at -20 °C until analysis.

GC-FID

An Agilent GC 6890 series equipped with a 7683 autosampler, a DB5 (30 m length, 0.25 mm internal diameter, film thickness 0.25 µm, J&W Scientific Inc, Folsom, CA, USA) column and a flame ionization detector (FID) was used for quantitative analysis. The injector temperature was set to 230 °C, an injection volume of 4 µl, a split ratio of 1:20 and a carrier gas (N_2) flow rate of 1.2 ml/min. The oven temperature program began at 60 °C with a ramp rate of 3 °C/min. The final temperature was set to 240 °C which was held for 5 min making a total run time of 65 min/sample. The FID detector temperature was set to 250 °C. The GC-FID was controlled by GC Chemstation software version B.04.01 (Agilent Technologies Inc, Santa Clara, CA, USA).

GC-MS

GC-MS analysis was performed on an Agilent 7890A series gas chromatograph equipped with a 7693 autosampler, an HP5-ms column (30 m length, 0.25 mm internal diameter, film thickness 0.25 µm, Agilent Technologies Inc, Santa Clara, CA, USA), and a single quadropole mass spectrometer 5975C. The MS source was set to 230 °C, the single quad temperature was 150 °C, and the transfer line temperature was set to 280 °C. The GC-column was linked to the MS via a quickswap (Agilent Technologies Inc, Santa Clara, CA, USA) and restrictor (0.11 mm internal diameter, Agilent Technologies Santa Clara USA). The injector temperature was 230 °C with an injection volume of 2 µl, a split ratio of 1:20, and a carrier gas (He) flow rate of 1.2 ml/min. The oven temperature program was the same as the GC-FID. The mass range analyzed by the mass spectrometer was 50-500 amu. The GC-MS was controlled by Enhanced Chemstation software version E.02.00.493 (Agilent Technologies Inc,

Santa Clara, CA, USA). The NIST library version 2.0f (Standard Reference Data Program of the National Institute of Standards and Technology, Distributed by Agilent Technologies) was used to assist compound identification.

Standards preparation

Mono and sesquiterpenoid references were weighed to 50 mg in a tarred volumetric flask using a Satorius analytical balance A200S 0.01 mg (Satorius Mechatronics, Utrecht, The Netherlands). Volume was brought to 25 ml with EtOH to make 2 mg/ml stock solutions. Stock solutions were used to make dilutions for standard curves. Stocks were stored at -20 °C in sealed glass vials in the dark until needed. Cannabinoid references were supplied already quantified in EtOH. References were diluted in EtOH to make standard curves. Cannabinoid references were stored at -20 °C in amber sealed glass vials in the dark until needed.

Method validation

Reproducibility

Intra-day reproducibility was determined by injecting an aliquot of a cannabis extract 5 times from the same vial in a single day and a reference sample of γ-terpinene (1 mg/ml) 5 times from the same vial in a single day (n = 5). Inter-day reproducibility was determined by taking a fresh aliquot of the same cannabis extract and γ-terpinene reference and injecting 5 times for an additional two days (n = 15) using fresh aliquots on each day. All injections performed on the GC-FID.

Extraction efficiency

Three 1 g samples of a batch of Bedrocan that had been used in previous studies (Fischedick et al., 2010) and stored for 7 months at 4 °C in the dark were extracted with the procedure outlined above. After 3 extractions a 4th extraction was performed on each Bedrocan sample with an additional 25 ml of ethanol and analyzed for residual compounds by GC-FID.

Accuracy

Accuracy was determined by checking the recovery of the extraction method with spiked monoterpenoids and sesquiterpenoids. Five 1 g samples of a batch of Bedrobinol used in previous studies (Fischedick et al., 2010) and stored for 7 months at 4 °C in the dark were extracted as outlined above. Five 1 g samples of the same batch of Bedrobinol were spiked with 50 µl of the pure references of β-pinene, linalool and β-caryophyllene while in falcon tubes then extracted as described above. Five volumetric flasks were spiked with 50 µl of the pure references of β-pinene, linalool, and β-caryophyllene and brought to 100 ml with EtOH. All samples analyzed by GC-FID. Percent recovery was calculated by subtracting the peak area of each terpenoid from the spiked samples minus the un-spiked controls. This number was then divided by the peak area of pure references diluted in 100 ml ethanol and multiplied by 100.

Linear range, LOD, LOQ, and RF

The linear range was determined empirically by injecting standard compounds in a range of 0.01 mg/ml to 2 mg/ml. The LOD and LOQ were determined empirically and using signal to noise calculations with Chemstation software. The detector response for monoterpenoids, sesquiterpenoids, and cannabinoids was determined by running standard curves (0.02 mg/ml – 1.0 mg/ml) of γ-terpinene, limonene, α-pinene, β-pinene, (*S*)-limonene, camphor, linalool, 1,8-cineol, β-caryophyllene, humulene, caryophyllene oxide, Δ^9-THC, Δ^8-THC, CBD, CBN, and CBG in duplicate.

Instrumental precision

The variation in peak area of the internal standard 1-octanol for all cannabis samples was used to determine precision of the GC-FID.

Data analysis

Principal component analysis (PCA) was performed on SIMCA-P+ version 12.0.0.0 (Umetrics, Umeå Sweden). Unit variance scaling was used. Hierarchical clustering analysis was also done on SIMCA-P+ software and used PC's 1-6 with the Ward method sorted by size.

Results and discussion

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 bred 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* (ie. hybrid indica/sativa) or having traits of both but mostly having traits representative of one of the morphotypes (ie. 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.

Table 1: Cannabis plant information and growth conditions. Vegetative and flowering columns show the number of days each sample was grown in each stage under standard conditions.

Variety	Vegetative	Flowering	Morphological type	Growth conditions
AG (1,2,3)	37	54	hybrid indica/sativa	1 and 2- standard 3- veg[a] +1 wk[b], fl[c] +1 wk
AE (1,2,3)	37	54	hybrid mostly sativa	1 and 2- standard 3- veg +1 wk, fl +1 wk
Ai94 (1,2,3)	37	54	hybrid mostly sativa	1 and 2- standard 3- veg +1 wk, fl +1 wk
AO (1,3,5,6,7)	37	47	indica	1,3,5,6- standard(4 seeds) 7- fl +1 wk
AN	37	54	indica	standard
AD	37	47	indica	standard
AM	37	40	indica	standard
AF	37	54	indica	standard
Bedropuur (A,B,C,D)	37	40	indica	A- veg -1 wk fl +1 wk B- standard C- veg +1 wk D- veg +1 wk fl +1 wk
Bedrocan (C)	37	54	hybrid indica/sativa	bedrocan- standard C- lower branches clipped
Bediol	29	54	indica/sativa/ruderalis	standard

[a]Veg = vegetative, [b]wk = week, [c]fl = flowering.

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 5 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 3 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 2 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 4 batches at the same time with 1 batch grown under standard conditions and the other 3 batches grown with deviations from standard conditions (Table 1).

Method validation

Results of GC method validation are summarized in Table 2. For precision the percent relative standard deviation (RSD) of the peak area of 1-octanol from the 120 cannabis samples analyzed was calculated. The low RSD of 1-octanol (2.8%) indicates that the method was precise in terms of needle injections and FID response over the duration of the analytical period. This period consisted of 130 h of GC time excluding calibration curves and other validation analyses. Reproducibility was determined by comparing peak areas of each compound in a Bedropuur extract for both intraday and interday analyses. All compounds had a RSD of <5%. The reproducibility of a pure compound, γ-terpinene had a RSD of <2%. These low RSD values indicate that the method is reproducible for the analysis of cannabis terpenoids and cannabinoids.

