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Direct NMR analysis of cannabis water extracts and tinctures and semi-quantitative data on Δ^9-THC and Δ^9-THC-acid

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Abstract

Cannabis sativa L. is the source for a whole series of chemically diverse bioactive compounds that are currently under intensive pharmaceutical investigation. In this work, hot and cold water extracts as well as ethanol/water mixtures (tinctures) of cannabis were compared in order to better understand how these extracts differ in their overall composition. NMR analysis and *in vitro* cell assays of crude extracts and fractions were performed. Manufacturing procedures to produce natural remedies can strongly affect the final composition of the herbal medicines. Temperature and polarity of the solvents used for the extraction resulted to be two factors that affect the total amount of Δ^9-THC in the extracts and its relative quantity with respect to Δ^9-THC-acid and other metabolites. Diffusion-edited ^{1}H NMR (1D DOSY) and ^{1}H NMR with suppression of the ethanol and water signals were used. With this method it was possible, without any evaporation or separation step, to distinguish between tinctures from different cannabis cultivars. This approach is proposed as a direct analysis of plant tinctures.

Keywords: Cannabis; Cannabis water extract; Tinctures; Nuclear magnetic resonance; Solvent signal suppression; Principal component analysis; NFκB

1. Introduction

The potential use of cannabis for medical purposes is currently under intensive investigation (Ben Amar, 2006). Around 500 cannabis metabolites are known (El Sohly and Slade, 2005). About 70 structures belong to the typical class of terpenophenolic derivatives known as cannabinoids; (−)-Δ^9-*trans*-(6a*R*,10a*R*)-tetrahydrocannabinol (Δ^9-THC) is probably the best studied cannabis constituent, but not the only one to have proven pharmacological activities (Barrett et al., 1985; Fairbairn and Pickens, 1987; Thomas et al., 2005; Wilkinson et al., 2003). In fact, Δ^9-THC-acid (Verhoeckx et al., 2006), cannabidiol (CBD) (Mechoulam et al., 2002), cannabigerol (CBG) and cannabinol (CBN) (Wilkinson and Williamson, 2007), for instance, also show important therapeutic effects without causing the undesired psychotropic activity typical of Δ^9-THC. Furthermore, the bioactivities of Δ^9-THC alone are different from those of a crude cannabis extract, as synergistic effects have been reported (Russo and Guy, 2006). The presence of alkaloids in very low amounts has been described in both roots and leaves of cannabis (El Sohly, 1985; Mechoulam, 1988). This crude alkaloid mixture is endowed with anti-inflammatory effects but at the moment there is no pharmacological data on the purified alkaloids.

This chemical and pharmacological complexity led to the re-introduction of cannabis crude extracts in clinics. Bedrocan®, a special cannabis variety with standardised content of Δ^9-THC and CBD is sold in Dutch pharmacies

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and Sativex®, a medicine consisting in the mixture of two cannabis extracts rich in Δ^9-THC and CBD, respectively, has been registered in Canada for the treatment of multiple sclerosis symptoms (Barnes, 2006). However, there is a need to develop medicinal cannabis with low contents of Δ^9-THC. This is the goal of an EU-funded research project coordinated by our research group. As part of this, we decided to study the chemistry and pharmacology of both the cannabis aqueous extracts (infusions) and hydroethanolic extracts (tinctures) in order to establish their metabolite profiles with emphasis on the relationship between the ratio Δ^9-THC/Δ^9-THC-acid and the extractive process.

Cannabis tea (water extract) is a popular remedy (Ware et al., 2005) while, prior to 1971, cannabis tinctures were available for prescription by British physicians (House of Lords, 1998) and can be considered as the traditional and officinal ways to consume medicinal cannabis products, respectively. However, they have not been investigated with modern techniques and the majority of the pharmacological and phytochemical data on cannabis analyze organic extracts without pharmaceutical relevance (e.g., chloroform, methanol, acetone).

Literature data on cannabis water extract are very scarce and certainly not up-to-date, although analgesic activity is reported for cannabis water extract (Segelman et al., 1974). Moreover, the use of cannabis tea has been stated to prolong and intensify significantly the psychotropic effects resulting from smoking marijuana (Segelman and Sofia, 1973; Segelman et al., 1974). More recently (Giroud et al., 2000), a case study with six healthy volunteers reported on the non-psychotropic activity of cannabis tea alone; this fact was linked to the low amount of Δ^9-THC in the cannabis tea. However, Δ^9-THC-acid, the main constituent of THC-rich *Cannabis sativa* L. cultivars, decarboxylizes into Δ^9-THC at temperatures around 103 °C. In this work, this effect was evaluated by extracting the plant with hot and cold water. On the other hand, tinctures are the most widely plant extracts produced in the pharmaceutical industry. Thus, the optimization of the analytical methods to determine the optimal alcoholic strength giving the best pharmacological activity with the least toxicity is an important issue. Moreover, the study of the traditional manufacturing procedures to prepare herbal medicines can make important contributions to the identification of new natural products as well as new synergistic effects (Politi et al., 2005, 2006a) because the way a natural medicine is prepared can strongly affect the final composition of the remedy. Two factors are of importance in the case of cannabis: the effect of the solvent polarity (different ethanol/water mixtures or water) and the temperature used for the extraction of the plant on the relative content of Δ^9-THC and Δ^9-THC-acid.

