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Cannabis through the looking glass: chemo- and enantio-selective separation of phytocannabinoids by enantioselective ultra high performance supercritical fluid chromatography†

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By using the Inverted Chirality Columns Approach (ICCA) we have developed an enantioselective UHPSFC method to determine the enantiomeric excess (ee) of (−)-Δ^9-THC in medicinal marijuana (Bedrocan®). The ee was high (99.73%), but the concentration of the (+)-enantiomer (0.13$_5$%) was not negligible, and it is worth a systematic evaluation of bioactivity.

Cannabinoids are one of the constituents of marijuana, the crude drug derived from the plant *Cannabis sativa* L. The term phytocannabinoids has been coined just to emphasize their plant-derived origin[1] with respect to synthetic cannabinoids (*e.g.*, nabilone,[2a] dexanabinol,[2b] or ajulemic acid[2c]) and endogenous cannabinoid receptor ligands (namely, endocannabinoids).[3] The most abundant and psychologically active compound of the class is (−)-Δ^9-*trans*-tetrahydrocannabinol (Δ^9-*trans*-THC),[4,5] which has indeed been the subject of thousands of papers.[6,7] Furthermore, we are currently witnessing a remarkable breakthrough in the recreational use of cannabis, with respect to the mode of consumption, short-term effects, chronic health consequences and cannabis use disorders.[8]

Another fascinating feature of phytocannabinoids is that most of them are chiral, and are produced, in the plant, in a single-enantiomer format, as typically occurs for natural products, with some exceptions.[9] As a result, restricted stereochemical requirements are present for the interaction of cannabinoids with the cannabinoid (CB) receptors,[10] and this has led numerous groups to investigate the possibility of separating the undesirable psychotropic effects from the desirable effects by suitable changes in stereochemistry. For example, the (−)-enantiomer of the synthetic cannabinoid dexanabinol (HU-210) is one of the most potent psychotropically active cannabinoids known, while the corresponding (+)-enantiomer (*i.e.*, HU-211) is devoid of the THC-like psychotropic effects.[11] It becomes evident, therefore, the importance of the stereochemical efficiency of synthetic pathways to unnatural cannabinoid analogues, as well as the efficiency of the determination of the enantiomeric purity (namely, enantiomeric excess, ee) in naturally occurring samples, in both single and in more complex mixtures.

We became interested in investigating the composition of Cannabis plant extracts from a stereochemical point of view; in fact, while stringent rules have been established by the FDA for the content determination of THC and cannabidiol (CBD) in medicinal marijuana,[12] the stereochemical features of such molecules and the determination of the enantiomeric purity have not yet been put into the foreground by the agency.

Furthermore, although the point of the chromatographic resolution of *chiral cannabinoids*[13a] has already been raised in 1993 and more recently addressed for synthetic cannabinoids,[13b] analytical approaches to crude plant extracts have been developed by just taking into account the single cannabinoids, irrespective of their stereochemistry.[14]

We started the investigation by focusing our attention on the phytocannabinoids collected in Fig. 1. Apart from the typical three-letter acronyms,[4,5] we included a bold number for each molecular entity, for the sake of clarity in the identification of the enantiomerically correlated molecules. In fact, it is well known that all the naturally occurring cannabinoids exist in the single-enantiomer format, *i.e.*, the (*R*,*R*)-form, which displays a negative optical rotation and a *trans*-configuration at the cyclohexene ring. Nevertheless, we have designed, for each structure, its mirror image (*i.e.*, the unnatural dextrorotatory enantiomer) to get a full idea of any implicit stereochemical scenario in cannabinoids.[15]

Cannabidivarin (CBDV, **1**) and cannabidiol (**2**), which feature two chiral centres, belong to the CBD-type subgroup of cannabinoids,[4] the only difference between them being the length of the alkyl side chain at C3′ (propyl and pentyl, respectively). From the

