Identification of Commercial Plant Materials

Macro-/microscopic Analyses, DNA barcoding, and Phytochemical Analyses

Seon Beom Kim, Charlotte Simmler, Guido F. Pauli

Chosen Plant Materials

Plant Name "Pharmacopoeial name"	Common Name	Rank[1]	Plant Part	Class of Metabolites
Marrubium vulgare* L.** ***"Marrubi herba"	Horehound	1	Stems, leaves	Terpenoids (e.g. marrubiin)[2] Flavonoids (e.g, apigenin, luteolin)[3] Phenolic acids, and phenylethanoids[4] Tannins Chlorophylls
Pausinystalia yohimbe* (K. Schum.) Pierre** ***"Yohimbe cortex"	Yohimbe	12	Bark	Alkaloids (e.g. corynanthine, yohimbine)[5, 6] Tannins
Trigonella foenum-graecum* L.** ***"Foenugraeci semen"	Fenugreek	22	Seeds	Saponins (e.g. diosgenin, yamogenin)[7] Flavonoids (e.g., apigenin, luteolin)[8, 9] Pyridine-type alkaloids (e.g.,trigonelline, gentianin)[10] Carbohydrates (Galactose-mannose gum) Amino acids Fatty acids[10, 11]
***Epidemedium* sp.[12, 13]**	Horny Goat Weed	29	Aerial parts	Terpenoids Flavonoids (e.g icariin)[14, 15] Lignans[16] Chlorophylls
Cassia senna* L, Cassia angustifolia VAHL** ***"Senna folium"	Senna	34	Leaves	Anthraquinones (e.g. sennosides)[17, 18, 19] Flavonoids, phenolic aicds[19] Tannins Chlorophylls Fatty acids

Chosen Plant Materials

These five plants materials were chosen according to:

- Their mainstream **usage** by the U.S. public
- Their **phytochemical diversity**
- The diversity in the parts of the plants **traditionally used**:
  - Leaves
  - Aerial parts
  - Bark
  - Seeds
- The presence of compounds that could **interfere** with bioassays or DNA barcoding analyses including:
  - Polymeric tannins
  - Fatty acids
  - Chlorophylls
  - Polysaccharides

Few Bibliographic References

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General Experimental Procedures

- **Microscopic analyses:**
  - The acquired plant powder were prepared with chloral hydrate clearing solution on a microscopic slide
  - The slides were analyzed by the Leica DMIRB microscopy (Leica, Germany) system with LAS V4.6 microscope Imaging software
  - The collected microscopic features were compared to the information collected in the literature

- **DNA barcoding** (See related and shared protocol at https://doi.org/10.7910/DVN/M8CW8Z,)

- **Extraction (see scheme next slide):**
  - Using the DIONEX ASE 350, botanical plant materials were successively extracted by
    - 1st_ *n*-Hexane
    - 2nd_ 50% $CHCl_3$/MeOH
    - 3rd_ 50% MeOH/Water

    - Each extract obtained from the steps 1st-3rd, were combined to yield a crude extract containing metabolites of all polarity ranges.
    - To evaluate the reproducibility of the process, each plant material was extracted three times and codified #A, #B and #C
    - The extraction yields and phytochemical fingerprints were compared between each replicate
    - The extraction parameters were as follows:
      - Sample weight (10 g); cell type (100 ml); temperature (60°C); heat (5min); static time (15min); cycles (1 time); rinse volume (30%); purge (200s)