The processing of the complex mixtures of plant metabolites for analytical purposes may also give erroneous interpretations of the original composition and the ideal approach would be their analysis without neither preparative nor chromatographic steps. The selection of the most suitable analytical technique is generally a compromise between speed, selectivity and sensitivity (Sumner et al., 2003). Several spectroscopic techniques such as NMR, MS, HPLC-UV/PDA and IR, currently also named "metabolomics tools", are available for the direct analysis of plant metabolites (Dunn et al., 2005). NMR is a rapid and selective tool but has low sensitivity, while MS offers good sensitivity and selectivity but relatively longer analysis times. For the present investigation it was our goal to acquire information on aqueous and hydroethanolic cannabis extracts in a fast and direct way. Therefore, this work is mostly based on 1D NMR experiments, also used for semi-quantitative analysis (Pauli et al., 2005), and comparison with the recent NMR-based literature on cannabinoids and cannabis extracts (Choi et al., 2004a,b; Hazekamp et al., 2004). Cell assays and principal component analysis of the NMR data were also included in the investigation.

2. Result and discussion

2.1. Qualitative phytochemical analysis of cannabis aqueous extracts

The first strategy adopted to abate the content of the psychoactive Δ^9-THC was by producing a cannabis water extract. Fig. 1 shows the ^{1}H NMR spectra of three extracts obtained from three aliquots of THC-rich cannabis material after maceration in deuterated chloroform, methanol and water. The typical cannabinoid proton signals of the extracts in chloroform (Fig. 1a) and methanol (Fig. 1b) emerge in particular in the NMR region between 6-6.5 ppm. These are mostly due to Δ^9-THC (**1**) and

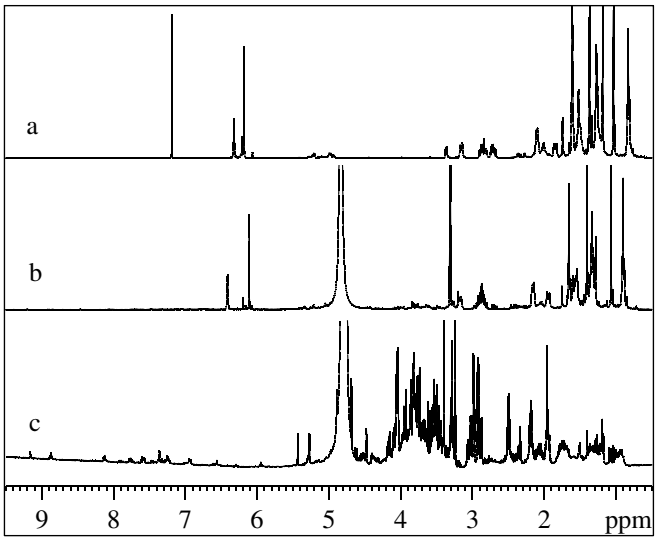

Fig. 1. ^{1}H NMR spectra (400 MHz) of crude cannabis extracts obtained after overnight maceration of three aliquots of 70 mg aerial parts in 1 ml of the following deuterated solvents: (a) chloroform; (b) methanol; (c) water.

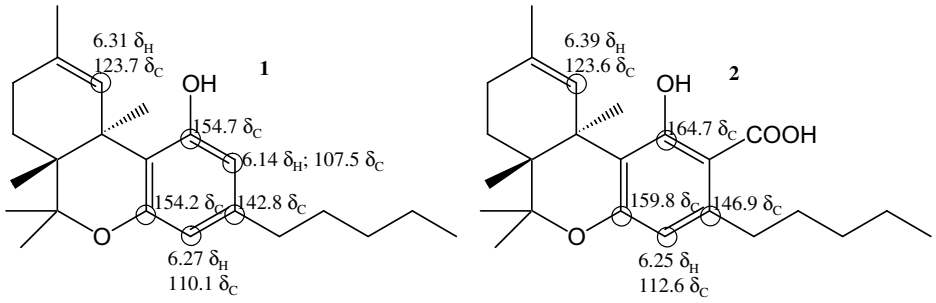

Fig. 2. Structures of Δ^9-THC (**1**) and Δ^9-THC-acid (**2**). Atoms with protons and carbons chemical shifts values measured in deuterated chloroform are circled.

Δ^9-THC-acid (**2**) (see Fig. 2). These proton signals are not detected in the water extract (Fig. 1c) indicating that the content of both cannabinoids in cannabis water extract are at least greatly reduced.