The extraction method chosen for this study had previously been demonstrated to be exhaustive and exhibit a high recovery for the quantitative analysis of cannabinoids by HPLC (Hazekamp, 2007). Therefore we sought to determine whether or not the extraction procedure utilized for cannabinoids was also exhaustive for the terpenoids present in cannabis. Bedrocan was selected because it has been shown to contain high levels of Δ^9-THC and terpenoids (Fischedick et al., 2010). By the fourth ethanol extract only 2% Δ^9-THC compared to the total peak area of Δ^9-THC in the first 3 extracts remained. This was consistent with previous results concerning the recovery of cannabinoids with this extraction method (Hazekamp, 2007). No other residual compounds were detected in the fourth extract indicating that the method is also exhaustive for the extraction of terpenoids in cannabis. Accuracy of the extraction method was demonstrated by determining the recovery of spiked terpenoids. We selected Bedrobinol plant material for this experiment because in previous studies (Fischedick et al., 2010) this plant material was shown to have low levels of β-pinene and β-caryophyllene with no detectable levels of linalool. All terpenoids were completely recovered indicating that the method is accurate for the analysis of cannabis terpenoids (Table 2).

Linear standard curves (r^2> 0.99) for all compounds tested could be generated in the range of 0.01 mg/ml to 1 mg/ml. For Δ^9-THC linear standard curves up to 2 mg/ml could be generated. The limit of quantification (LOQ) for monoterpenoids with a molecular weight (M_r) of 136 was 0.4 mg/g, monoterpenes with oxygen was 0.5 mg/g, sesquiterpenoids with a M_r of 204 was 0.4 mg/g, sesquiterpenoids with oxygen was 0.5 mg/g, and for cannabinoids was 0.6 mg/g. The signal to noise ratio was greater then 1:10 for all compounds present at a concentration above their LOQ. A signal to noise ratio of 1:5 was selected as the limit of detection (LOD) and is therefore half that of the LOQ for each compound group. The response factor (RF) for each compound is shown in table 2. The low variability in RF among compounds with similar mass and chemical structure using the FID is consistent with other research done on the quantification of essential oils with GC-FID (Bicchi et al., 2008). Therefore terpenoids or cannabinoids for which no reference is available could be accurately quantified with components of similar or identical molecular mass/formula.

Qualitative and quantitative analysis of cannabis varieties

Compounds were identified by comparing their mass spectra, and retention times with authentic references as well as literature reports (Adams, 1989; Hillig, 2004; Komori et al., 1968; Ross and ElSohly, 1996; Rothschild et al., 2005). The NIST library was also used to assist in compound identification. A summary of quantitative data for all compounds in all 11 cannabis varieties is shown in Table 3. The compounds γ-terpinene, limonene, α-pinene, β-pinene, linalool, β-caryophyllene, humulene, Δ^9-THC, *trans*-(−)-Δ^8-tetrahydrocannabinold (Δ^8-THC), CBD, and cannabigerol (CBG) were quantified using their standard curve RF values. All other compounds were quantified using the average RF for compounds with the closest molecular mass/formula. In total 36 compounds were quantified.

The sesquiterpenoids δ-guaiene and bulnesol are reported as putatively identified because they have not been reported in cannabis previously and a reference compound was not available for structural confirmation. Five compounds could not be identified so we report their characteristic mass ions. Unknown monoterpenoid TP(1) *m/z*: 152 [M^+], 91, 84, 69 (base). The unknown sesquiterpenoids (SQ) SQ(1) *m/z*: 204 [M^+], 161 (base), 133, 105; SQ(2) *m/z*: 236 [M^+], 204, 161, 119, 93 (base); SQ(3) *m/z*: 204 [M^+], 161 (base), 122, 93. The unknown cannabinoid CB(1) *m/z*: 356 [M^+], 313, 297 (base), 243, 231. The monoterpenoid TP(1), we suspect is oxygen substituted due to its M_r of 152. The unknown sesquiterpenoids SQ(1) and SQ(3) appear to have been reported as unknowns in previous studies (Hillig, 2004; Ross and ElSohly, 1996). SQ(2) is a sesquiterpenoid with unknown substitution. No detectable levels of the Δ^9-THC breakdown product CBN were detected in any samples. This indicates that the drying and storage process used in this study resulted in no significant amount of Δ^9-THC degradation except perhaps that of *trans*-(-)-Δ^9-tetrahydrocannabinolic acid A (THCA) into Δ^9-THC.

Figure 1 Correlation of cannabinoid versus terpenoid levels

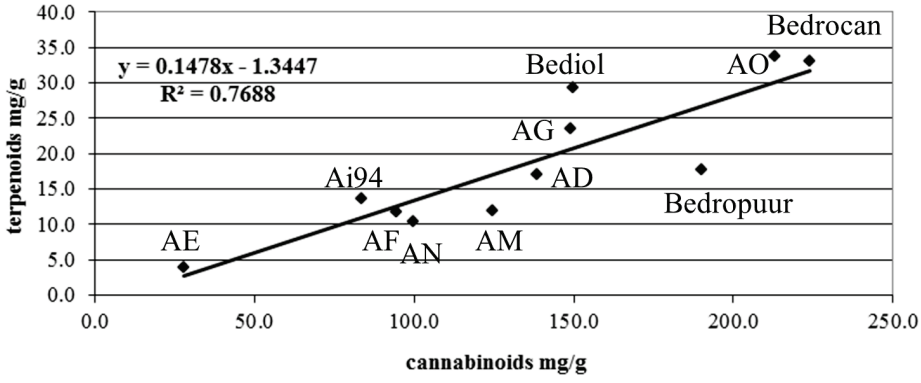


Table 2: Validation results.

Reproducibility			**Comparison RF**	
Extract	**Intraday (n=5) RSD**	**Interday (n=15) RSD**	**TP[a] M_r = 136**	**RF**
β-pinene	0.4	0.5	γ-terpinene	4604
myrcene	0.5	0.4	(*R*)-limonene	4598
limonene	0.7	0.5	α-pinene	4625
1-octanol	1.3	0.6	β-pinene	4753
linalool	1.2	0.6	(*S*)-limonene	4649
β-caryophyllene	1.3	0.5	average	4646
humulene	1.3	0.5	RSD	1.4
δ-guaiene	4.2	1.6	**TP with oxygen**	
SQ(1)	1.9	0.6	camphor	3894
SQ(2)	4.9	1.1	linalool	3936
elemene	1.7	0.6	1,8-cineol	3827
guaiol	1.5	0.7	average	3886
γ-eudesmol	1.4	1.3	RSD	1.4
THCV	1.1	1.6	**SQ[b] M_r = 204**	
CBC	2.6	1.3	β-caryophyllene	4754
CBGM	1.9	0.4	humulene	4661
Δ^9-THC	1.6	0.9	average	4708
CBG	2.0	0.8	RSD	1.4
Pure compound			**SQ with oxygen**	
γ-terpinene	0.5	1.9	caryophyllene-oxide	4285
Precision	**RSD (n=120)**		**Cannabinoids**	
1-octanol	2.8		Δ^9-THC	3490
Extraction efficiency	**% Remaining (n=3)**		Δ^8-THC	3674
Δ^9-THC	2		CBD	3502
Accuracy	**% Recovery (n=5)**	**RSD**	CBN	3521
β-pinene	102	3.2	CBG	3614
linalool	102	2.2	average	3560
β-caryophyllene	100	3.5	RSD	2.2

[a]TP = terpenoid. [b]Sesquiterpenoid.

Table 3: Quantitative data for cannabis strains (mg/g plant material). Compounds with no ± had standard deviations of < 100 µg.