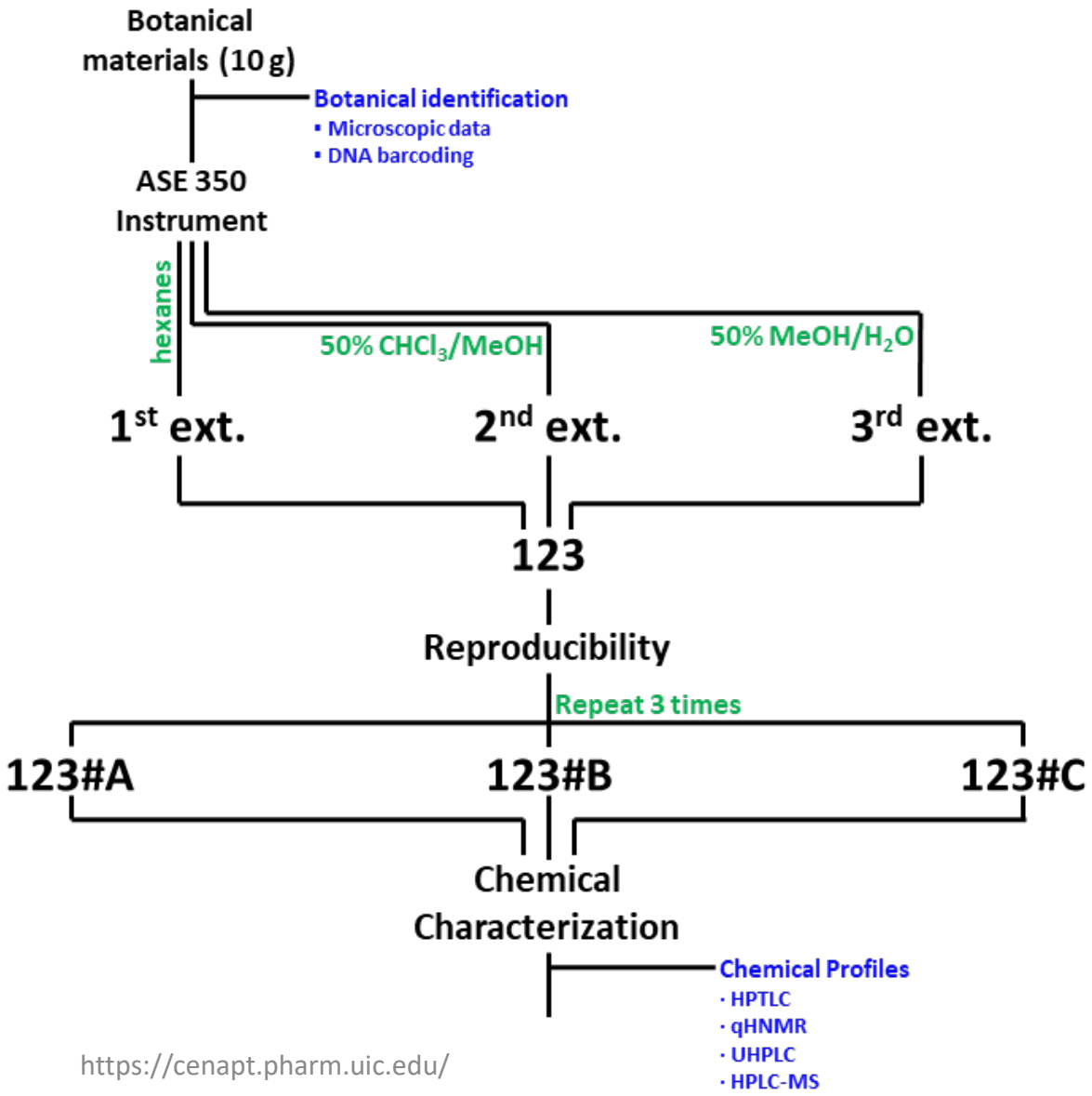
- **Phytochemical fingerprinting**
  - The chemical fingerprints of plant materials were performed by HPTLC, UHPLC-UV, and ^{1}H NMR
  - The composition of each botanical extract was compared with the corresponding specific phytochemical marker.

Schematic Overview:
From Botanical Identification to Extraction and Phytochemical Fingerprinting

[ASE_350 extraction instrument]

ASE_350 Extraction parameter

Extraction Condition	Value
Sample Weight (g)	10.0
Cell Type (ml)	100
Temperature ('C)	60
Heat (min)	5
Static Time (min)	15
Cycles	1
Rinse Volume (%)	30
Purge (s)	200



Botanical Information (*Epimedium sagittatum*)

https://www.ncbi.nlm.nih.gov/Taxonomy/

Tropicos.org. Missouri Botanical Garden. 29 Nov 2018
http://www.tropicos.org/Image/100112682