In a second experiment, 5 g of the same batch of THC-rich *C. sativa* (labelled CS) were used to produce the hot water extract (CShw) and other 5 g to produce the cold water extract (CScw) (see experimental). Twenty-five milliliters of both water extracts were extracted with *n*-hexane obtaining 13 mg of the organic fraction from the hot water extract (CShw_c) and 5 mg from the cold water extract (CScw_c). Fig. 3 shows the NMR analysis of both organic fractions dissolved in deuterated chloroform. Both mixtures contain mostly Δ^9-THC and Δ^9-THC-acid. Integration of the Δ^9-THC signal at 6.14 ppm and of the Δ^9-THC-acid signal at 6.39 ppm allowed us to calculate the ratio of these cannabinoids. In the organic fraction from the hot water extract (CShw_c), the content of Δ^9-THC is 1.52 times that of Δ^9-THC-acid while in the organic fraction from the cold water extract (CScw_c) the relative amount of Δ^9-THC is reduced to 0.09 times that of Δ^9-THC-acid. The higher amount of Δ^9-THC in the hot water extract is due to the known decarboxylation of Δ^9-THC-acid into Δ^9-THC that occurs approaching 103 °C (Segelman and Sofia, 1973). Based on the solubility of Δ^9-THC in water (2.8 mg/l), from 25 ml of water a maximum of 0.07 mg of Δ^9-THC can be expected. However, from 25 ml of the hot water extract 13 mg of a mixture containing more than 50% of Δ^9-THC was obtained. This result indicates that the use of high temperature or the presence of other compounds from cannabis favour the extraction and solubilisation of Δ^9-THC in water.

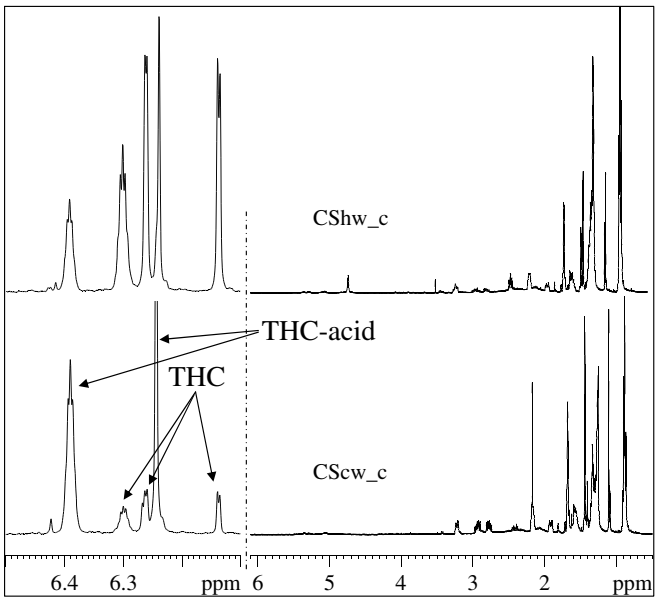

Fig. 3. ^{1}H NMR spectra in deuterated chloroform (400 MHz) of the organic fraction from the hot water extract (CShw_c) and the organic fraction from the cold water extract (CScw_c). On the left part of the spectra the signal intensity has been magnified. Δ^9-THC and Δ^9-THC-acid protons signals in the region of 6–6.4 ppm are indicated with arrows.

In order to acquire further data on the cannabis water extract this crude mixture was fractionated by precipitation in methanol/water 4:1 obtaining a soluble fraction and a precipitate (see Section 4). Fig. 4 shows the ^{1}H NMR analysis of the crude extract compared with both fractions. The precipitate CShw_p presents broad signals in the carbohydrate region that indicate the presence of polysaccharides. A polysaccharide from the aqueous extract of cannabis with potential anti-glaucoma activity has been described (Hodges et al., 1985). This mixture had shown a very potent intraocular pressure-lowering activity (antiglaucoma) when tested by i.v. injection into rabbits. Partial purification with gel exclusion chromatography yielded very active material (lowers intraocular pressure maximally at 1 mg/animal) with an estimated molecular weight of 500 kDa; Rhamnose, galactose and uronic acid were the major sugar constituents identified. Further effort could be made in the future in order to assess if such bioactive polysaccharide corresponds to that one detected in this work.

Previously, Cannabis polypeptides have also been reported as water soluble derivatives (Hillestad et al., 1977; Tang et al., 2006). It is known that folding of polypeptides can occur in presence of methanol or other organic solvent in water solution (Chakraborty et al., 2005; Zloh et al., 1998). It is therefore theoretically possible that, due to the use of methanol in the fractionation step, the refolding of polypeptides may be responsible of the

