Optimisation of protein extraction from medicinal cannabis mature buds for shotgun bottom-up proteomics

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Abstract

Medicinal cannabis is used to relieve the symptoms of certain medical conditions, such as epilepsy. Cannabis is a controlled substance and until recently was illegal in many jurisdictions. Consequently, the study of this plant has been restricted. Proteomics studies on Cannabis sativa reported so far have been primarily based on plant organs and tissues other than buds, such as roots, hypocotyl, leaves, hempseeds, and flour. As far as we know, no optimisation of protein extraction from cannabis reproductive tissues has been attempted. Therefore, we set out to assess different protein extraction methods followed by mass spectrometry-based proteomics to recover, separate and identify the proteins of the reproductive organs of medicinal cannabis, apical buds and isolated trichomes. Database search following shotgun proteomics was limited to C. sativa protein sequences available from UniprotKB. Our results demonstrate that a buffer containing the chaotrope reagent guanidine-hydrochloride recovers many more proteins than a urea-based buffer. In combination with a precipitation with trichloroacetic acid, such buffer proved optimum to identify proteins using a trypsin digestion followed by nLC-MS/MS analyses. This is validated by focusing on enzymes involved in the cannabinoid pathway.

Context

4000BC	Cannabis sativa plants cultivated and used as a food, textiles, ropes, paper, and medicinal applications for millennia in Asia.
1838	Rapid adoption among the Western medicinal community.
1900	Cannabis therapeutical uses diminish due opiates.
1937	Marijuana Tax Act abolishes medicinal applications in the U.S.A.
1961	Single Convention on Narcotic Drugs prohibits production and supply of cannabis.
1967	Australian Narcotic Drugs Act regulates the cultivation of cannabis for medicinal and related scientific purposes.
1989	Australian Therapeutic Goods Act ensures standards and prompt access.
2016	**Victoria is the first Australian jurisdiction to legalise access to medicinal cannabis by enacting the Medicinal Cannabis Act 2016.**
2018	Australian Narcotic Drugs Amendment Regulations for export.

Experimental design

Extraction Solutions

TCA/DTT/A solution: 10% TCA, 10mM DTT, acetone	TCA/DTT/EtOH solution: 10% TCA, 10mM DTT, EtOH
Urea buffer: 6M urea, 10mM DTT, 10mM Tris-HCl pH 8.0, 75mM NaCl, 0.05% SDS.	Gnd-HCl buffer: 6M Gdn-HCl, 10mM DTT, 5.37 mM sodium citrate tribasic 2H2O, 0.1 M Bis-Tris.

Extraction number	TCA/DTT/A precipitation	TCA/DTT/E precipitation	Urea buffer resuspension	Gnd-HCl buffer resuspension	Extraction Name
Extraction 1	no	no	yes	no	AB1
Extraction 2	no	no	no	yes	AB2
Extraction 3	yes	no	yes	no	AB3
Extraction 4	yes	no	no	yes	AB4
Extraction 5	no	yes	yes	no	AB5
Extraction 6	no	yes	no	yes	AB6

Extraction number	Urea buffer resuspension	Gnd-HCl buffer resuspension	Extraction Name
Extraction 1	yes	no	T1
Extraction 2	no	yes	T2

6 trichome extracts

18 apical bud extracts

24 plant protein extracts

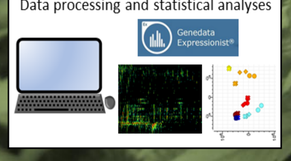
BCA protein assay (triplicates)

+ BSA

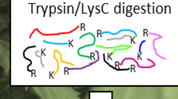
Top-down proteomics Intact protein analysis UPLC-MS (duplicates)

48 top-down LC-MS files

Data processing and statistical analyses



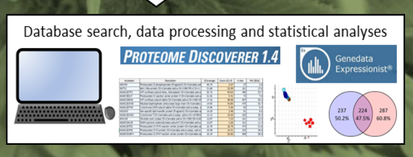
Trypsin/LysC digestion



Bottom-up proteomics Tryptic peptides analysis nLC-MS/MS (duplicates)

