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Cannabinoids Analysis of Laser-Microdissected Trichomes of *Cannabis sativa* L. by LC-MS and Cryogenic NMR

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Abstract

Trichomes of *Cannabis sativa* have been reported as the main site of cannabinoids production. A comprehensive study of cannabinoids was performed on capitate-stalked and capitate-sessile trichomes, and on capitate-stalked glands and stems harvested by laser microdissection (LMD) during flowering time (week 4 - 8). LC-MS and cryogenic NMR analysis were used for qualitative and quantitative assessment of cannabinoids in the collected cells. Δ^9-tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA) and cannabigerolic acid (CBCA) were identified as the major constituents in the all tested samples, while Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabigerol (CBG) were present in less quantity. Cannabichromene (CBC) and cannabinol (CBN) were detected as minor compounds only in intact capitate-stalked trichomes in week 8. Based on the cannabinoids levels, discrimination of capitate-stalked and capitate-sessile trichomes at flowering time was possible. The study demonstrated the possibility of other spots for cannabinoids production besides the gland of the capitate-stalked trichomes. In particular the presence of cannabinoids in the stem of capitate-stalked trichomes is reported for the first time. The combined use of LMD, LC-MS and cryogenic NMR constitutes a valuable method for the comprehensive assessment of plant metabolites at the cellular level.

Introduction

Figure 1. Trichomes. A: trichomes on the bract of *C. sativa*, B: capitate-stalked trichome, C: capitate-sessile trichome, D: bulbous trichomes

Cannabis sativa has long been used as a medicine in Asia, mainly in India, before the Christian era [1]. The most responsible compounds for biological activities of *Cannabis* are cannabinoids, a unique group of terpenophenolic compounds possessing alkylresorcinol and monoterpene moieties in their molecular structures.

Recently, new insights into the cellular localization of the cannabinoids production were revealed, focusing on trichomes [2-4], small protrusions of epidermal on the surfaces of leaves and other organs of plants. In *C. sativa*, trichomes are the main site of cannabinoids production, and classified into three types, namely capitate-stalked, capitate-sessile and bulbous trichomes. Capitate-stalked trichome contains highest cannabinoids and consists of two parts, the gland (head) and the stem. The head contains disc cells presumed to be the site of cannabinoid production and surrounded by the storage cavity [2], while the stem is formed by stipe cells and basal cells and is not yet functionally characterized.

Laser microdissection (LMD) is a powerful tool for isolating specific tissues, cell types and even organelles from sectioned biological specimen in a manner conducive to the extraction of RNA, DNA, protein or secondary metabolites [5]. LMD use a narrow beam uv laser to circumscribe and cut out the cells of interest from surrounding cells and tissues. The wavelength of uv laser is 337-340 nm. It is slightly higher than the absorption peak of proteins and nucleic acids but not make heat-damage on the neighbour cells [5,6].