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† Electronic supplementary information (ESI) available: Chemo- and enantio-selective separation of seven phytocannabinoid standards by eUHPSFC on the (*S*,*S*)-Whelk-O1 column; simultaneous UV and CD detection for racemic CBC on the (*R*,*R*)-Whelk-O1 column. See DOI: 10.1039/c7cc06999e

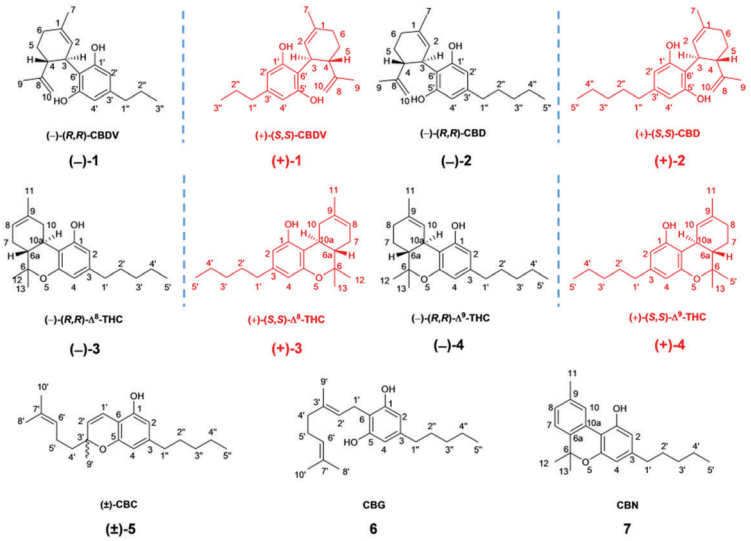

Fig. 1 Chemical structures of the main naturally occurring chiral phytocannabinoids and of their mirror images. The picture also includes two achiral cannabinoids, namely CBG (**6**) and CBN (**7**).

THC-type subgroup of cannabinoids,[4] we extracted both the Δ^8-THC-type (**3**) and the Δ^9-THC-type (**4**) cannabinoids, which are double-bond position isomers of THC, the former being less psychologically potent than the latter. Another fascinating chiral phytocannabinoid is cannabichromene (CBC, **5**), that presents only one chiral centre, and it is typically reported as a rare case of a natural racemate.[4,5] This cannabinoid has no psychotropic effect, but interesting pharmacological activities such as anti-inflammatory, antifungal and antimicrobial properties have been reported.[16] We have included, in the investigation, also two achiral cannabinoids, *i.e.*, cannabigerol (CBG, **6**), which is the biosynthetic precursor of CBC, and cannabinol (CBN, **7**), whose concentration in Cannabis products (*i.e.*, marijuana, hashish and hash oil) has been shown to increase during the storage of these materials. Thus, it was necessary to develop an analytical method, which had to be enantio- and chemo-selective at the same time.

The first problem we faced in stereoselective analysis of Cannabis plant extracts was that vegetable extracts are highly enriched complex mixtures and often the minor enantiomers, or the racemates, are not available as reference samples. To overcome this limitation, our group has previously developed a method for the identification and accurate quantification of the minor enantiomer in trace analysis of natural products, named the ''Inverted Chirality Columns Approach'' (ICCA).[17a,b] This method is based on the switching between two Chiral Stationary Phases (CSPs) having the same bound selector with an opposite configuration, to reverse the elution order of a given enantiomeric pair, according to the reciprocal principle of selectand–selector-systems.[17c] This technique is very useful when the minor enantiomer follows the major one and it is partially hidden by the tailing of the leading enantiomer: on the CSP with opposite configuration the trace enantiomer is eluted first, thus enabling a more precise and accurate quantification by peak area integration.