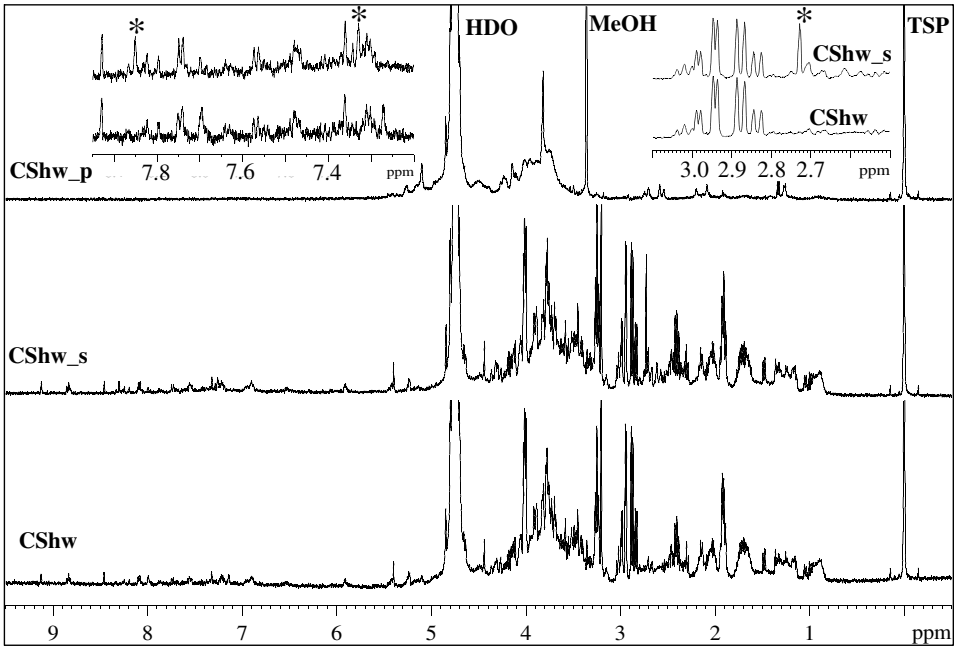

Fig. 4. ^{1}H NMR spectra in deuterated water (400 MHz) of crude hot water extract (CShw) and the corresponding soluble (CShw_s) and precipitate (CShw_p) fractions. TSP indicates the signal from the internal standard while MeOH and HDO referred to residual solvent signals. Symbol "*" indicates protons signals from the artefact detected in the CShw_s spectrum.

differences observed between the spectrum of the soluble fraction CShw_s and that of the original crude extract CShw (appearance of an intense signal at 2.73 ppm and other changes in the aromatic chemical shift area, see expanded region in Fig. 4).

^{1}H NMR analysis of the six fractions obtained from size exclusion chromatography of fraction CShw_s is shown in Figure S1 (see supplementary data). 2D NMR experiments (not shown) were performed on the fractions with the specific aim to detect the presence of (+)-cannabisativine, one of the better described cannabis alkaloids (Kuethe and Comins, 2004; Turner et al., 1976) that was found in roots and leaves (El Sohly, 1985). However, probably due to the relatively low sensitivity of the NMR method or to the extraction and fractionation procedures adopted in this case, we could not identify this alkaloid in the hot water extract.

2.2. Qualitative phytochemical analysis of cannabis tinctures

Different cannabis tinctures (ethanol/water mixtures used to extract the plant) using 20%, 40% and 80% v/v of ethanol were prepared. Different cannabis cultivars were used including THC-rich (CS and Bed) and THC-free chemotypes (CBD and NC) as well as a confiscated illicit marijuana sample (IM) that was provided by the British Home Office. To achieve their direct NMR analysis a novel strategy was used, namely ^{1}H NMR experiments with triple suppression of the ethanol and water signals (see experimental). With this technique, the analysis of the tinctures (Fig. 5) were performed without any evaporation or separation step by adding 0.05 ml of deuterated water to 0.65 ml of the samples. This experiment has been used previously for the characterization of beer (Duarte et al., 2002). In the 80% tinctures, the typical cannabinoids signals appear around 6 ppm in the spectra of the THC-rich plants (CS and Bed) and the illicit material (IM). While these signals in the CS and Bed tinctures are similar, the IM tincture presents a different profile for these cannabinoids signals as well as other signals at 4.35 ppm, 6.90 ppm and 8.22 ppm that are unique to this tincture. Both THC-free cultivars (CBD and NC) do not show these cannabinoid signals within the spectra and they are clearly distinguishable from the other tinctures. In the 40% tinctures from both THC-rich plants, cannabinoids signals appear greatly reduced around 6 ppm while other aromatic signals appear in the spectra at 7.45 ppm and 6.75 ppm. These are not detected in any of the other 40% tinctures and they represent a marker for the THC-rich plants in this analysis. Other minor protons are observed between 5 ppm and 9 ppm for all the 40% tinctures and they can be used to compare in detail the various samples. For the 20% tinctures we note that a set of proton signals around 2.55 ppm is characteristic of the IM and NC samples, while a similar set of protons is detected around 2.75 ppm only for CS and Bed samples.

^{1}H NMR experiments with suppression of the ethanol and water signals and diffusion-edited ^{1}H NMR (1D DOSY) for the direct analysis of cannabis tinctures were compared (Fig. 6). Depending on the values of the diffusion time and gradient strength used to acquire the 1D DOSY spectra, the signals from the low molecular weight compounds contained in a mixture can be partially filtered out (Politi et al., 2006b). In this case, water and ethanol