Experiments

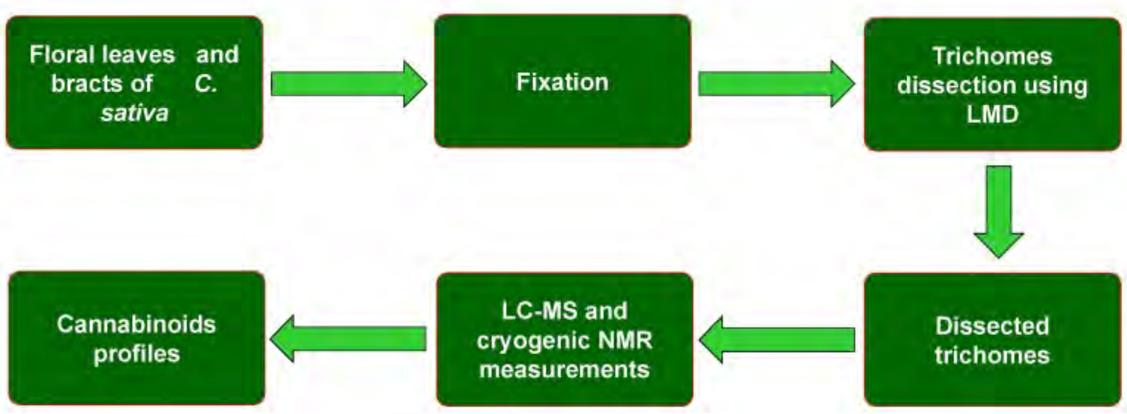

Figure 2. Experimental flowchart

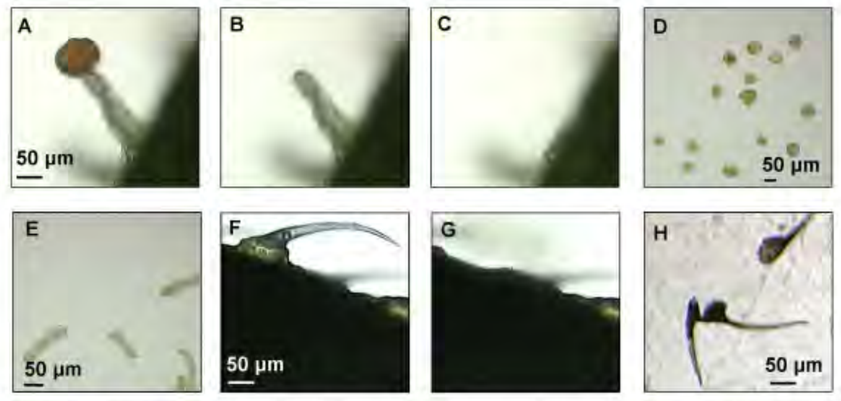

Figure 3. Microdissection of trichomes of medicinal *Cannabis*. A: intact capitate-stalked trichome before dissection, B: capitate-stalked trichome after dissection of the head cells, C: capitate-stalked trichome – complete dissection, D: dissected head cells, E: stem cells after dissection, F: capitate-sessile trichome before dissection, G: view after dissection of the capitate-sessile trichome, H: dissected capitate-sessile trichomes.

Results and discussion

- Cannabinoid profiles in all the investigated samples were qualitatively similar. THCA, CBDA, CBGA, THC, CBD, and CBG were detected in all samples during the flowering stages, while CBC and CBN were only identified in the intact capitate-stalked trichomes, and their heads at week 8.
- THCA, CBDA, and CBGA were present in high concentrations in all tested samples relative to other cannabinoids. Meanwhile, THC, CBD, and CBG were detected at low concentrations.
- CBCA, the presence of which could not be ascertained by LC-MS as its reference compound was unavailable, was effectively confirmed by ´cryogenic NMR.
- The discovery of cannabinoids in the stem of capitate stalked is new and might indicate the existence of additional sites of cannabinoid production, besides the trichome heads

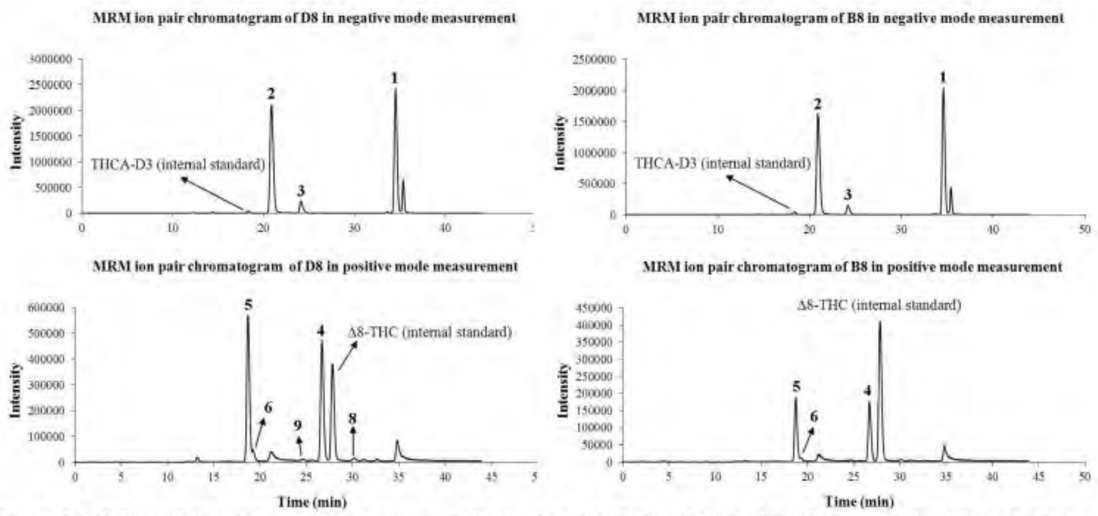

Figure 4. Total ion chromatograms of ion pair multiple reaction monitoring (MRM) of identified cannabinoids and internal standards, LC-MS analysis at week 8. D: heads of capitate-stalked trichomes, B: stems of capitate-stalked trichomes, trichomes; 1: THCA, 2: CBDA, 3: CBGA, 4: THC, 5: CBD, 6: CBG, 8: CBC, 9: CBN.

Samples	N	THCA/N (ng/ml)	CBDA/N (ng/ml)	CBGA/N (ng/ml)	THC/N (ng/ml)	CBD/N (ng/ml)	CBG/N (ng/ml)	CBC/N (ng/ml)	CBN/N (ng/ml)	OA/N (ng/ml)
ST4	76	250	100	31	3.5	1.5	0.2	ND	ND	ND
ST5	83	38	46	6	1.8	1.4	0.1	ND	ND	ND
ST6	92	733	416	88	7	7	0.6	ND	ND	ND
ST7	93	388	156	31	2.0	2.9	0.2	ND	ND	ND
ST8	90	866	571	58	11	14	0.6	0.3	0.1	ND
D4	121	126	58	18	0.8	0.4	0.1	ND	ND	ND
D5	101	220	145	38	2.0	1.2	0.1	ND	ND	ND
D6	126	272	164	43	3.0	2.0	0.3	ND	ND	ND
D7	143	141	55	13	1.5	0.7	0.1	ND	ND	ND
D8	95	841	665	71	15	21	0.9	0.9	0.1	ND
B4	43	196	116	24	2.2	1.3	0.1	ND	ND	ND
B5	88	292	164	41	3.0	1.8	0.2	ND	ND	ND
B6	74	207	87	10	2.4	1.1	0.1	ND	ND	ND
B7	94	349	114	27	3.5	1.4	0.1	ND	ND	ND
B8	75	783	530	49	6	7	0.3	ND	ND	ND
SE4	25	53	11	6	0.4	ND	ND	ND	ND	ND
SE5	45	40	13	6	0.3	ND	ND	ND	ND	ND
SE6	52	74	23	4	1.6	0.5	ND	ND	ND	ND
SE7	53	93	45	11	0.5	0.4	ND	ND	ND	ND
SE8	60	313	214	33	3	1.9	ND	ND	ND	ND