Compound	RR_t[a]	AO	Bedropuur	Bedrocan	Bediol	AG	AE	Ai94	AN	AF	AM	AD
number of samples		25	20	10	5	15	15	15	5	5	5	5
α-pinene	0.26	6.7±2.4	0.5	0.8±0.1	1.7±0.1	2.8±0.4	Tr[c]	0.8±0.5	Tr	0.5	3.2±0.1	ND[b]
β-pinene	0.31	1.9±0.6	0.7±0.1	1.2±0.2	0.8±0.1	1.4±0.2	Tr	0.5±0.1	Tr	0.7	1.2	Tr
myrcene	0.33	14.8±7.3	1.6±0.7	6.1±1	19±1.9	13±1.6	0.4±0.1	7.1±0.8	0.8±0.2	3.4±0.1	6.7±0.6	13.8±0.9
α-phellandrene	0.35	ND	ND	0.6±0.1	Tr	ND	Tr	Tr	ND	Tr	ND	ND
Δ3-carene	0.36	ND	ND	0.5±0.2	Tr	ND	Tr	Tr	ND	Tr	ND	ND
α-terpinene	0.36	ND	ND	0.4	Tr	ND	Tr	Tr	ND	Tr	ND	ND
β-phellandrene	0.38	ND	ND	2.1±0.3	0.7±0.1	ND	Tr	0.5	ND	1.0	ND	Tr
limonene	0.38	2.5±0.7	4.9±0.8	ND	ND	2.4±0.4	ND	ND	2.3±0.3	ND	0.7±0.1	ND
cis-ocimene	0.40	1.4±0.4	ND	3.9±0.6	1±0.1	0.6±0.1	0.7±0.1	1±0.1	ND	1.0±0.1	ND	ND
terpinolene	0.47	ND	ND	11.3±1.7	3.7±0.4	ND	1.9±0.9	2.9±0.3	ND	5.4±0.2	ND	ND
linalool	0.48	0.9±0.1	1.2±0.2	Tr	ND	Tr	ND	Tr	Tr	Tr	Tr	Tr
terpineol	0.63	Tr	Tr	0.7±0.1	0.6	ND	Tr	Tr	Tr	Tr	Tr	ND
TP(1)	0.86	ND	ND	0.7±0.2	Tr	ND	Tr	ND	ND	Tr	ND	ND
β-caryophyllene	1.00	3.5±2.4	2.6±0.2	1.6±0.2	0.8±0.1	1.4±0.4	0.5±0.3	0.8±0.4	1.4±0.2	0.5	0.5	2.0±0.1
α-guaiene	1.03	ND	Tr	Tr	Tr	0.5	ND	Tr	ND	Tr	Tr	Tr
humulene	1.05	1.2±0.9	0.8±0.1	0.9±0.1	0.6±0.1	0.7±0.1	Tr	0.7±0.1	Tr	Tr	ND	0.6
δ-guaiene[P]	1.13	ND	0.7±0.1	0.8±0.1	0.8	0.7±0.2	ND	ND	ND	ND	ND	0.6
SQ(1)	1.18	0.5±0.1	0.6±0.1	0.6±0.1	Tr	Tr	ND	ND	1.3±0.1	Tr	ND	ND
SQ(2)	1.19	0.6±0.2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SQ(3)	1.19	ND	1.0±0.2	0.6±0.1	Tr	0.5±0.1	Tr	ND	1.8±0.1	ND	ND	ND
elemene	1.21	1.1±0.3	2.3±0.4	1.3±0.3	Tr	0.6±0.1	0.8±0.2	ND	2.7±0.6	Tr	Tr	ND

guaiol	1.27	0.6±0.2	0.6	ND	ND	ND	0.4	ND	ND	Tr	ND	ND
γ-eudesmol	1.30	0.7±0.2	0.6	ND	ND	ND	0.4	ND	ND	Tr	ND	ND
β-eudesmol	1.34	0.4	Tr	ND	ND	ND	Tr	ND	ND	Tr	ND	ND
agarospirol	1.35	0.5±0.1	Tr	ND	ND	ND	Tr	ND	ND	Tr	ND	ND
bulnesol[P]	1.37	0.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
α-bisabolol	1.39	0.5±0.1	Tr	ND	ND	ND	ND	ND	0.5±0.1	ND	ND	Tr
CBDV	2.04	ND	ND	ND	Tr	ND	ND	1.5	ND	ND	ND	ND
THCV	2.15	1.3±0.4	1.3±0.3	1.3±0.2	Tr	0.7±0.1	Tr	ND	0.6	0.7±0.1	1±0.1	0.6
CBD	2.27	Tr	Tr	Tr	79.8±1.8	ND	ND	73.6±2.1	ND1	ND	Tr	ND
CBC	2.27	2.1±0.4	2.1±0.3	2.3±0.1	5.4±0.1	1.4±0.1	1.6±0.8	4.6±0.3	0.9±0.1	0.9	1.4±0.1	2.2±0.2
CB(1)	2.32	0.8±0.5	1.1±0.2	1.6±0.3	1.2	0.7	ND	ND	Tr	Tr	Tr	Tr
CBGM	2.32	ND	1.1±0.1	ND	ND	ND	ND	ND	1.8±0.3	2.6±0.1	Tr	Tr
Δ8-THC	2.33	Tr	0.7±0.1	Tr	Tr	ND	ND	1.2±0.1	ND	0.6	Tr	Tr
Δ9-THC	2.37	199.2 ±33	181 ±21	207.5 ±19	61.5 ±1.9	144.1 ±16	26 ±7	3.4 ±0.6	95.2 ±6.7	87.2 ±3.4	120.1 ±5.5	134.9 ±6.6
CBG	2.42	10±1.6	4.1±1.3	11.2±2	1.7±0.2	2.8±0.6	1±0.4	ND	1.9±0.2	3.0±0.3	2.1±0.1	1.2±0.1

[a]*RRt* relative retention time to β-caryophyllene. [b]ND = not detected (< LOD). [c]Tr = trace levels detected (< LOQ). [p]Putative identification.

Higher levels of cannabinoids were positively correlated to higher levels of terpenoids (Figure 1). Both the cannabinoids and terpenoids of cannabis are localized primarily in glandular trichomes (Malingre et al., 1975; Taura et al., 2007). This may explain why in some varieties their levels are correlated, however it does not prove that their biosynthesis is necessarily correlated. This is demonstrated by the Bedropuur variety whose Δ^9-THC levels are high but its terpenoid levels are similar to the varieties AD and AG. This suggests that it is possible to breed cannabis that contains high levels of Δ^9-THC but not necessarily higher levels of terpenoids.

Figure 2 PCA of all cannabis varieties. PC1 versus PC2, scatter plot (top) and loading plot (bottom). (1) AO, (2) Bedropuur, (3) Bedrocan, (4) Bediol, (5) AG, (6) AE, (7) Ai94, (8) AN, (9) AF, (10) AM, and (11) AD.