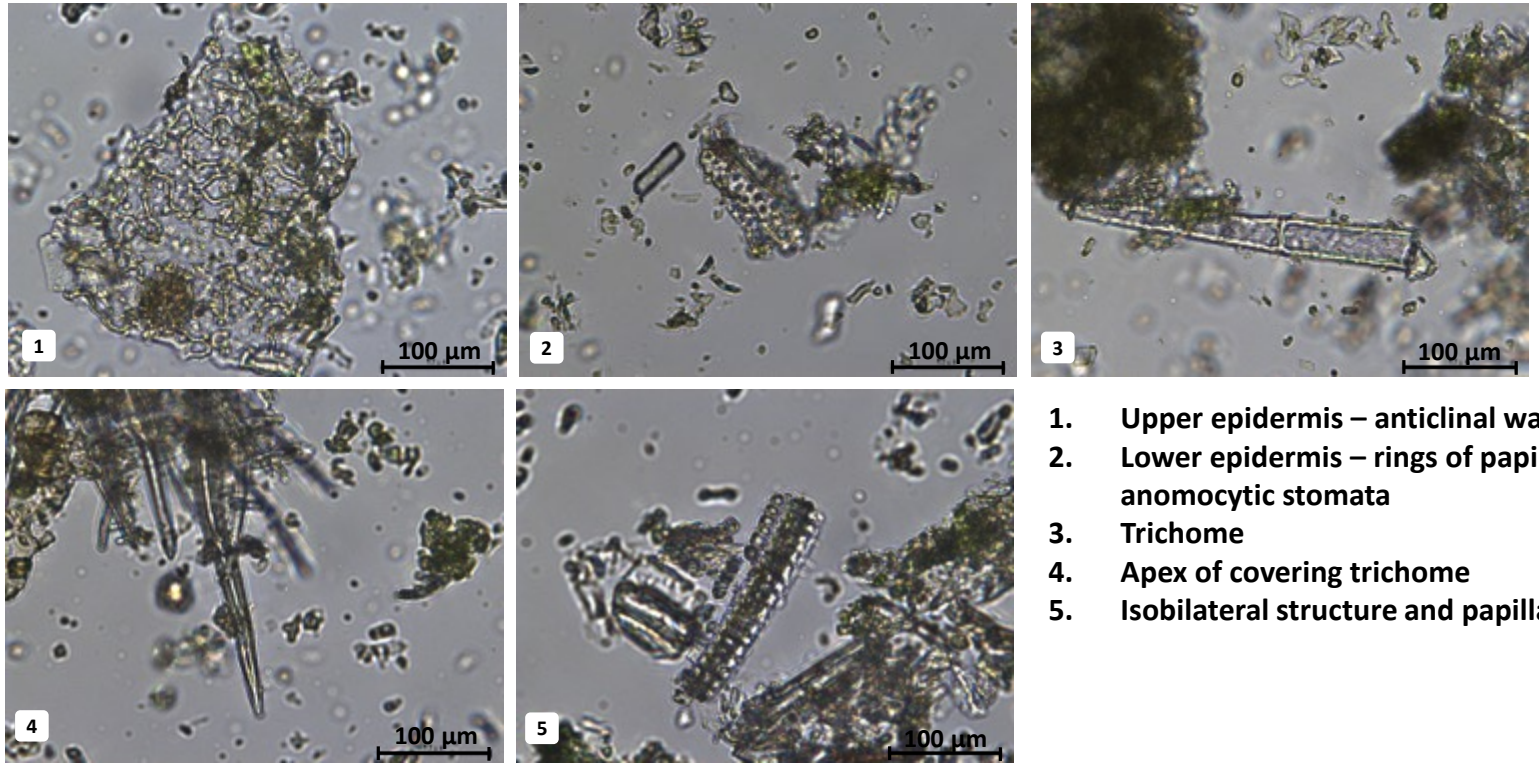
- **Taxonomy ID:** 253616 (for references in articles please use NCBI:txid253616)
- **Scientific name:** *Epimedium sagittatum* (Siebold & Zucc.) Maxim.
- **Inherited blast name:** eudicots
- **Rank:** species
- **Genetic code:** Translation table 1 (Standard)
- **Mitochondrial genetic code:** Translation table 1 (Standard)
- **Plastid genetic code:** Translation table 11 (Bacterial, Archaeal and Plant Plastid)
- **Lineage (full)**

Cellular organisms; Eukaryota; Viridiplantae; Streptophyta; Streptophytina; Embryophyta; Tracheophyta; Euphyllophyta; Spermatophyta; Magnoliophyta; Mesangiospermae; eudicotyledons; early-diverging eudicotyledons; Ranunculales; Berberidaceae; Berberidoideae; Epimedium

- **English common name for *Epimedium* sp.:** Horny Goat Weed
- **Part of the plant traditionally used:** Leaves
- **Major Phytochemicals:** Flavonoids (Icariin[14, 15]), lignans[16]

Macroscopic and Microscopic Analyses (*Epimedium species*)

- **Microscopic analyses[19]**



1. Upper epidermis – anticlinal walls
2. Lower epidermis – rings of papillae, anomocytic stomata
3. Trichome
4. Apex of covering trichome
5. Isobilateral structure and papillae

Epimedium sagitattum
Commercial powder

DNA-based Botanical Identification (*Epimedium species*)

ITS-2 sequence

485pb

AGGCTGGGACTCGAGTCTTTGACGCAAGTTGCGCCCAAGGCCATTAGGTCGAGGGCACGTCTGCCTGGGCGTCACGCACAGCGTCGCTCCCACCATTATGCCTTTG
TTCTCTTATCGGGCAACTGCAACGTGGCTTGGGAAGCGGATATTGGCCCCCCGTACCTTTGTAGGCGCGGCCGGCCTAAAATTCGGCCCTCGGCGACGAGCGTCAC
GATCAGTGGTGGTTGAATAACCCCTTTGTCATAGACCGGTATCGTGTTGTTTCGTCGTCTATTTGGGCCACATGGACCCTTGCGTGTCGTATAAACGACATTCACTCTG
CGACCCCAGGTCAGGCGGGACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAGAAGAAACTTACAAGGATTCCCTTAGTAACGGCGAGCGAACCGGGATC
AGCCCAGCTTGGGAATCGGGCGACTTCGTTGTCCGAATTGTAGTCTGGAAAAAGCGTCA

Significant alignment in Genbank with: ***Epimedium wushanense*** isolate horny_goat_weed 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. Sequence ID: KU724206.1