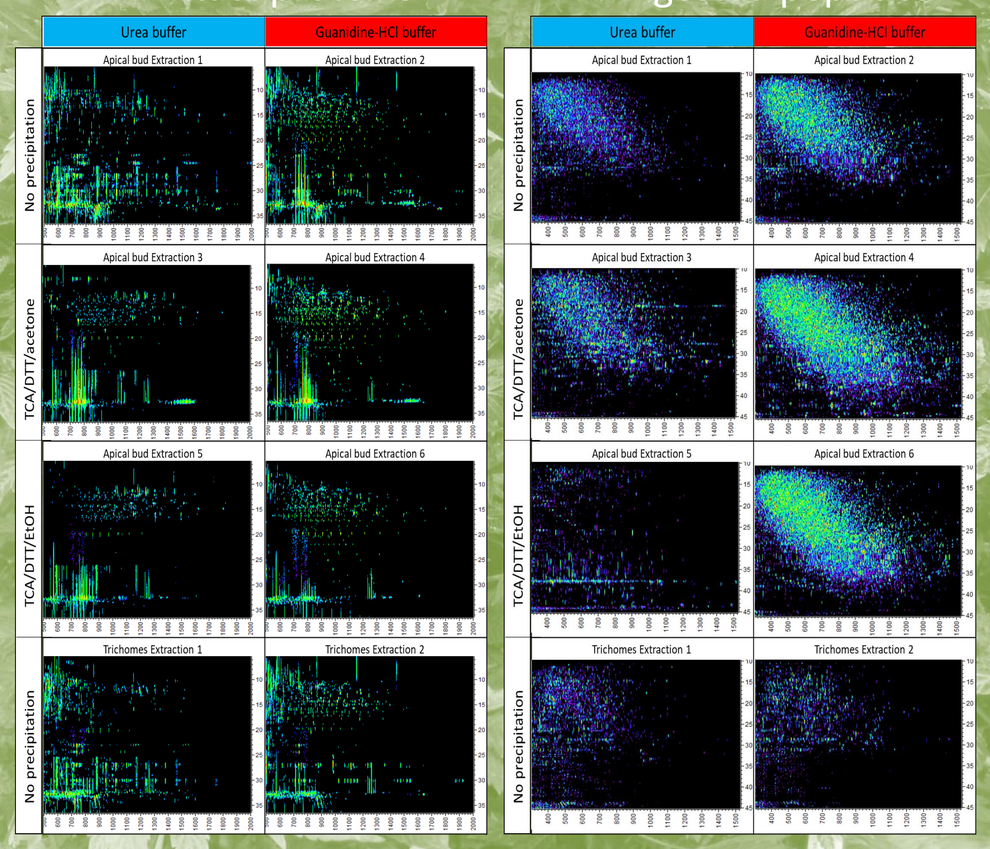
50 bottom-up LC-MS/MS files

Database search, data processing and statistical analyses



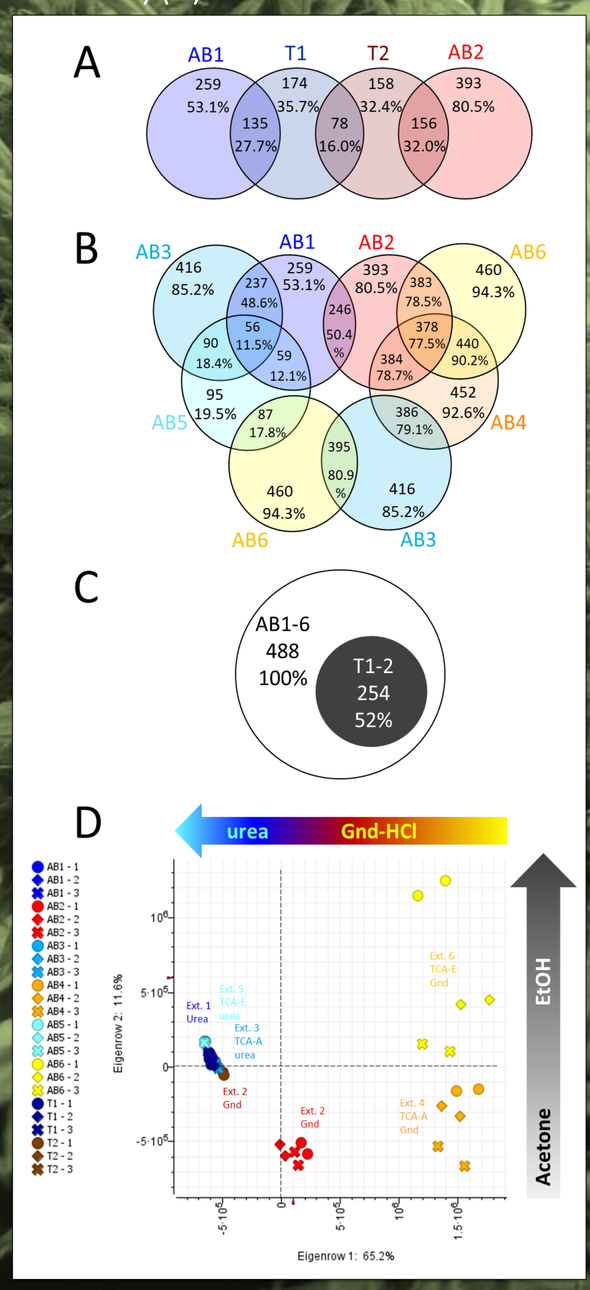
LC-MS patterns

LC-MS patterns of intact proteins (A) and tryptic digests (B) are complex. In apical buds, guanidine-HCl-based extraction methods (2, 4, and 6) generate many more peaks than urea-based methods (1, 3, and 5). In trichomes, extraction methods 1 and 2 yield comparable patterns, albeit with less peaks than in apical buds.