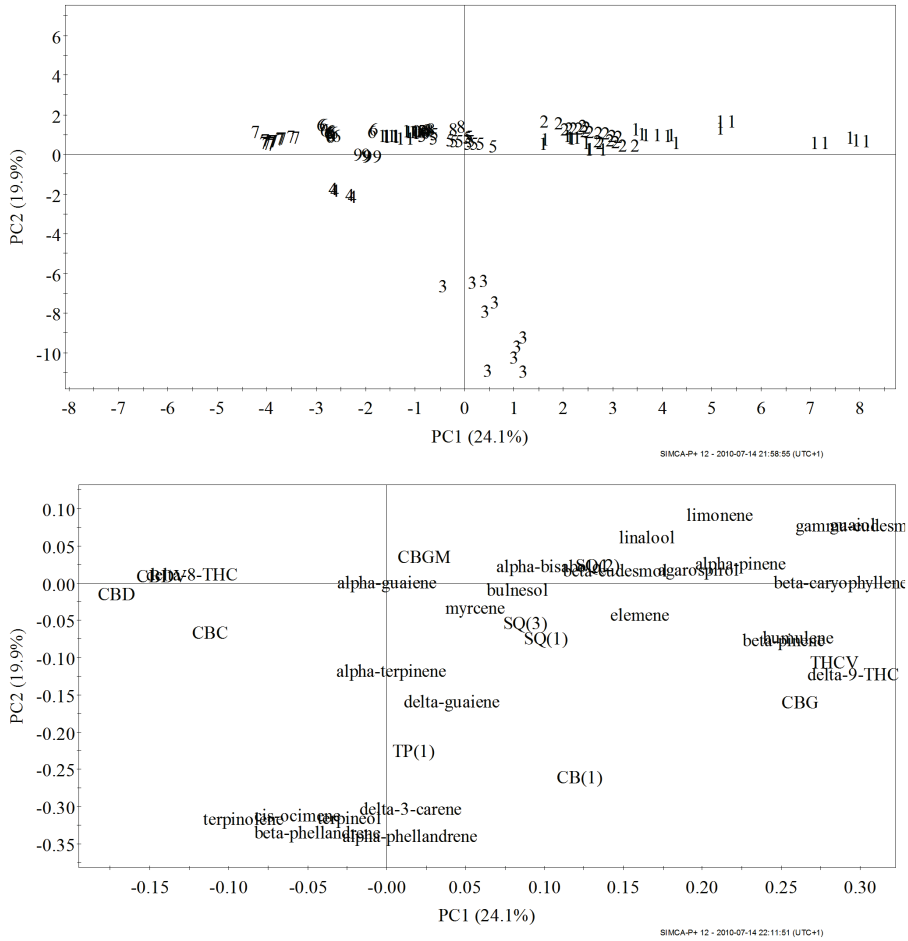
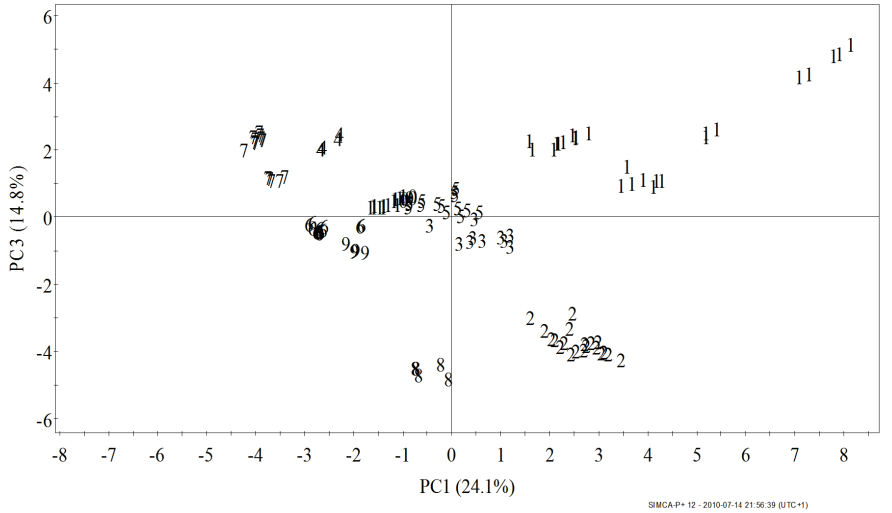
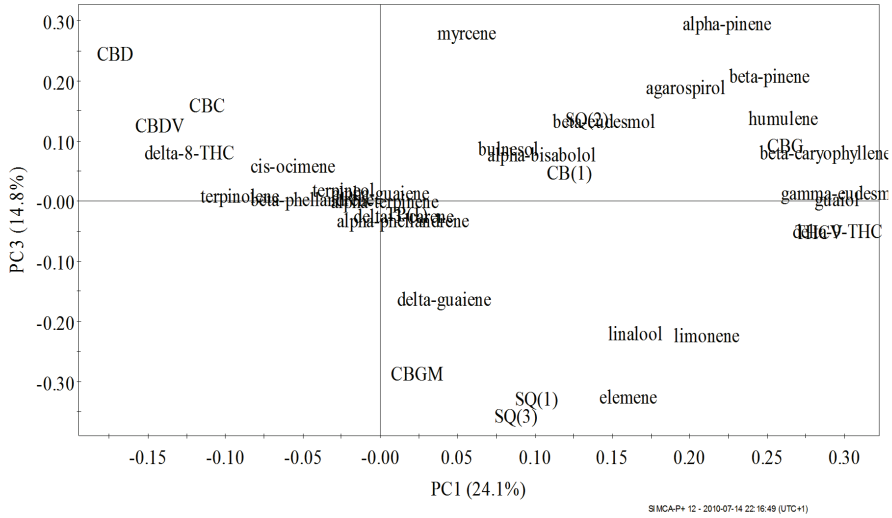


Figure 3 PCA of all cannabis varieties. PC1 versus PC3, scatter plot (top) and loading plot (bottom). (1) AO, (2) Bedropuur, (3) Bedrocan, (4) Bediol, (5) AG, (6) AE, (7) Ai94, (8) AN, (9) AF, (10) AM, and (11) AD.





Metabolic fingerprinting of cannabis

It's clear from the data that each cannabis variety is both qualitatively and quantitatively different (Table 3). The levels of Δ^9-THC ranged from 20.8% (Bedrocan) to 0.3% (Ai94). Bedrocan, Bedropuur, and AO all contained high levels of Δ^9-THC (>15%). AG, AM, AD, AN, and AF all contained a medium level of Δ^9-THC (<15%, >5%). Bediol also contained a medium level of Δ^9-THC (6%) however its relatively

high level of CBD (8%) makes it unique compared to the other varieties. AE and Ai94 contained low amounts of Δ^9-THC (<5%). Ai94 contained a relatively high level of CBD (7.4%) compared to the other varieties. Ai94 also contained the C_3 side chain variant of CBD, (-)-cannabidivarin (CBDV). Bediol only contained trace levels of CBDV. The levels of propyl side chain analogues of cannabinoids have been reported to be of chemotaxonomic significance. It has been hypothesized that the enzymes involved in enhancing the levels of these compounds originate from *C. indica* and are not commonly present in *C. sativa* subtype (Hillig and Mahlberg, 2004). CBD was only detected in trace amounts (<0.6 mg/g) or not at all among the high, medium, and low Δ^9-THC varieties making them representatives of the drug/Δ^9-THC chemotype. Bediol is representative of the intermediate chemotype and Ai94 is representative of the fiber/CBD chemotype. The levels of Δ^9-THC and CBD alone do not chemically distinguish the high or medium Δ^9-THC containing varieties well from one another.

Therefore to further chemically classify cannabis principle component analysis (PCA) was used. PCA is a multivariate projection method which extracts and displays systemic variation from a set of matrix data consisting of observations and variables (Eriksson et al.,, 2006). The 36 compounds were the variables and their mg/g levels the observations. Initially all the cannabis samples were analyzed by PCA (Figure 2). Principal component 1 (PC1) and PC2 explains 44% of the variance. The highest Δ^9-THC containing varieties Bedropuur, Bedrocan, and AO are separated along the positive PC1. The Bedrocan variety was also well separated along negative PC2. The compounds responsible for making Bedrocan different according to the loading plot are terpinolene, β-phellandrene, α-phellandrene, terpineol, *cis*-ocimene, and Δ^3-carene. Bedrocan contained higher levels of these compounds compared with other varieties. Terpinolene was a very dominant monoterpenoid (11.3 mg/g) in the Bedrocan variety (Table 3). Bediol partially separated along the negative PC2 also contained terpinolene, β-phellandrene, α-phellandrene, terpineol, and Δ^3-carene but in lower levels than Bedrocan. This observation is interesting because the Bediol variety was bred by hybridizing the Bedrocan variety with higher CBD containing varieties.