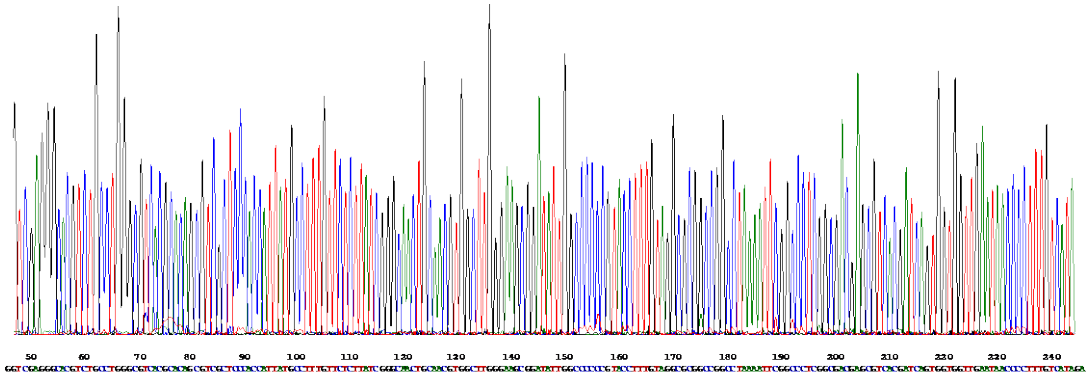
Score	Expect	Identities	Gaps	Stand
846 bits(458)	0.0	461/462 (99%)	1/462(0%)	Plus/Plus

But also with ***Epimedium simplicifolium*** isolate Y1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence, Sequence ID: KC494651.1

Score	Expect	Identities	Gaps	Stand
791 bits(428)	0.0	460/475 (97%)	3/475(0%)	Plus/Plus

Significant alignment with ***Epimedium koreanum*** genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, Sequence ID: AB758657.1

Score	Expect	Identities	Gaps	Stand
652 bits(353)	0.0	356/357 (99%)	1/357(0%)	Plus/Plus



DNA-based Botanical Identification (*Epimedium species*)

psbA-trnH sequence

371 pb

AATATTTTTTGCTTCAAACTCACATAGGCATCTTTTTGCTTTTTCAGGAAAAGAAAAAAAAGCATTACTATAACTACCGGATTCATTCTAGATTATCCTAATGTTGTATATC
TTCGTCAAAATTGTTTCTAATAATTTTTTTATCAACTTAAAGTGAAAGTTCACTAATTCGTATTAGTGTATTGTTTTCGAATGAATAGTCTGTCAATTTTTCTTTACTTTGC
AAAAAAGTATAAAGAAAAACGAAATGAAAAAAGTCATGTAATACTAAGTAGAGTGGTAGAGTGGAAATTTCATTCTTTTGGGTTAAGAAGTTGAGAAATTAAAGGG
GCGGATGTAGCCAAGTGGATTAAGGCAGTGGATTGTGAATC

Significant alignment in Genbank with ***Epimedium koreanum*** chloroplast, complete genome, Sequence ID: KM207675.1

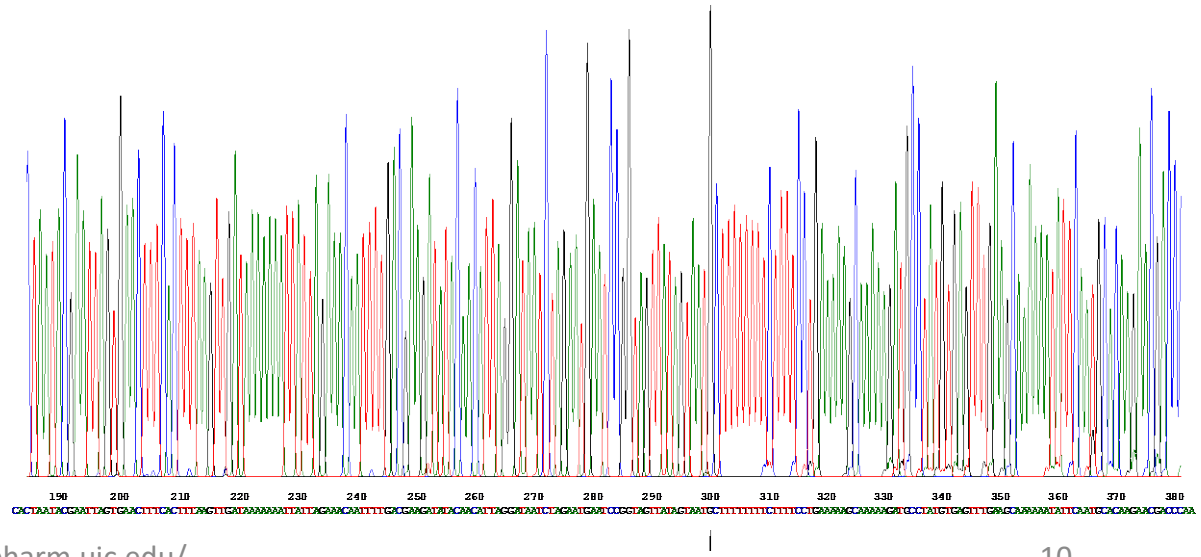
Score	Expect	Identities	Gaps	Stand
641 bits(347)	4e-180	369/378(98%)	8/378(2%)	Plus/Minus

But also with ***Epimedium acuminatum*** chloroplast, complete genome, Sequence ID: KU522469.1

Score	Expect	Identities	Gaps	Stand
641 bits(347)	4e-180	364/372(98%)	1/372(0%)	Plus/Minus