Pirkle-type CSPs (such as the DACH-DNB and the Whelk-O1) are the ideal instruments for the application of the ICCA approach, because they exist in both the enantiomeric versions.[17a,b] From 2010, a clear trend towards the development of CSPs in the sub-2 μm format has been reported for the chromatographic separation of chiral analytes to enhance both analysis speed and column efficiency.[18] This has opened new frontiers in the field of enantioselective ''e'' Ultra-High Performance Liquid Chromatography (eUHPLC)[19] and enantioselective ''e'' Ultra-High Performance Supercritical Fluid Chromatography (eUHPSFC).[20] SFC has indeed recently undergone an outstanding revival in the enantioseparation of pharmaceuticals, particularly in the initial screening stage of the drug discovery process.[21]

In this work, we successfully applied, for the chemo- and enantio-selective separation of phytocannabinoids, the ICCA method using, as the inverted chirality columns, those based on the (*S*,*S*)- and (*R*,*R*)-Whelk-O1 CSPs in the sub-2 μm format, under eUHPSFC conditions. It must be acknowledged that UHPSFC technology proved recently to be an alternative and orthogonal solution for the separation of synthetic cannabinoids.[13b,14]

The two UHPC-Whelk-O1 sub-2 μm CSPs were prepared according to a previously described procedure[19c] starting from Kromasil 1.8 μm silica particles, and slurry packed into 100 × 4.6 mm I.D. stainless steel columns. To evaluate the separation ability of the Whelk-O1 CSP towards different cannabinoids, a mixture containing seven standards (*i.e.*, (−)-**1**, (−)-**2**, (−)-**3**, (−)-**4**, (±)-**5**, **6** and **7**, whose structures are shown in Fig. 1) was prepared starting from the commercially available stock solutions (0.1–1.0 mg ml^{-1}) in methanol. All the standards have been well separated within 12 minutes using CO_2/MeOH, 98 : 2 as the mobile phase, at a flow-rate of 3.5 ml min^{-1}, under isocratic and isoconfertic conditions (see Fig. S1 of the ESI†). This result was taken as a proof of the optimal chemoselectivity of the Whelk-O1 CSP, in particular for the couple Δ^8-THC (**3**) and Δ^9-THC (**4**), which are simply two positional isomers.

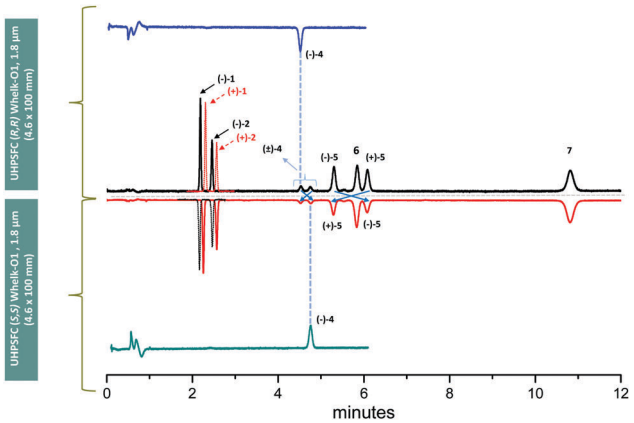

Fig. 2 Demonstration of the chemical equivalence of the two 1.8 µm UHPC-(*R*,*R*)- and (*S*,*S*)-Whelk-O1 columns (100 × 4.6 mm I.D.) upon separation of a six-component cannabinoid standard mixture. The dotted lines represent the (+)-enantiomers of compounds **1** and **2** predicted by the ICCA method. Mobile phase: CO_2/MeOH = 98 : 2; flow-rate: 3.5 ml min^{-1}; *T* = 30 °C; ABPR = 1500 psi; detection: UV at 214 nm. For the chemical structures of **1–7**, see Fig. 1.

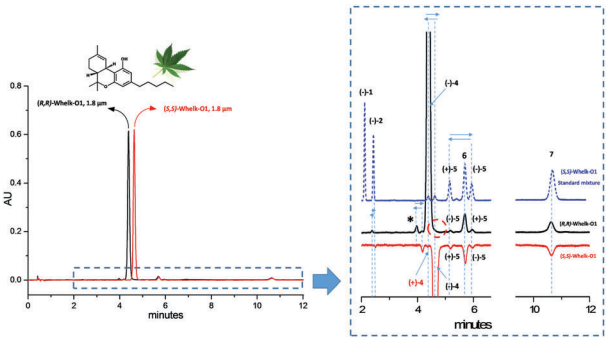

Fig. 3 A Bedrocan® ethanol extract analyzed by applying the ICCA protocol. For peak identification, the chromatogram of a six-component cannabinoid standard mixture has been shown in the inset as a dotted line trace. The asterisk denotes a chiral unknown impurity.