The loading plot along the positive PC1 and positive PC2 shows that Bedropuur and AO contained more of the sesquiterpene alcohols guaiol, γ-eudesmol, β-eudesmol (Figure 2). The study by Hillig (2004) reported that guaiol, γ-eudesmol, and β-eudesmol were characteristic terpenoid compounds of the *C. indica* varieties originating from Afghanistan. These sesquiterpenoid alcohols appear to be important in distinguishing *C. indica* varieties from one another because the AG, AN, AM, and AD varieties which are also *C. indica* morphotypes did not contain detectable levels of these compounds. AF another *C. indica* only contained trace amounts of these compounds (Table 3). In order to distinguish Bedropuur and AO further PC1 and PC3 were compared (Figure 3). PC3 was able to explain an additional 15% of the variance. The Bedropuur variety contained higher levels of limonene as well as the sesquiterpenoid elemene while the AO variety contained higher levels of myrcene and α-pinene. Also along PC3 information was obtained about the AN variety which contains a medium level of Δ^9-THC (95.2 mg/g) but higher levels of the sesquiterpenoids SQ(1), SQ(3), and elemene when compared to all other varieties.

The medium Δ^9-THC varieties AG, AF, AM, and AD were not well separated along PC1, 2, or 3. Therefore these varieties were reanalyzed by PCA with all other varieties excluded (Figure 4). AG and AD had higher and very similar levels of Δ^9-THC, myrcene, and β-caryophyllene compared with AM and AF. AG and AD were distinguished along PC2. AG contained more α-pinene, β-pinene, limonene, α-guaiene, elemene, and SQ(3). AD however contained low levels of monoterpenoids and sesquiterpenoids in general and only slightly higher levels (<1.0 mg/g) of myrcene, β-caryophyllene, and CBC compared to AG. AF contained the highest levels of cannabigerol monomethyl ether (CBGM) while AM contained higher levels of myrcene and α-pinene.

Hierarchical cluster analysis (HCA) was used to confirm the PCA analysis (Figure 5). The AO batches are all clustered together and each genotype (different seed) is also grouped together. The Bedropuur batches are clustered together and AN was the next similar variety. Both Bedropuur and AN were separated along the negative PC3 (Figure 3) because of the presence of the cannabinoid CBGM as well as numerous similarities in monoterpenoids and sesquiterpenoids (Table 3). Bedrocan was in its own group which is consistent with PCA analysis. The clipped Bedrocan batch exhibits some differences according to HCA when compared to unclipped. Bediol and Ai94 are related due to higher levels of CBD but each clustering on their own. Ai94(2) however was clustered closer too AE and AF most likely because of small differences caused by growing this variety in different batches. The medium Δ^9-THC varieties are all clustered close to one another except AF. AF was closer to AE and Ai94(2) most likely because it has the lowest amount of Δ^9-THC compared to the other medium varieties. Each medium Δ^9-THC variety is clustered with itself except AG because it was grown in different batches which caused small differences in chemical profile. The morphotype of each medium Δ^9-THC variety does not seem important in relating these varieties.

These observations represent a significant improvement compared with other methodologies, discussed in the introduction, using chemotaxonomy to discriminate cannabis varieties. By using quantitative data on cannabinoid and terpenoid levels it was possible to chemically distinguish each variety from one another with the aid of PCA. Both Hillig (2004) and Hillig and Mahlberg (2004) had difficulty discriminating drug type cannabis accessions from one another. Furthermore the conclusion in the study of Hillig (2004) that sesquiterpenoids were more important than monoterpenoids in chemically differentiating cannabis varieties is not accurate. In this study monoterpenoids were able to distinguish varieties which had similar sesquiterpenoid levels and similar cannabinoid levels such as AO and Bedropuur as well as a number of the medium Δ^9-THC varieties.

Effect of growing cannabis in different batches and growth cycle deviations

The effect on chemical profile from growing cannabis varieties in separate batches about a month apart as well 1 week extra vegetative and flowering periods was studied in the AG, AE, and Ai94 varieties. A comparison of the compounds within the AG varieties batches is shown in Figure 6. The differences between each batch were

minor with no clear distinction between them. The largest differences are between AG(1) and AG(2) with the level of myrcene being 1.8 mg/g higher on average in AG(1) compared with AG(2), β-caryophyllene being 0.5 mg/g higher on average in AG(2) compared to AG(1), CBG being 0.8 mg/g higher in AG(2) compared to AG(1), and Δ^9-THC being 17.3 mg/g higher in AG(2) compared to AG(1). Most compounds in the AE batches did not differ much in concentration (<1.0 mg/g), except terpinolene and Δ^9-THC (Figure 7). The Ai94 batches also only had minor differences (Figure 8). These results importantly demonstrate that genetically identical cannabis plants grown in batches at separate times under standardized environmental conditions are reproducible in terms of terpenoid and cannabinoid concentrations.

Figure 4 PCA of the varieties AG, AD, AM, and AF. PC1 versus PC2 scatter plot (top) and loading plot (bottom).

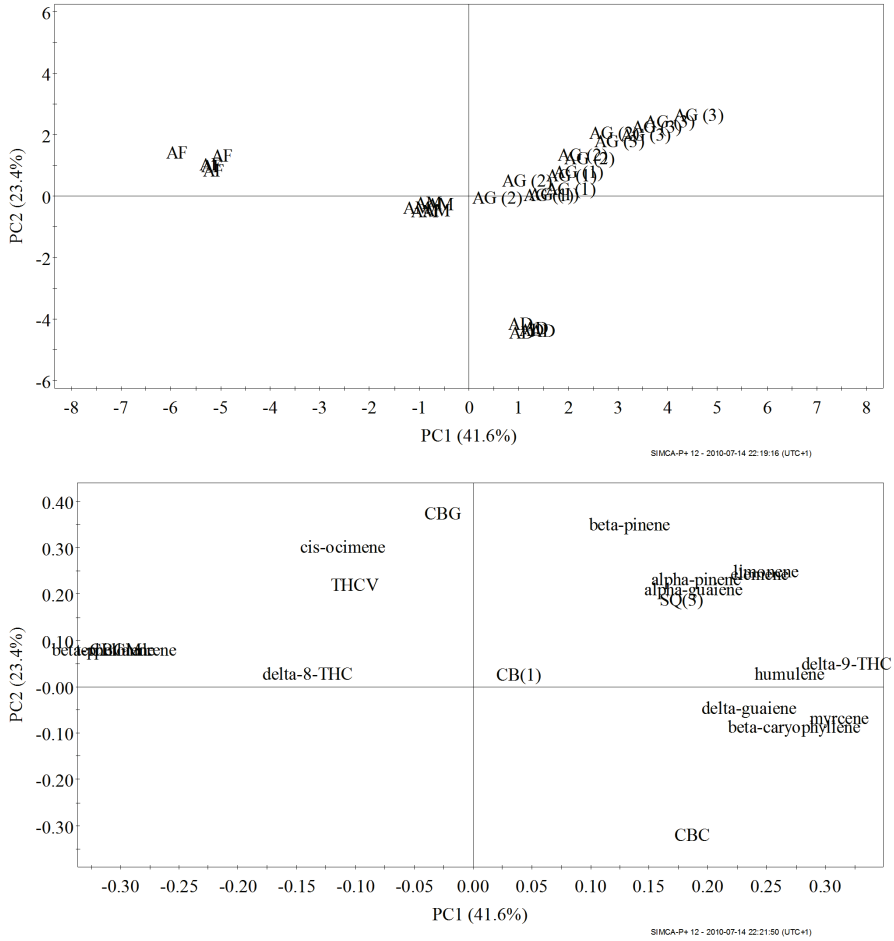
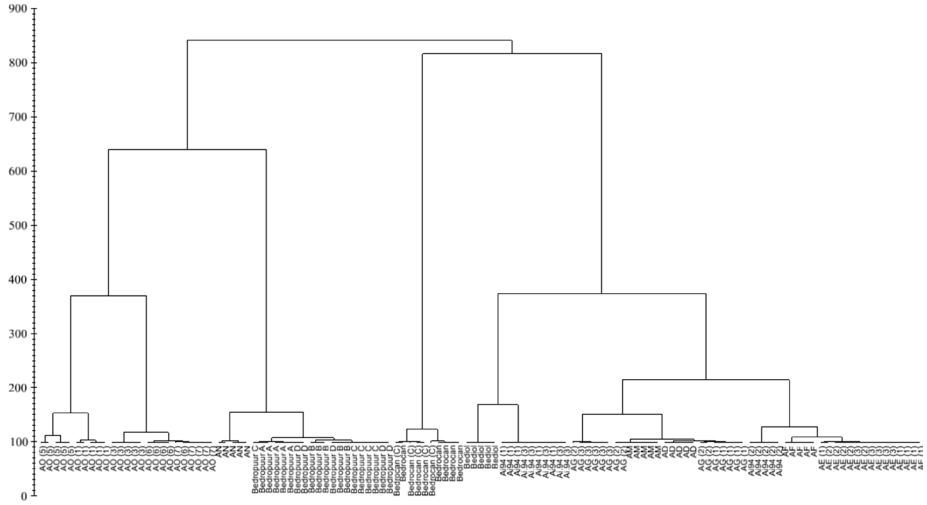