And with ***Epimedium sagittatum*** chloroplast, complete genome, Sequence ID: KU204899.1

Score	Expect	Identities	Gaps	Stand
641 bits(347)	4e-180	364/372(98%)	1/372(0%)	Plus/Minus



DNA-based Botanical Identification (*Epimedium species*)

rbcL sequence

718 pb

GGCGTTTGCATCAGCGGGTGTTAAGATTACAAATTGACTTATTATACTCCTGACTATGTAACGAAGGATACTGATATTTTGGCAGCATTCCGCGTCACTCCTCAACCTG
GAGTTCCACCTGAAGAAGCAGGGGCCGCTGTAGCTGCCGAATCTTCTACAGGTACATGGACAACCGTGTGGACCGATGGACTTACCAGTCTTGATCGTTACAAAGG
ACGGTGCTACCACATTGAGCCTGTTGCTGGAGAAGACAATCAATATATTTGTTACGTAGCCTATCCTTTAGACCTTTTTGAAGAGGGTTCTGTTACTAACATGTTTACTT
CTATTGTGGGTAATGTATTTGGGTTCAAAGCGCTGCGCGCTCTACGTCTGGAGGATCTGCGAATTCCTGTTGCTTATGTTAAAACTTTCCAAGGCCCGCCTCATGGTAT
CCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCTCTATTAGGATGTACTATTAAACCAAAATTGGGATTATCCGCTAAGAACTATGGTAGAGCGGTTTATGAA
TGTCTCCGCGGTGGGCTTGATTTTACCAAGGATGATGAGAACGTGAACTCCCAGCCATTTATGCGTTGGAGAGATCGTTTCCTATTTTGTGCCGAAGCTATTTATAAAT
CACAGGCGGAAACAGGTGAAATCAAAGGACATTACTTGAATGCTACTGCAGGTAACATGGCGAAAT

Significant alignment in Genbank with ***Epimedium koreanum*** chloroplast, complete genome Sequence ID: KM207675.1

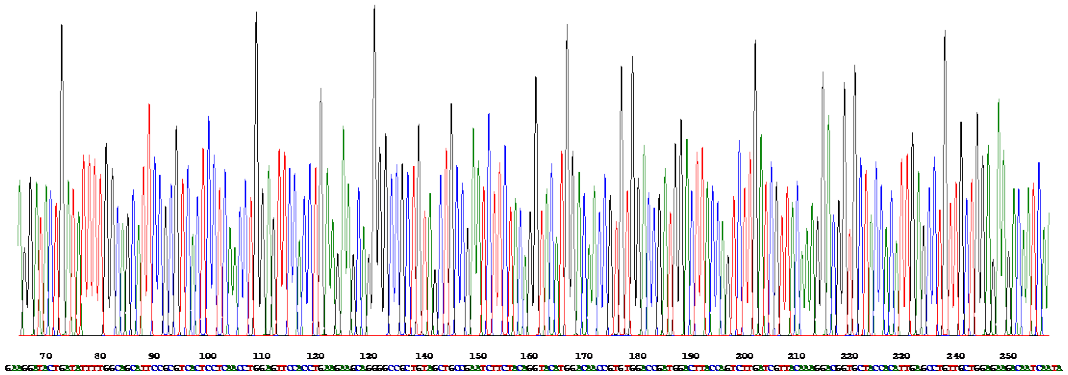
Score	Expect	Identities	Gaps	Stand
1277 bits(691)	0.0	694/695 (99%)	1/695(0%)	Plus/Plus

But also with ***Epimedium dolichostemon*** chloroplast, complete genome,
Sequence ID: KU522470.1

Score	Expect	Identities	Gaps	Stand
1266 bits(685)	0.0	692/695(99%)	1/695(0%)	Plus/Plus

And with ***Epimedium sagittatum*** chloroplast, complete genome,
Sequence ID: KU204899.1

Score	Expect	Identities	Gaps	Stand
1260 bits(682)	0.0	691/695(99%)	1/695(0%)	Plus/Plus



DNA-based Botanical Identification (*Epimedium species*)

matK sequence

849 pb

CTACCCCATCCATTTTGAACTCTTGATTCAAACCCTTCGCTATTGGATACAGGATACCCCCGCTTTGCATTTATTACGATTCTTTCTCTACGAGTCTCAGAATTCGAATAAT
CTGATTACTCAAAAAAAAAGAGATATTTCGCATTTTTCAAATCAGAATCAAAGATTTTTCTTGTTCCTATATAATATTCATATATATGAATGCGAATCCATATTCGTTTTTCT
CCGTAAACAATCTGTTCATTTACGATCAAGATCGTATAGAGCCCTTCTTGAGCGAACACATTTTTATCGAAAAATAGACAAGTTTTTCTTCATTTTTCATAAAAATTTTCA
GACCACCTTATGGTTGTTCAAGGATCCTTTCATGAATTATGTCAGATATCAAGGAAAAGCCATTCTGGCTTCAAAAGGAACACCTCTTCTGATAAAAAAATGGAAGTAT
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AGAAATCTTTTTCATTTTTTTAGTGGATCCGCAGAAAAAAATACTTTGTTTCGAGTAAAGTACATACTTCGACT