The analysis of a further standard mixture obtained by replacing the (−)-**4** standard with the corresponding commercially available racemate (*i.e.*, (±)-**4**) has provided evidence of the enantioselective capability of the phase towards "THC" as well, the (+)-enantiomer eluting before the naturally occurring (−)-**4** on the (*S*,*S*)-Whelk-O1 column. Furthermore, the column proved to be capable of separating the two enantiomers of CBC (compound **5**), which was added to the mixture of standards as the racemate, and whose absolute configuration at C3′ has not yet been determined.[4] The identification of the two CBC enantiomers was made by checking their elution order in the (*R*,*R*)-Whelk-O1 column under standard HPLC conditions (see Fig. S2 of the ESI†) and simultaneous UV and CD detection at 280 nm (the first eluting enantiomer is the negative peak).

Afterwards, the two UHPC-Whelk-O1 sub-2 µm columns were tested in order to check their full equivalence, both from a chemical (*i.e.*, retention and selectivity) and a geometrical (*i.e.*, packing efficiency) point of view, as required by the ICCA method.[17a] For this purpose, a six-component standard mixture was analyzed for both the (*S*,*S*)- and (*R*,*R*)-Whelk-O1 columns under the same UHPSFC conditions (see Fig. 2), and we could confirm that just the enantiomerically correlated peaks have been inverted by switching from one column to the other, whereas the achiral molecules (*i.e.*, **6** and **7**) were not affected by the variation, and eluted from the two columns with identical retention behaviours (see Table S1 of the ESI†).

By applying the ICCA protocol to the above-mentioned standard mixture, we were able to simulate a virtual racemate for those compounds that were available just as single enantiomers (*i.e.*, (−)-**1** and (−)-**2**). The simulation (see dot lines in Fig. 2) has allowed us to predict the retention factor (k'), the enantioselectivity (α) and the resolution (R_s) for the virtual (−)-**1**/(+)-**1** and (−)-**2**/(+)-**2** couples (see Table S1 of the ESI†).

As a final investigation, we analyzed an ethanol extract from Bedrocan® (medicinal marijuana from *Cannabis sativa* L. strains) which is claimed to contain "THC" as the major component (Fig. 3). Indeed, by expanding the chromatogram baseline, we have detected, besides "THC", also "CBC", "CBG" and "CBN" in smaller amounts (see Table S2 of the ESI†), together with a chiral unknown impurity. Moreover, thanks to the ICCA method, we made it possible to elute the minor enantiomeric impurity (+)-**4** before the major one (*i.e.*, (−)-**4**) and thus we were able to measure an ee equal to 99.73% on the UHPC-(*S*,*S*)-Whelk-O1 column (see Table 1). Notably, for "CBC", we found that, instead of being a racemate, the ee is about 25% (equally measurable on both the columns), which means that it occurs as a scalemic mixture.

We can conclude that the chromatographic system we have developed proved useful in the chemo- and enantio-selective separation of all the tested cannabinoid samples, and it has been demonstrated to be reliable for the determination of extreme ee of Δ^9-THC in pharmaceutical formulations. Notably, it is the first time that the trace (+)-enantiomer of "THC" has been quantified in medicinal marijuana. Such a feature is fundamental both for further evaluation of the bioactivity and in the case of single-molecule cannabinoid pharmaceutical products (*e.g.*, Marinol®), whose stereochemistry must be accurately controlled.