Figure 5 Hierarchical clustering analysis of all cannabis samples.



A detailed look into the chemical variation among the Bedropuur batches is shown in Figure 9. Batches A, C, and D differed in the concentrations of certain compounds compared with the standard batch B. The levels of limonene were lower in A, C, and D. Myrcene was lower in A and D compared with B and C. The levels of Δ^9-THC were about 30 mg/g higher in Bedropuur C compared to the other 3 batches. Bedropuur D had the lowest amount of Δ^9-THC. Bedropuur A had lower concentrations of the β-caryophyllene, elemene, and CBG when compared to batches B and C. These results demonstrate that alterations in growth cycle time can cause changes in the chemical profile of cannabis plants grown under environmental conditions that were otherwise the same. Alterations in growth cycle time appear to cause more differences in a cannabis varieties chemical profile then growing the plant material in different batches. However more experiments with more varieties, grown with more deviations in growth cycle time, and more replicates would be needed to confirm these observations.

Clipping the lower branches on the Bedrocan variety caused some compounds to be present at lower concentrations (Figure 10). These compounds include myrcene, *cis*-ocimene, β-caryophyllene, elemene, CBG, and Δ^9-THC. This suggests that by clipping the lower branches, which would allow more water and nutrients to flow to the upper parts of the plant closest to the light, does causes some changes in the chemical profile. Further experiments would be needed to determine if this represents a consistent pattern and explain why it occurs.

The different AO batches exhibited the greatest quantitative differences in chemical profile compared with all other varieties (Figure 11). AO batches exhibited a wide range of concentrations for α-pinene, myrcene, β-caryophyllene, and Δ^9-THC. The different AO batches could even be clearly distinguished by PCA (Figure 12). This observation shows that by metabolically profiling cannabis strains based on cannabinoid

and terpenoid levels it is also possible to distinguish separate genotypes of the same variety.

Overall these experiments demonstrate that the best way to grow reproducible batches of cannabis is by using genetically identical plant material grown from clones, under standardized environmental conditions, with the same growth cycle. Deviations in growth cycle and clipping of lower branches can cause quantitative differences, although minor in absolute terms, in chemical profile. These deviations can obscure their chemical classification as was observed in the HCA. Cannabis plants from seeds representing different genotypes but the same variety can differ considerably in quantitative chemical profile. Future research should aim to determine if cannabis could be grown in such a reproducible manner for many years. As a preliminary indication of chemical profile reproducibility a previous study in our laboratory using similar methodology analyzed the Bedrocan variety. This plant material was grown about 1 year previously to the batches analyzed in this study. This batch had similar levels of the main compounds observed in the present study (Fischedick et al., 2010).

Conclusions

In this study a simple quantitative GC-FID method was validated for the quantitative analysis of cannabis monoterpenoids, sesquiterpenoids, and cannabinoids. Quantitative GC data was used to chemically discriminate cannabis varieties with the aid of principal component analysis. Our results show for the first time using validated methodology the absolute (mg/g) levels of cannabinoids and terpenoids in cannabis simultaneously. This data can be useful for guiding pharmacological or clinical studies that want to examine the potential interactions of the volatile constituents of cannabis. The chemical profile of cannabis varieties could potentially be more closely correlated to therapeutic effectiveness. The reported methodology could be implemented in the quality control of medicinal cannabis. Our methodology also appears to be able to overcome the difficulties in chemotaxonomic analysis of cannabis observed by other researchers in distinguishing drug type cannabis varieties from one another. These techniques should be applied on a wider range of cannabis samples representing both geographically and morphologically distinct varieties. By combining genomic approaches with metabolic fingerprinting it may be possible to elucidate exactly which biochemical pathways differ in various cannabis varieties and how these differences lead to the observed chemical profile.

Figure 6 Comparison of AG batches. Compounds that are missing in certain batches were present at levels < LOQ.

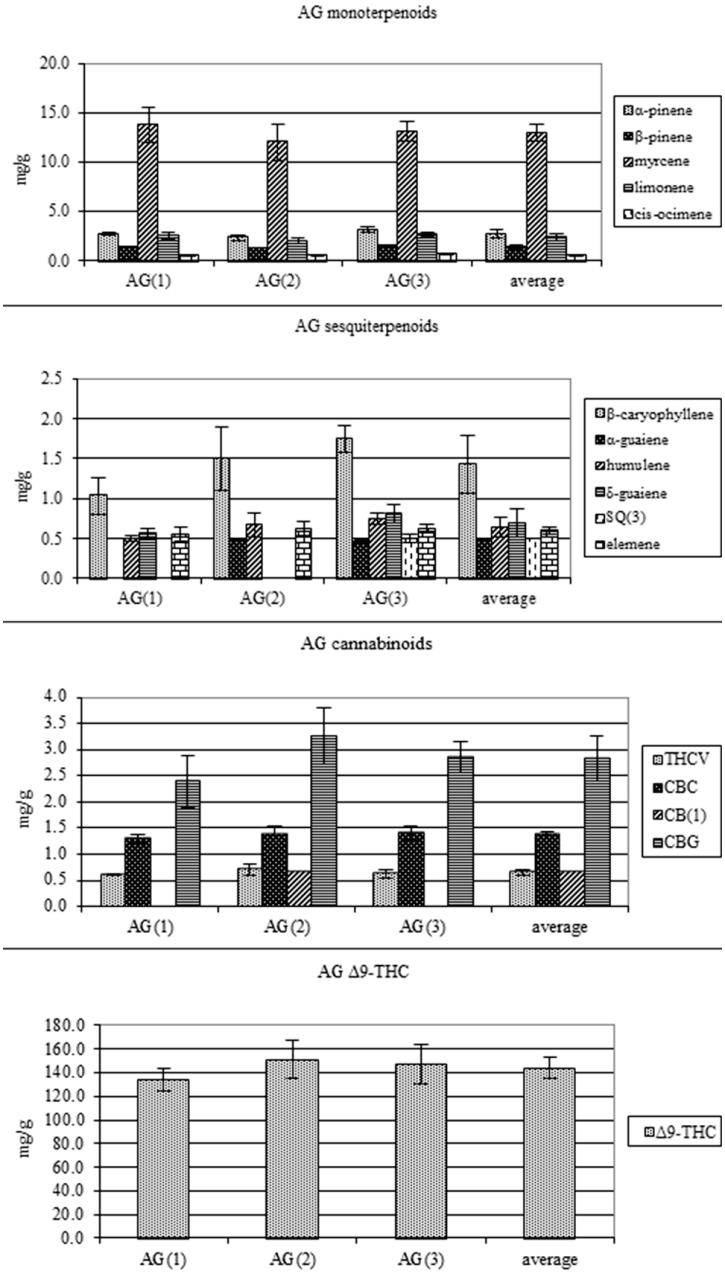


Figure 7 Comparison of AE batches. Compounds that are missing in certain batches were present at levels < LOQ.