Significant alignment in Genbank with ***Epimedium koreanum*** chloroplast, complete genome Sequence ID: KM207675.1,

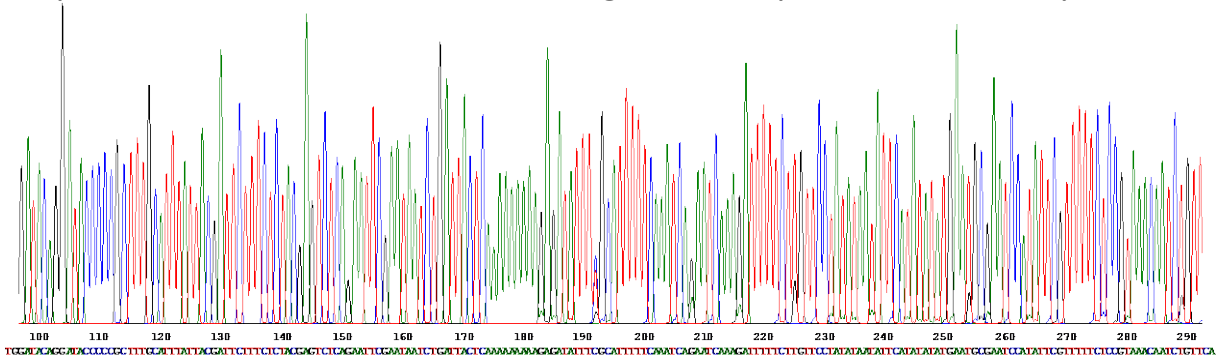
Score	Expect	Identities	Gaps	Stand
1557 bits(843)	0.0	847/849(99%)	0/849(0%)	Plus/Minus

…But also with ***Epimedium grandiflorum*** tRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene, complete cds; chloroplast, Sequence ID: JN010335.1

Score	Expect	Identities	Gaps	Stand
1552 bits(840)	0.0	846/849(99%)	0/849(0%)	Plus/Plus

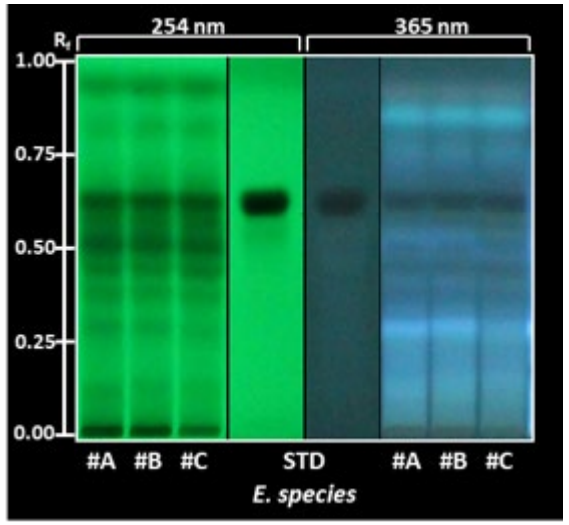
…And with ***Epimedium sagittatum*** chloroplast, complete genome, Sequence ID: KU204899.1

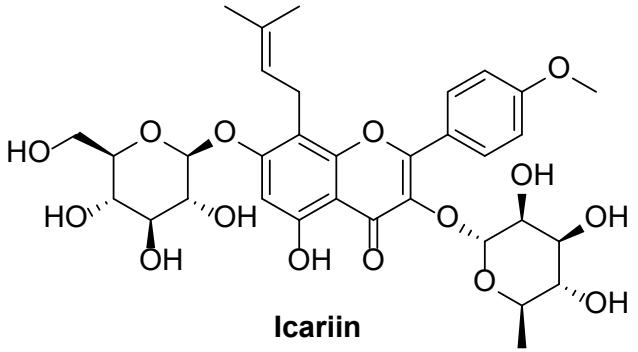
Score	Expect	Identities	Gaps	Stand
1546 bits(847)	0.0	845/849(99%)	0/849(0%)	Plus/Minus



Phytochemical Fingerprinting by HPTLC (*Epimedium species*)

[A] HPTLC





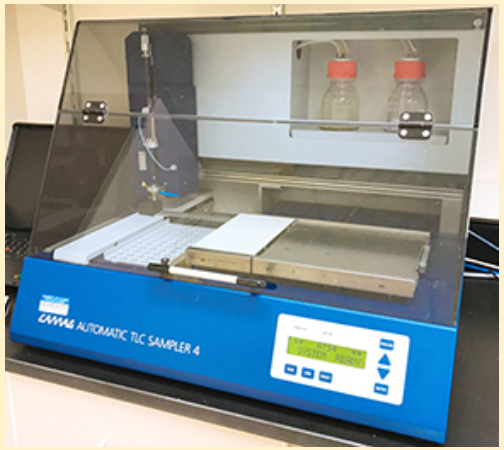
Icariin

▸ **Sample Preparation**
· Crude extracts: 10 mg/ml in the 50% CH_3CN/H_2O
· Reference compound: 1 mg/ml in the 50% CH_3CN/H_2O