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Table 1 Determination of the enantiomeric excess (ee) for "THC" (**4**) and CBC (**5**) in the Bedrocan® ethanol extract on the UHPC-Whelk-O1 sub-2 µm columns under eUHPSFC conditions

CSP config.	Peak name	Area (µV s)	EF[a] (%)	ee (%)
(*R*,*R*)	(−)-**4**	3 694 825	—[b]	—[b]
	(+)-**4**	—[b]	—[b]	
(*S*,*S*)	(+)-**4**	4917	0.13_5	99.73
	(−)-**4**	3 642 648	99.86_5	
(*R*,*R*)	[CD(−)280]-**5**[c]	18 766	37.42	25.16
	[CD(+)280]-**5**[c]	31 383	62.58	
(*S*,*S*)	[CD(+)280]-**5**[c]	31 346	62.81	25.62
	[CD(−)280]-**5**[c]	18 559	37.19	

[a] EF = enantiomeric fraction. [b] On the (*R*,*R*)-Whelk-O1 column the (+)-**4** peak is eluting on the tailing of the major (−)-**4** component and thus it is not quantifiable. [c] Plus and minus signs refer to the signs of the circular dichroism (CD) band at the indicated wavelength.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

1 (*a*) J. Gertsch, R. G. Pertwee and V. Di Marzo, *Br. J. Pharmacol.*, 2010, **160**, 523; (*b*) A. Ligresti, L. De Petrocellis and V. Di Marzo, *Physiol. Rev.*, 2016, **96**, 1593.
2 (*a*) A. Ward and B. Holmes, *Drugs*, 1985, **30**, 127; (*b*) R. Mechoulam, N. Lander, A. Breuer and J. Zahalka, *Tetrahedron: Asymmetry*, 1990, **1**, 315; (*c*) S. H. Burstein, C. A. Audette, A. Breuer, W. A. Devane, S. Colodner, S. A. Doyle and R. Mechoulam, *J. Med. Chem.*, 1992, **35**, 3135.
3 (*a*) V. Di Marzo and A. Fontana, *Prostaglandins, Leukotrienes Essent. Fatty Acids*, 1995, **53**, 1; (*b*) V. Di Marzo, M. Bifulco and L. De Petrocellis, *Nat. Rev. Drug Discovery*, 2004, **3**, 771; (*c*) L. O. Hanuš, *Chem. Biodiversity*, 2007, **4**, 1828.
4 M. A. ElSohly and D. Slade, *Life Sci.*, 2005, **78**, 539.
5 L. O. Hanuš, S. M. Meyer, E. Muñoz, O. Taglialatela-Scafati and G. Appendino, *Nat. Prod. Rep.*, 2016, **33**, 1357.
6 C. M. Andre, J.-F. Hausman and G. Guerriero, *Front. Plant Sci.*, 2016, **7**, 1.
7 R. Mechoulam, *Curr. Pharm. Des.*, 2000, **6**, 1313.
8 K. Solymosi and A. Köfalvi, *Mini-Rev. Med. Chem.*, 2017, **17**, 1223.
9 J. M. Finefield, D. H. Sherman, M. Kreitman and R. M. Williams, *Angew. Chem., Int. Ed.*, 2012, **51**, 4802.
10 G. Appendino, G. Chianese and O. Taglialatela-Scafati, *Curr. Med. Chem.*, 2011, **18**, 1085.
11 (*a*) E. Shohami and R. Mechoulam, *Drug Dev. Res.*, 2000, **50**, 211; (*b*) A. I. Maas, G. Murray, H. Henney 3rd, N. Kassem, V. Legrand, M. Mangelus, J. P. Muizelaar, N. Stocchetti and N. Knoller, *Lancet Neurol.*, 2006, **5**, 38 and Pharmos TBI investigators.