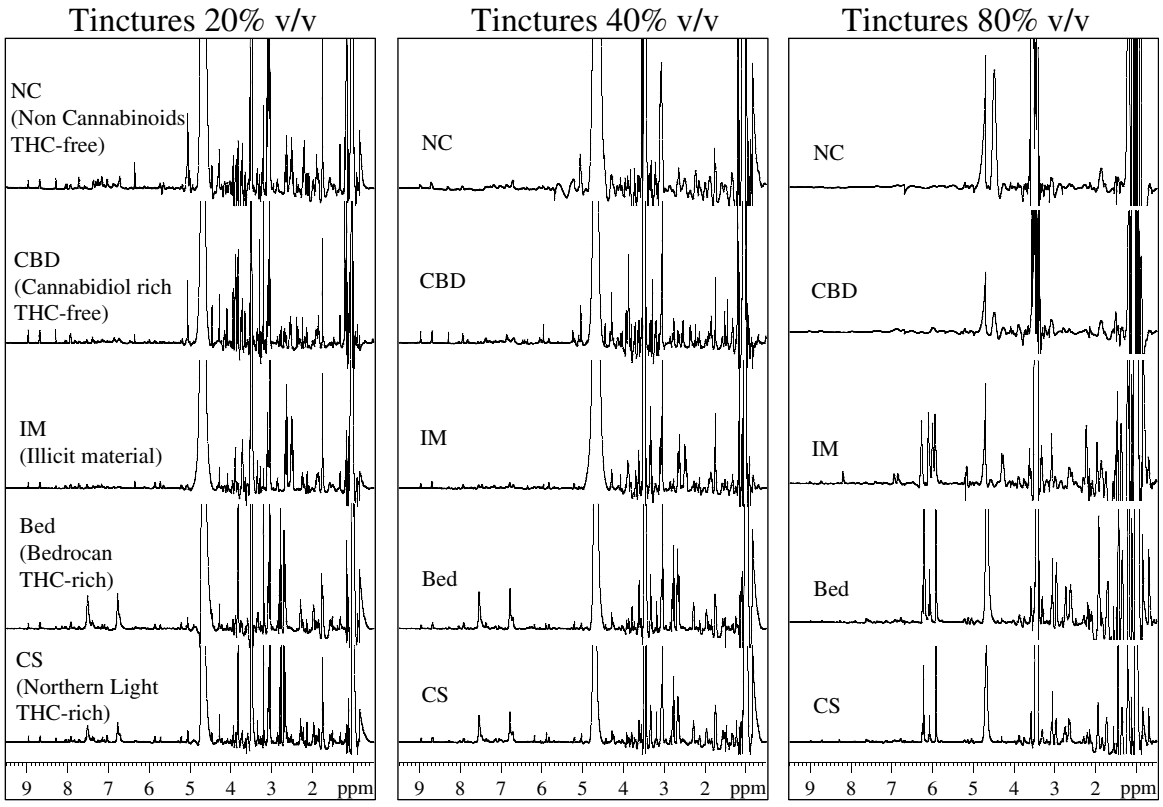

Fig. 5. ^{1}H NMR experiments (500 MHz) with suppression of the ethanol and water signals. Comparison of three types of tinctures (20%, 40% and 80% v/v) from five different cannabis cultivars (CS = Northern Lights 5 crossed with Haze, THC-rich; Bed = Bedrocan, THC-rich; IM = illicit material; CBD = cannabidiol-rich, THC-free; NC = non-cannabinoids, THC-free).

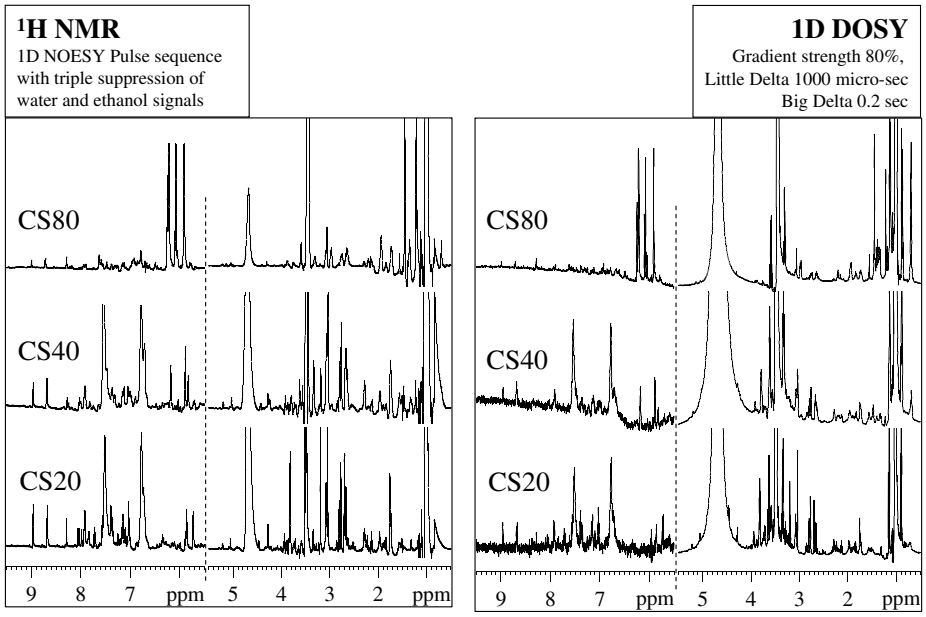

Fig. 6. Comparison of the ^{1}H NMR experiments with suppression of the ethanol and water signals with the diffusion-edited ^{1}H NMR (1D DOSY) experiments (500 MHz) in the analysis of CS tinctures. On the left part of the spectra the signal intensity has been magnified.

represent the lowest molecular weight molecules in the samples. Reducing the solvents signals in the spectra allows the detection of protons from cannabis metabolites. Under the conditions used (see Section 4), the experiments with solvent suppression take around 25 min while the 1D DOSY takes only 8 min, using 64 scans in both cases.