▸ **HPTLC Plate**
· HPLTC Silica gel F_{254} [EMD]

▸ **HPTLC Solvent System**
· EtOAc/acetic acid/formic acid/water (100/11/11/26)

▸ **HPTLC Image Capture**
· UVP MultiDoc-It Digital Imaging system (254 nm, 365nm)

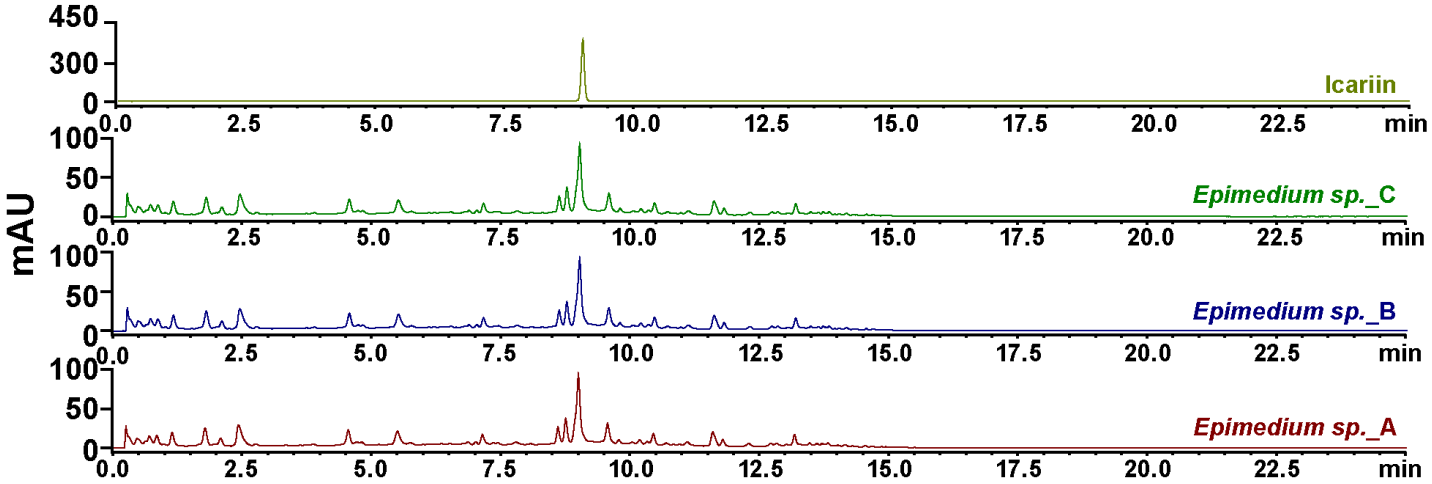


▸ **HPTLC condition**

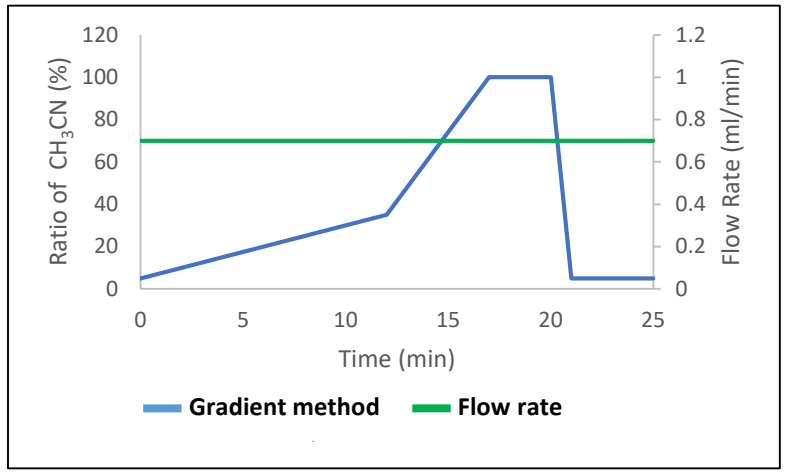
Instrument: CAMAG Automatic TLC Sampler 4
Band Length (mm): 4.0
Application volume (µl): 5.0
Filling speed (µl/s): 11.0
Predosage volume (nl): 200
Retraction volume (nl): 200
Dosage speed (nl/s): 100
Rinsing vacuum time (s): 6
Filling vacuum times (s): 0
Gas: Air
TLC size (cm): 5*10

Phytochemical Fingerprinting by UHPLC (*Epimedium species*)

[B] UHPLC (254 nm)