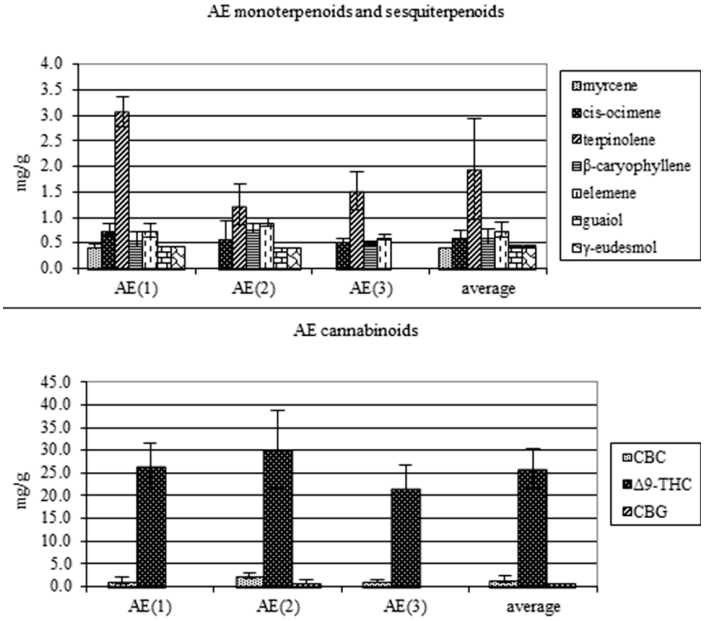


Figure 8 Comparison of Ai94 batches. Compounds that are missing in certain batches were present at levels < LOQ.

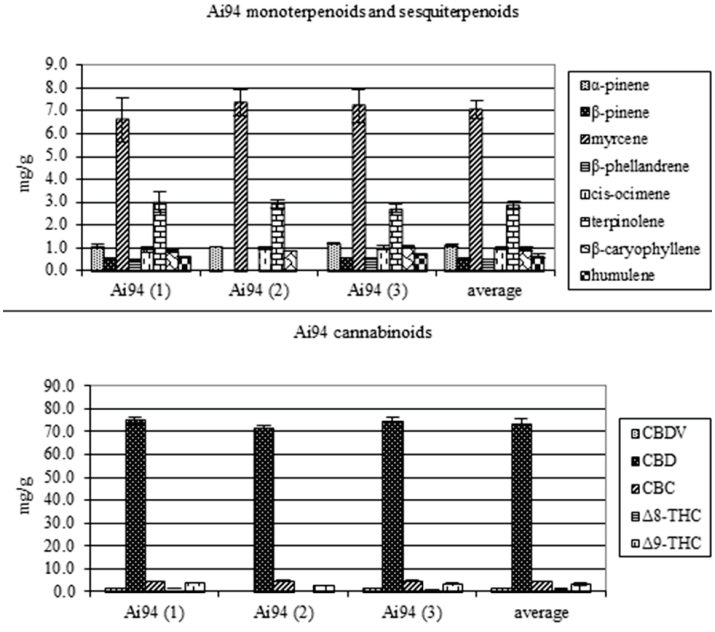


Figure 9 Comparison of Bedropuur batches. Compounds that are missing in certain batches were present at levels < LOQ.

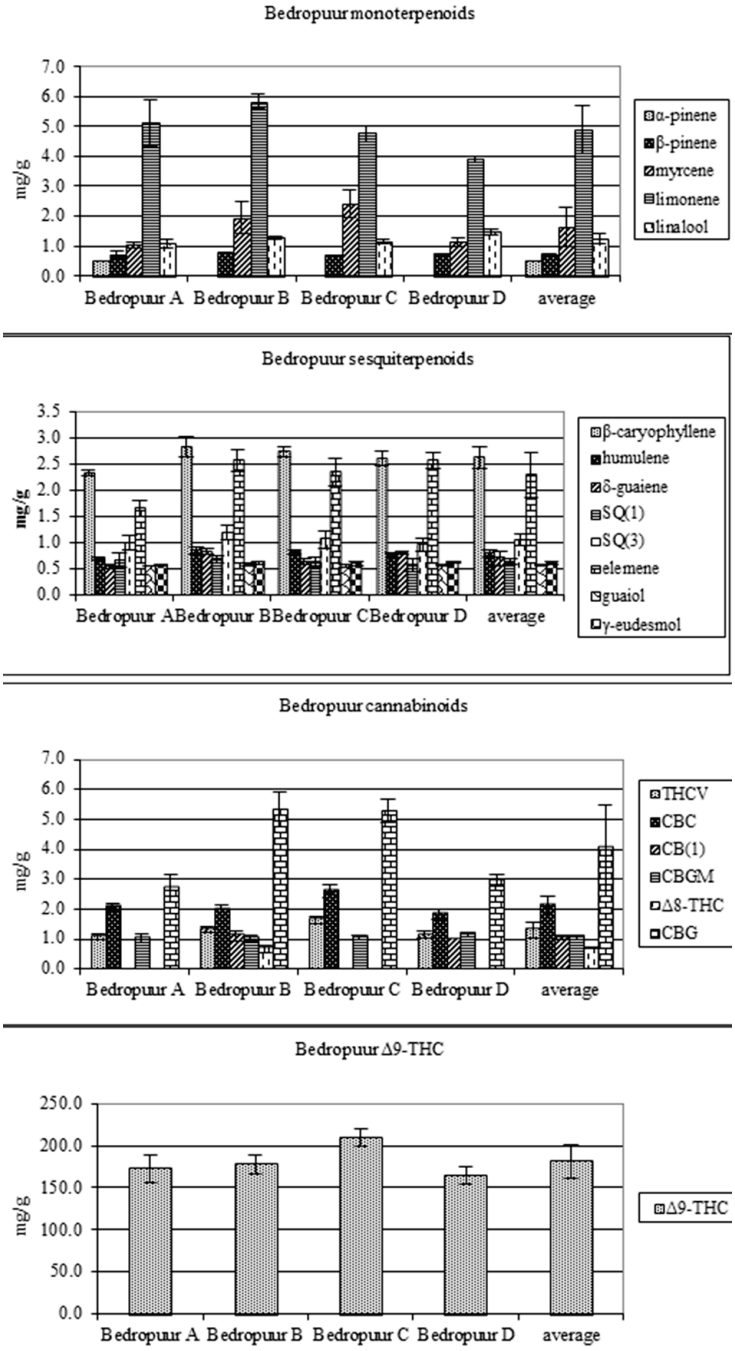


Figure 10 Comparison of Bedrocan batches. Compounds that are missing in certain batches were present at levels < LOQ.