12 (*a*) D. C. Throckmorton, FDA Regulation of Marijuana: Past Actions, Future Plans, Joint Meeting, April 12, 2016 can be found under http://www.fda.gov; (*b*) A. Mead, *Epilepsy Behav.*, 2017, **70**, 288.
13 (*a*) S. Levin, S. Abu-Lafi, J. Zahalka and R. Mechoulam, *J. Chromatogr. A*, 1993, **654**, 53; (*b*) T. Toyo'oka and R. Kikura-Hanajiri, *Chem. Pharm. Bull.*, 2015, **63**, 762.
14 (*a*) M. Wang, Y. H. Wang, B. Avula, M. M. Radwan, A. S. Wanas, Z. Mehmedic, J. Van Antwerp, J. Parcher, M. A. ElSohly and I. A. Khan, *Planta Med.*, 2016, **82**, PA28; (*b*) M. Wang, Y. H. Wang, B. Avula, M. M. Radwan, A. S. Wanas, Z. Mehmedic, J. van Antwerp, J. Parcher, M. A. ElSohly and I. A. Khan, *J. Forensic Sci.*, 2017, **62**, 602.
15 (*a*) M. A. Schafroth, G. Zuccarello, S. Krautwald, D. Sarlah and E. M. Carreira, *Angew. Chem., Int. Ed.*, 2014, **53**, 13898; (*b*) M. A. Schafroth and E. M. Carreira, *Prog. Chem. Org. Nat. Prod.*, 2017, **103**, 37.
16 H. N. Elsohly, C. E. Turner, A. M. Clark and M. A. Elsohly, *J. Pharm. Sci.*, 1982, **71**, 1319.
17 (*a*) E. Badaloni, W. Cabri, A. Ciogli, R. Deias, F. Gasparrini, F. Giorgi, A. Vigevani and C. Villani, *Anal. Chem.*, 2007, **79**, 6013; (*b*) E. Badaloni, W. Cabri, A. Ciogli, I. D'Acquarica, R. Deias, F. Gasparrini, F. Giorgi, D. Kotoni and C. Villani, *J. Chromatogr. A*, 2010, **1217**, 1024; (*c*) V. Schurig, *Molecules*, 2016, **21**, 1535.
18 (*a*) I. D'Acquarica, D. Kotoni, A. Ciogli, M. Pierini, J. Kocergin, H. Ritchie, C. Villani and F. Gasparrini, *LC-GC Europe*, 2014, **27**, 128; (*b*) A. Cavazzini, N. Marchetti, R. Guzzinati, M. Pierini, A. Ciogli, D. Kotoni, I. D'Acquarica, C. Villani and F. Gasparrini, *TrAC, Trends Anal. Chem.*, 2014, **63**, 95.
19 (*a*) G. Cancelliere, A. Ciogli, I. D'Acquarica, F. Gasparrini, J. Kocergin, D. Misiti, M. Pierini, H. Ritchie, P. Simone and C. Villani, *J. Chromatogr. A*, 2010, **1217**, 990; (*b*) D. Kotoni, A. Ciogli, C. Molinaro, I. D'Acquarica, J. Kocergin, T. Szczerba, H. Ritchie, C. Villani and F. Gasparrini, *Anal. Chem.*, 2012, **84**, 6805; (*c*) D. Kotoni, A. Ciogli, I. D'Acquarica, J. Kocergin, T. Szczerba, H. Ritchie, C. Villani and F. Gasparrini, *J. Chromatogr. A*, 2012, **1269**, 226.
20 (*a*) A. Grand-Guillaume Perrenoud, C. Hamman, M. Goel, J.-L. Veuthey and D. Guillarme, *J. Chromatogr. A*, 2012, **1266**, 158; (*b*) L. Sciascera, O. Ismail, A. Ciogli, D. Kotoni, A. Cavazzini, L. Botta, T. Szczerba, J. Kocergin, C. Villani and F. Gasparrini, *J. Chromatogr. A*, 2015, **1383**, 160.
21 (*a*) K. De Klerck, D. Mangelings and Y. Vander Heyden, *J. Pharm. Biomed. Anal.*, 2012, **69**, 77; (*b*) K. De Klerck, C. Tistaert, D. Mangelings and Y. Vander Heyden, *J. Supercrit. Fluids*, 2013, **80**, 50.