Although in the 1D DOSY the signal to noise ratio is lower, all the major protons previously described for CS tinctures were also detected with this faster NMR experiment. With both experiments parts of the spectra (around 4.77 pm, 3.65 ppm and 1.17 ppm) appear unresolved due to the incomplete suppression of the solvent signals, but useful information can still be acquired; the region of the spectra between 5 and 12 ppm, for instance, is well resolved and it affords crucial information for natural product identification. The proton chemical shift values usually vary for a single component analysed in different solvents. Therefore, with this NMR approach, only tinctures with the same ethanolic strength can be used for a comparative fingerprinting analysis.

2.3. Quantitative phytochemical analysis of cannabis tinctures

In order to better acquire semi-quantitative data on Δ^9-THC and Δ^9-THC-acid, and to compare our data with those from the literature (Choi et al., 2004a,b; Hazekamp et al., 2004) the dried residues from the tinctures were extracted with chloroform and methanol as described in Section 4. Fig. 7 shows the comparison between the proton spectra acquired in deuterated chloroform of fractions CS80_c, CS40_c and CS20_c. The first two contain mostly Δ^9-THC and Δ^9-THC-acid while in the latter other proton signals appear in the spectrum (indicated with the asterisks). The Δ^9-THC content was 0.46 times that of Δ^9-THC-acid in fraction CS80_c, 0.28 times in fraction CS40_c, and only 0.10 times in fraction CS20_c. For the first two cases, quantitative ^{13}C NMR analysis confirms these observed ratio values (see supplementary data and Figure S3). The small amount of fraction CS20_c obtained did not allow a quantitative ^{13}C NMR analysis of this mixture. In the CS20_c sample other cannabinoid signals between 6 and 6.5 ppm appear in the spectra with a relative high intensity with respect to the content of Δ^9-THC and Δ^9-THC-acid. Other protons possibly belonging to cannabinol and cannflavins are also detected around 6.9–7.6 ppm.

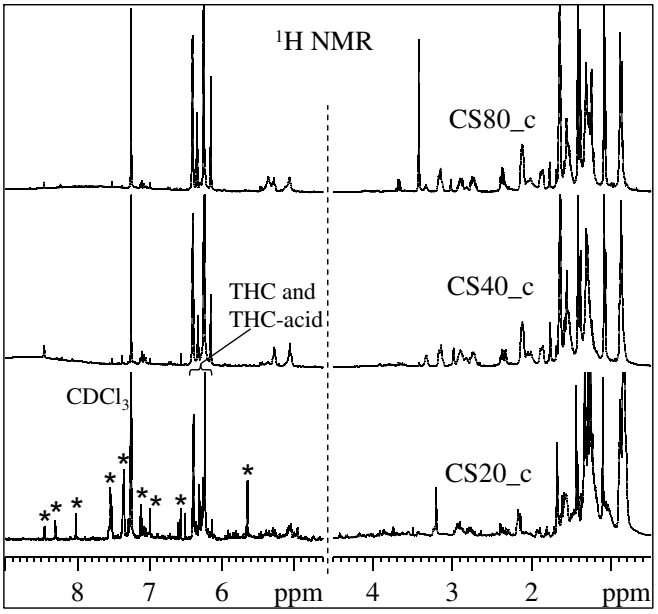

Fig. 7. ^{1}H NMR in deuterated chloroform (400 MHz) of fractions CS20_c, CS40_c, and CS80_c. On the left part of the spectra the signal intensity has been magnified. Asterisks indicate proton signals that are relatively more abundant with respect to Δ^9-THC and Δ^9-THC-acid signals in CS20_c spectrum compared to CS40_c and CS80_c ones.

Minor quali-quantitative differences between the methanol and water soluble fractions are detected within the overall NMR regions of the corresponding spectra (Fig. S4 and S5 in supplementary data).

As shown, this technique is still semi-quantitative, and further work must be done to allow the quantitation of single compounds in the mixture. The strength of this approach resides in the fast acquisition of data from tinctures without virtually any pre-processing of the samples. Furthermore, the resulting fingerprint reflects the real composition of the mixture and multivariate analysis can be applied to the NMR data if necessary. We have already applied this method for batch-to-batch variability and stability test on different commercial tinctures (Politi et al., unpublished). However, if a quantitative standardisation of certain markers or bioactives of the tincture is needed, then LC–UV–MS in any of its variants is the instrumental technique of choice. The development of different conditions of elution and processing of the samples is time consuming but the analytical results give accurate quantitative data of selected metabolites, even if they are present in minute quantities. Examples are the application of LC–MS to the control of cannabinoids in crude extracts (Stolker et al., 2004) or in forensic samples (Yang and Xie, 2006).

2.4. Pharmacological data

All water soluble samples including the crude hot water extract CShw, fractions CShw_s and CShw_p, and fractions 1–6 from the size exclusion chromatography (see experimental) were tested in the NF-κB assay in HeLa cells (Bremner et al., 2004) (see Section 4). At a concentration of 0.1 mg/ml, neither NF-κB inhibitory nor cytotoxic effects were observed in the model. However, the EtOH 100% extracts from the same THC-rich plant material (CS) at the same concentration resulted in toxicity for the HeLa cells as evidenced by morphological changes induced on the cells during incubation (cells with normal phenotype $< 10\%$).