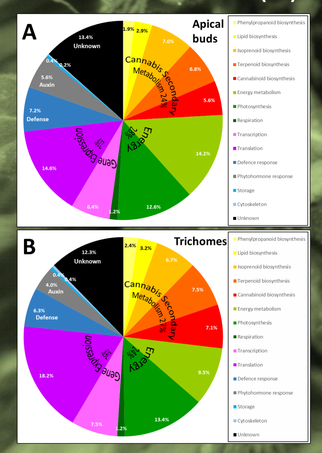
Method comparison

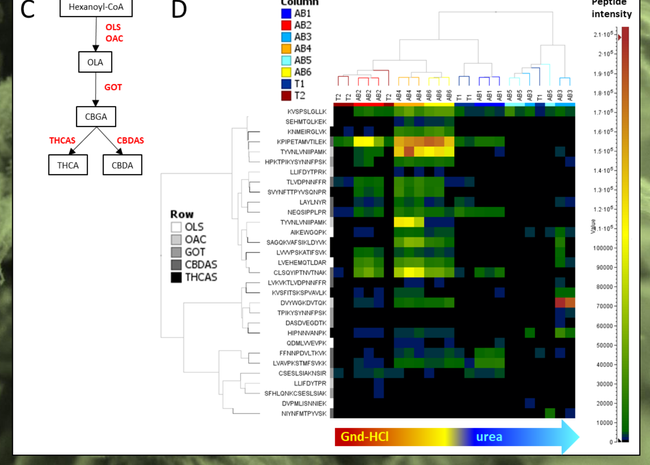
Venn diagrams shows the complementarity of methods 1 and 2 in trichomes with little overlap (A) and confirm that methods 4, 6 and 2 yield the greatest number of database hits in apical buds (B). All peptides identified in trichomes were also identified in apical buds (C). PCA illustrates the separation of guanidine-HCl based-methods from urea-based methods along PC 1 (65.2% variance), and the distinction between acetone (method 4) and ethanol (method 6) precipitations along PC 2 (11.6% variance) (D).



Protein identification

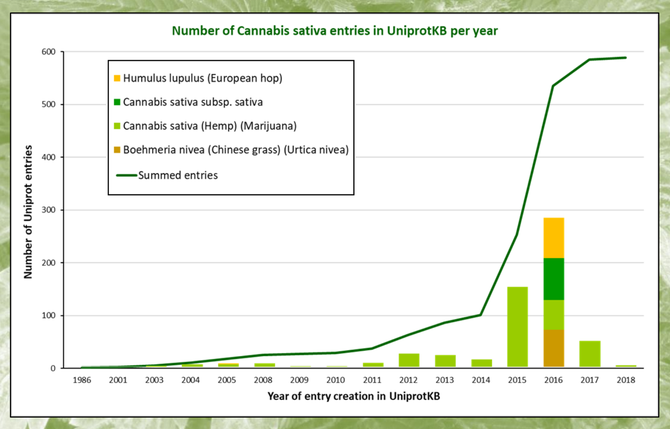
Most proteins belong to the cannabis secondary metabolism (24% in apical buds (A) and 27% in trichomes (B)), which encompasses the biosynthesis of phenylpropanoids, lipid, isoprenoids, terpenoids, and cannabinoids, the latter enriched in trichomes (B). Enzymes involved in phytocannabinoid metabolism (C) can only be identified using methods 4 and 6 (D).





Database search

We have retrieved all the entries referenced under the keyword “Cannabis sativa” in UniprotKB and produced a histogram of their distribution per year of creation. Most entries (81%) were created in 2015-2017, with only 10 created in 2018. Whilst ever-increasing, the number of sequences from *C. sativa* publicly available in Uniprot is far from sufficient, and the proteomics community still must rely on information from unrelated plants species, such as *Arabidopsis*, and rice, to identify cannabis proteins.



Conclusions – Future work

This is the first time protein extraction is optimised from cannabis reproductive organs, and a guanidine-HCl buffer used on *C. sativa* samples. Guanidine-HCl-based methods (2, 4, and 6) are best suited to recover proteins from medicinal cannabis buds and preceding this with a precipitation step in TCA/acetone (AB4) or TCA/ethanol (AB6), ensures optimum trypsin digestion followed by MS/MS. The optimum method will be applied to various cannabis cultivars. We are currently working on optimising top-down and middle-down proteomics on cannabis buds.