Table 1. Concentrations of cannabinoids in dissected trichomes samples based on LC-MS analysis. N: number of dissected trichomes, ST: intact capitate-stalked trichomes, D: heads of capitate-stalked trichomes, B: stems of capitate-stalked trichonmes, SE: intact capitate-sessile trichomes; number in the sample name represents the collection week; ND for CBD, CBG, CBN, OA: < 3 ng/mL in measured sample, ND for CBC: < 30 ng/mL in measured sample.

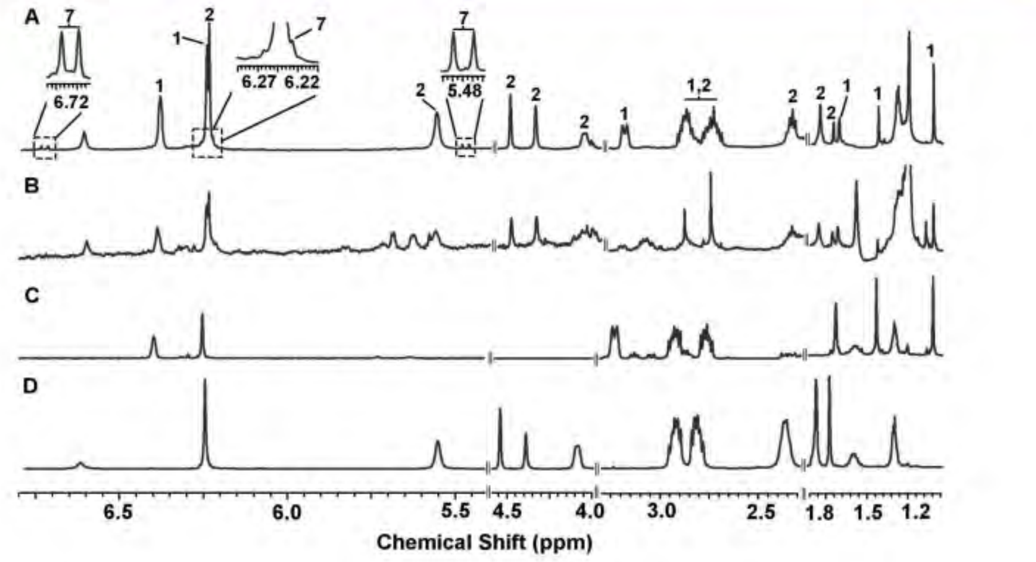

Figure 5. ^{1}H NMR spectra of dissected trichomes and reference compounds. A: capitate-stalked trichomes at week 8, B: capitate-sessile trichomes at week 8, C: reference THCA, D: reference CBDA. 1: THCA, 2: CBDA, 7: CBCA

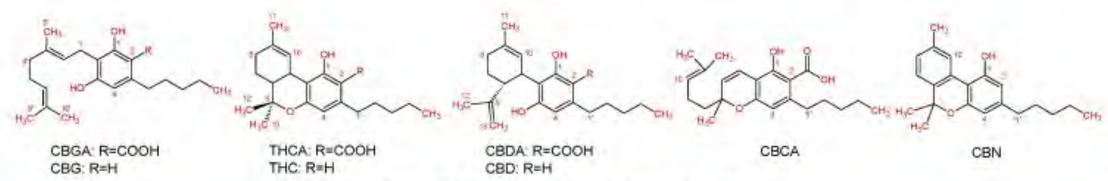

Figure 6. Structures of identified cannabinoids

Conclusions

In this research we demonstrated a promising method of cannabiniod analysis in trichomes of medicinal *Cannabis sativa* combining LMD, LC-MS, and cryogenic NMR. LMD allowed isolating specific trichomes and their individual parts without contaminations from neighbor cells or tissues. Subsequent application of LC-MS provided for effective detection and measurement of low amounts of secondary metabolites in dissected trichome samples. Finally, cryogenic NMR was used to confirm structures of the identified trichome-specific cannabinoids. Moreover, the successful detection of CBCA by the latter analytical method shows that cryogenic NMR can be efficiently applied in metabolite identification, even in absence of appropriate reference compounds.

Acknowlegments

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