With regard to the tinctures, the cannabinoid content augments increasing the concentrations of ethanol. In the same way, the maximum non-toxic concentration (see Section 4) of these extracts, measured with the MTT assay on HeLa cells, increases by reducing the ethanol strength used for extraction. Whether the non-toxicity of cannabis extracts is due to the low levels of cannabinoids remains unclear, but a positive correlation between increasing etha-

nol tincture strength (and therefore cannabinoid content) and cytotoxicity as measured in the MTT assay (Fig. S2 in supplementary data) was found. The maximum non-toxic concentrations for the three tinctures CS20, CS40 and CS80 were 20.83 μmol/ml, 1.47 μmol/ml and 0.03 μmol/ml, respectively, after 6 h incubation and 14.58 μmol/ml, 0.43 μmol/ml and 0.01 μmol/ml, respectively, after 24 h incubation as derived from the MTT assay on HeLa cells (Fig. S2 in supplementary data).

3. Conclusion

A novel approach based on NMR spectroscopy, ^{1}H NMR experiments with suppression of the ethanol and water signals and diffusion-edited ^{1}H NMR (1D DOSY), was developed and successfully applied for the fast and direct analysis of tinctures. Relative amounts of Δ^9-THC and Δ^9-THC-acid in the extracts analysed vary depending on the temperature (extraction with hot and cold water) and the polarity of the solvent used for the extraction (tinctures with different ethanol strength). To the best of our knowledge, this is the first time that the polarity of the solvent used for the extraction is described as a factor that can affect the relative quantity of Δ^9-THC respect to other cannabinoids in the extract. Fraction CS20_c from the 20% tincture fulfils the demand for cannabis extracts low in Δ^9-THC still containing other cannabinoids of medicinal interest. Further studies to maximize the yield of this promising mixture of cannabis products are ongoing. As for the aqueous extracts, this approach revealed the non-negligible presence of Δ^9-THC and Δ^9-THC-acid in infusions of cannabis which may, at least in part, explain the recreational and medicinal uses of this particular preparation. In the emerging metabolomic era, it is essential to monitor how manufacturing procedures affect the final content of natural products in herbal medicines.

4. Experimental

4.1. Plant material

All relevant Home Office licenses for growing, transproting and possession of cannabis have been in place at all times. The seeds of Northern Lights 5 crossed with Haze (labelled CS) were bought from the Pukka Seed Company, Guildford, UK. This cannabis cultivar was grown under hydroponic conditions. Plants grown in pods filled with ceramic beads where nutrient rich water was pumped to the base of the plant 4 times a day. Growing conditions were the following: 70% Humidity, 24 °C Temperature, 5.5–6 pH of water, 1300 ppm of CO_2, metal halides 600 W for vegetative cycle (18 h a day cycle), and sodium's 1000 W for blooming (18 h then slowly reduced to 12 h). The seeds were planted on 15/08/2005, the first males were removed on 27/09/2005 and the females were harvested on 02/12/2005. Bedrocan (labelled Bed) was received in 2006 from the Office of Medicinal Cannabis, Ministry of Health, Welfare and Sports, The Hague, The Netherlands. Illicit material (labelled IM) was provided in 2004 by the Home Office, UK. Cannabidiol-rich and non-cannabinoid cultivars (labelled CBD and labelled NC, respectively) were received in 2005 from the ISCI (Experimental Institute for the Industrial Crop), Rovigo, Italy.

4.2. Chemicals

Freshly deionised, ultrafiltered water was obtained with a Milli-Q system (Molsheim, France). Ethanol 99.7–100% (AnalaR, VWR, Poole, UK).

4.3. Extraction and fractionation

Three different aliquots of CS (70 mg each) were macerated overnight at room temperature in 1 ml of $CDCl_3$, MeOD, and D_2O and the extracts were directly analysed by NMR (Fig. 1).

Five grams of dried CS were extracted with 100 ml hot water (heating and boiling for 3 min) and after filtration approximately 50 ml of this decoction, labelled CShw, were recovered. Another 5 g sample of CS was extracted with 100 ml cold water (overnight under agitation, room temperature) and after filtration almost 50 ml of this extract labelled CScw were recovered. 25 ml of both water extracts were extracted with 5 ml of *n*-hexane (3 times) and these organic fractions, labelled CShw_c and CScw_c, were dried in the rotavap to give 13 mg and 5 mg, respectively.

Another CShw extract was produced from 150 g of CS in 2 l hot water (heating and boiling for 3 min). This extract was concentrated in a rotavap and finally freeze-dried to give 15 g of crude lyophilised material. Ten grams of CShw were dissolved in 20 ml of water and 80 ml MeOH were added obtaining a precipitate and a soluble fraction that were separated by centrifugation and labelled CShw_p (yield 30%) and CShw_s (yield 70%), respectively.