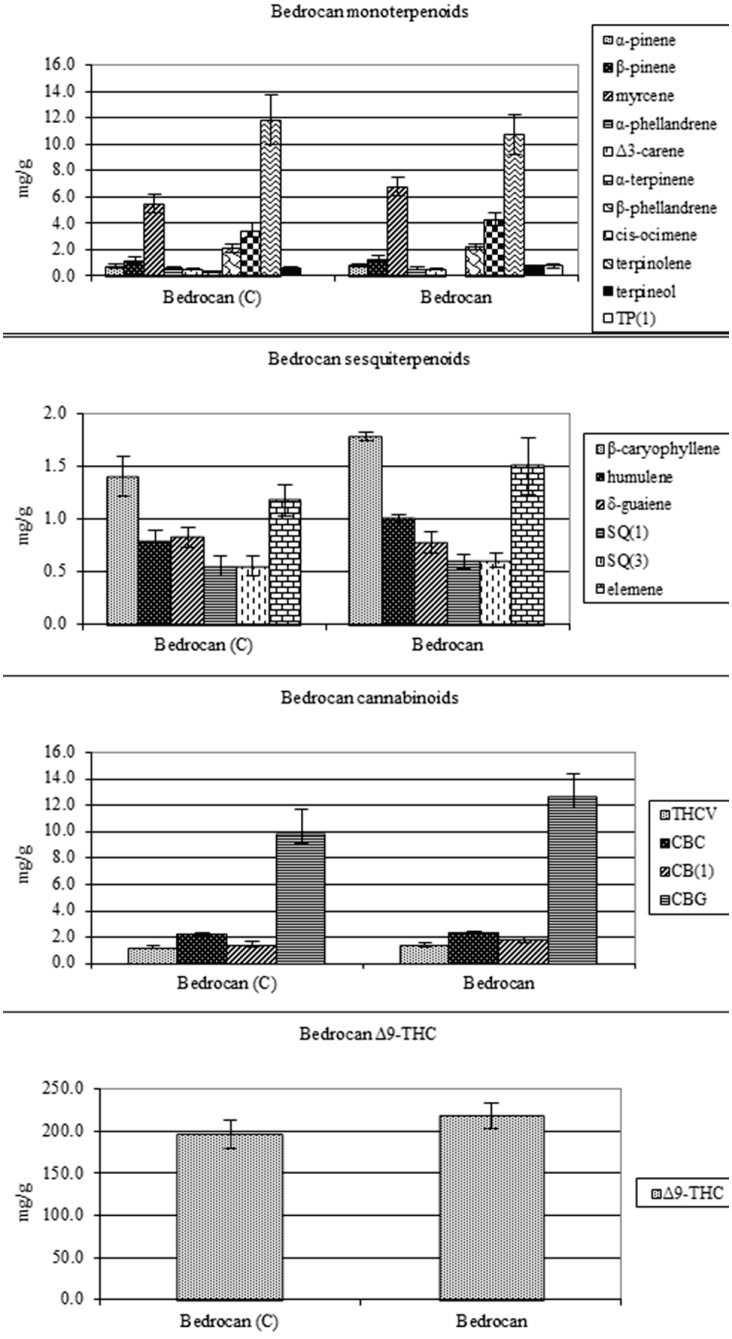


Figure 11 Comparison of AO seed batches. Compounds that are missing in certain batches were present at levels < LOQ.

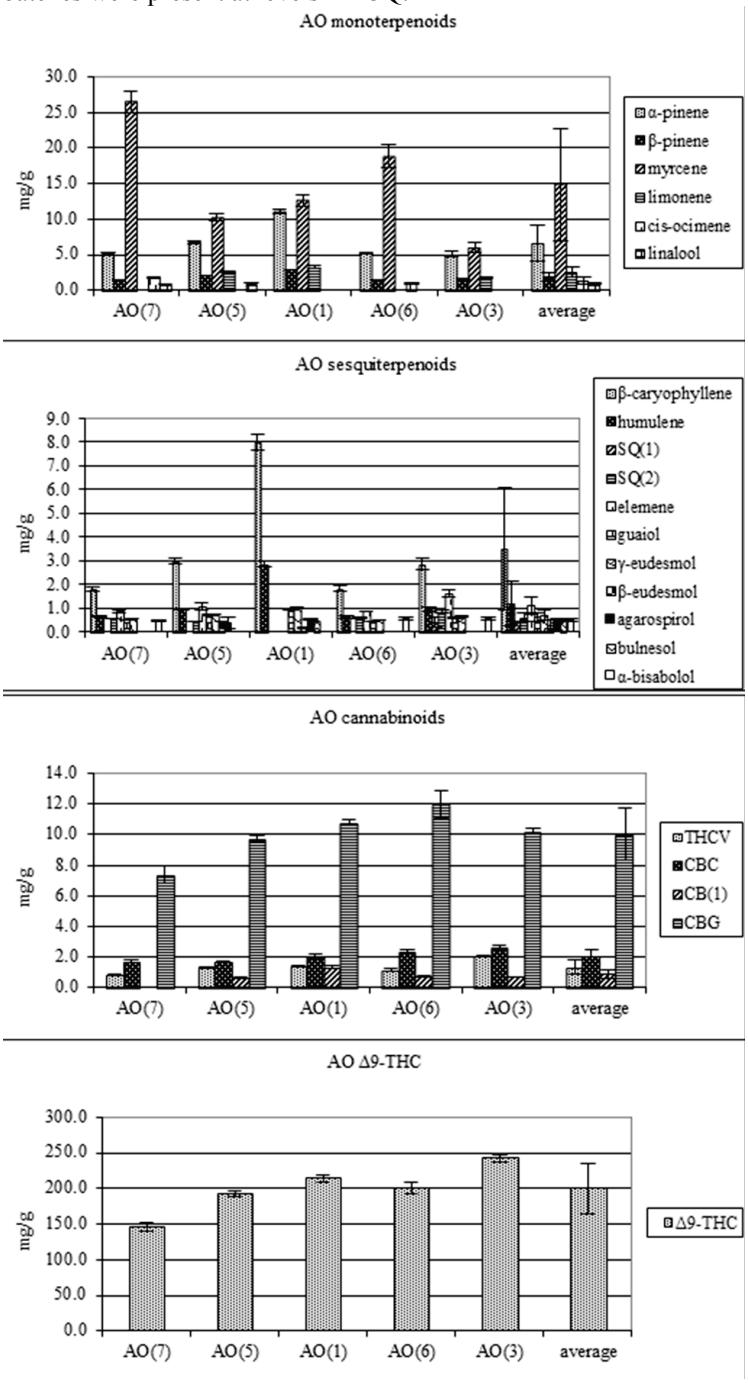
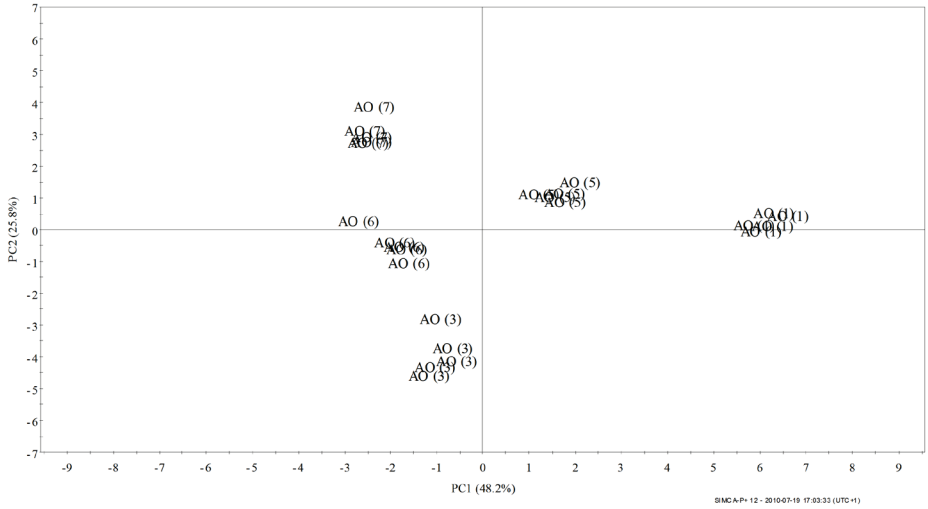


Figure 12 PCA loading plot PC1 versus PC2 of AO seed batches.



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