The five different plant cultivars; Northern Lights 5 crossed with Haze (CS), Bedrocan (Bed), illicit material confiscated and provided by the British home office (IM), cannabidiol-rich cultivar (CBD), and non-cannabinoid cultivar (NC) were used to produce the corresponding tinctures. In every single extraction, 10 g of each dried plant material were used. Hundred milliliters of different mixtures of ethanol/water (20%, 40%, and 80% v/v) were used to macerate the plants for 3 days in the dark under agitation. After filtration under vacuum, approximately 50 ml, 65 ml, and 75 ml were recovered from the 20%, 40%, and 80% tinctures, respectively. These different recovery levels were probably due to the absorption of water by the dried cannabis material. This absorbed water was not released during filtration. Fifty milliliters of the three tinctures from Northern Lights 5 crossed with Haze, labelled, respectively CS20, CS40, and CS80, were dried in the rotavap obtaining

1.4 g, 2.3 g, and 1.7 g. These residues were extracted with 100 ml of $CHCl_3$ and centrifuged to separate the soluble part from the insoluble one. Then the residues were extracted with 100 ml of MeOH and centrifuged. The residual materials contained only water-soluble constituents. From the 20% tincture the chloroform-soluble fraction labelled CS20_c (40 mg), the methanol-soluble fraction labelled CS20_m (371 mg), and the water-soluble fraction labelled CS20_w (438 mg) were obtained. From the 40% tincture fractions CS40_c (280 mg), CS40_m (738 mg), and CS40_w (612 mg) were obtained. From the 80% tincture fractions CS80_c (1315 mg), CS80_m (131 mg), and CS80_w (60 mg) were obtained.

4.4. NMR sample preparation

CShw_c (13 mg) and CScw_c (5 mg) were dissolved in 0.7 ml $CDCl_3$. The hot water extract CShw and both fractions CShw_p and CShw_s were all analysed at a concentration of 10 mg/ml using 0.7 ml of D_2O with 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP) as internal standard (0.2 mg/ml). All tinctures produced (CS20, CS40 and CS80, Bed20, Bed40 and Bed80, IM20, IM40 and IM80, CBD20, CBD40 and CBD80, NC20, NC40 and NC80) were directly analysed by adding 0.05 ml of D_2O as internal lock to 0.65 ml of each tincture using 1D DOSY experiments and the 1D NOESY pulse sequence with water and ethanol signals suppression. Approximately 40 mg of each chloroform, methanol and water fraction from the three tinctures CS20, CS40 and CS80 were dissolved in 0.7 ml of the corresponding deuterated solvents.

4.5. NMR analysis

NMR spectra of samples were obtained on Bruker AVANCE 400 and 500 MHz spectrometers equipped with a multinuclear probehead with z-gradient. The TOPSPIN v1.3 software was used for spectra acquisition and processing, The spectra were recorded in various solvent systems at 300 K and the chemical shift calibration was carried out either on the TSP signal or residual solvent peak. The size of all 1D spectra was 65 K and the number of transients varied for different types of spectra. The standard 1D ^1H NMR spectra were acquired with 30° pulse length and a relaxation delay of 2 s, while the ^1H NMR45 spectra were acquired with 45° pulse length and a relaxation delay of 60 s to enable an accurate quantification of peaks. Similarly ^{13}C NMR and ^{13}C NMR45 were acquired at a operating frequency of 100.6 MHz (30° pulse lengths and relaxation delay of 1 s and 45° pulse lengths and relaxation delay of 60 s, respectively).

The rest of the experiments were conducted on a 500 MHz instrument. 1D DOSY was obtained using a pulse sequence from Bruker library (led bpgp2s1d) using gradient strength (gpz6) 80, little delta (p30) 1000 μs and big delta (d20) 0.2 s. 1D ^1H NOESY pulse sequence (lc1pnfr) with multiple offset presaturation using frequency list was employed to suppress water and ethanol signals of the samples. Presaturation was carried out with a relaxation delay (d1 = 2 s) and mixing time (d18 = 0.8 s). In both cases the numbers of scans were 64.

4.6. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

A modification of the assay described previously (Mosmann, 1983) was used as a criterion of cytotoxicity. Briefly Cells were incubated at 37 °C and 5% CO_2 atmosphere for 6 h and 24 h in media supplemented with PBS at eight different concentrations between 0.1 and 100 μg/ml. Controls received vehicle only. The maximum non-toxic concentration (MNTC) was determined for each extract, i.e. cell viability >85% of the control. The dark blue formazan product was dissolved in DMSO/isopropanol and measured using a Anthos Lucy 1 luminometer/photometer (Anthos-Biochrome Ltd., Cambridge, UK) at 490 nm, and the data collected and processed using Stingray 1.5 software (Dazdaq Ltd., Brighton, UK).

4.7. IL-6/luciferase (IL-6/Luc) assay

We followed the protocol described previously (Bremner et al., 2004). Briefly, HeLa cells stably transfected with a luciferase reporter gene controlled by the IL-6 promoter. Cells were incubated in the presence of the compounds or plant extracts for 1 h and then stimulated (PMA, 50 ng/ml, final concentration). The cells were incubated for a further 7 h (37 °C/5% CO_2) and then lysed with luciferase lysis reagent (Promega, Madison, WI, USA). Lysates were transferred to 96-well plates and an Anthos Lucy 1 luminometer/photometer (Anthos-Biochrome Ltd., Cambridge, UK) was used both to add the luciferase substrate (Promega) and record the resulting luminometric readings following a reaction time of 10 s. Positive (stimulated cells without a sample) and negative (resting cells without stimulation) controls were included.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.07.018.

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