Gradient method

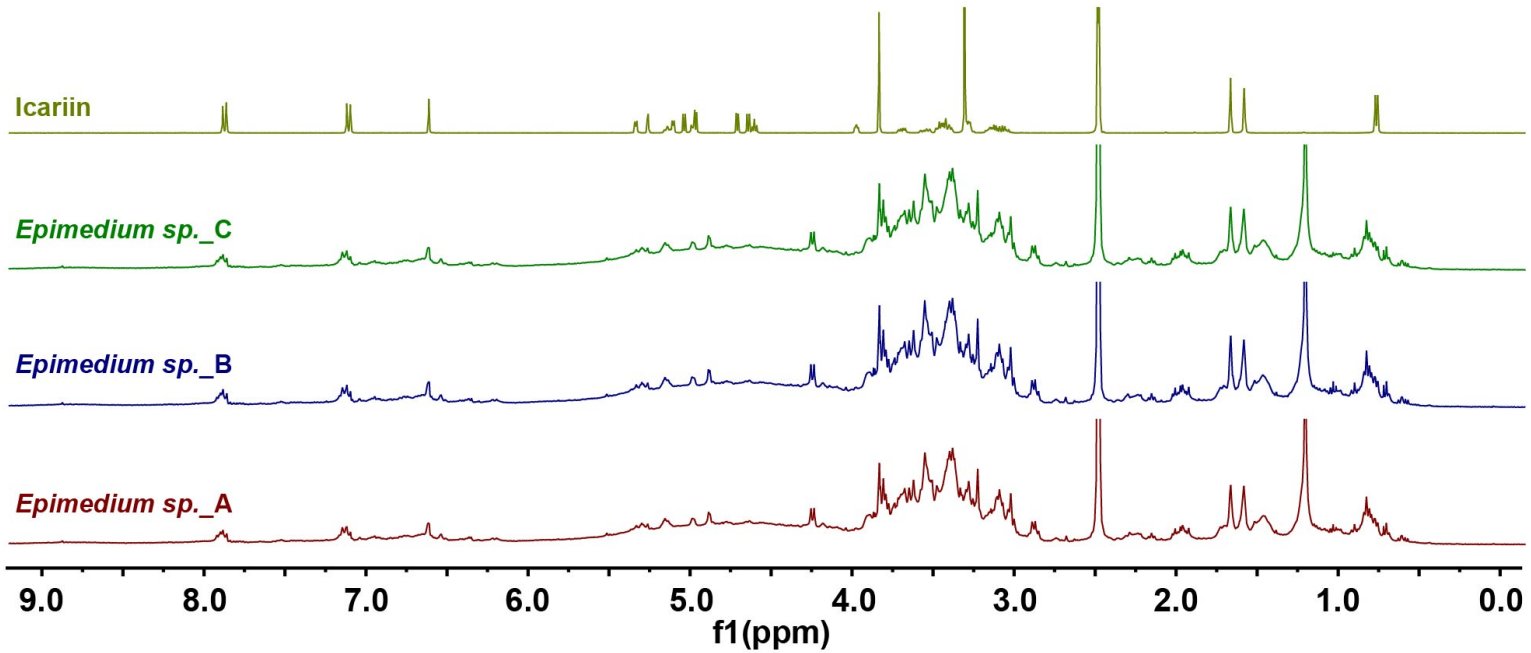


- **Instrument**
  - Shimadzu UFLC (Shimadzu Corp) with DAD and fluorescence detector
  - Column: Kinetex 1.7 µm XB-C18 100Å column (50.0 X 2.1 mm Phenomenex, USA)
  - Software: Shimadzu Labsolution software package
  - Solvent system: CH_3CN/Water gradient from 95% to 5% water with 0.1% formic acid

- **Sample preparation**
  - Crude extracts (10 mg/ml) and reference compound (1 mg/ml) prepared in 50% CH_3CN/water

Phytochemical Fingerprinting by ^{1}H-NMR (*Epimedium species*)

[C] ^{1}H-NMR



► **Sample Preparation**
- Crude extracts: 123#A: 7.32 mg/200 µl; 123#B: 7.50 mg/200 µl; 123#C: 7.72 mg/200 µl
- Icariin: 0.86 mg/200 µl
- Solvent: DMSO-d_6 (99.9%), Cambridge Isotope Laboratories, Inc. (Cas #: 2206-27-1, Lot #: 12G-464)

► **Instrument**
- Jeol ECZ 400 MHz in 3 mm NMR tube under the Ultra COOL probe.

► **Parameter (qNMR)**
- Temperature 25℃
- 90° single-pulse (relaxation delay: 60sec, receiver gain: 46, number of scan: 64)

Conclusions
Identification of a Commercial Plant Powder Declared as *Epimedium* Leaves

- The macroscopic and microscopic analyses confirm that our plant material contains grinded leaves of plant material.

- The DNA barcoding analyses confirm that the commercial sample contains (an) ***Epimedium species***. There was slightly lower alignment scores and sequence homologies with ***E. sagittatum*** as opposed to alignment scores with registered ***E. koreanum*** DNA sequences in GenBank.

- All acquired plants phytochemical fingerprints (HPTLC, UHPLC-UV and ^{1}H-NMR) confirm the presence of the characteristic marker Icariin in all extract replicates (A-C).

- There is no clear evidence that the commercial sample contains only ***E. sagittatum***. The commercial sample could contain a mixture of closely related ***Epimedium species***.

Botanical Information (*Marrubium vulgare*)

https://www.ncbi.nlm.nih.gov/Taxonomy/

Manfredonia (Italy), Dec 23, 2007
https://calphotos.berkeley.edu/cgi/img_query?seq_num=233678&one=T

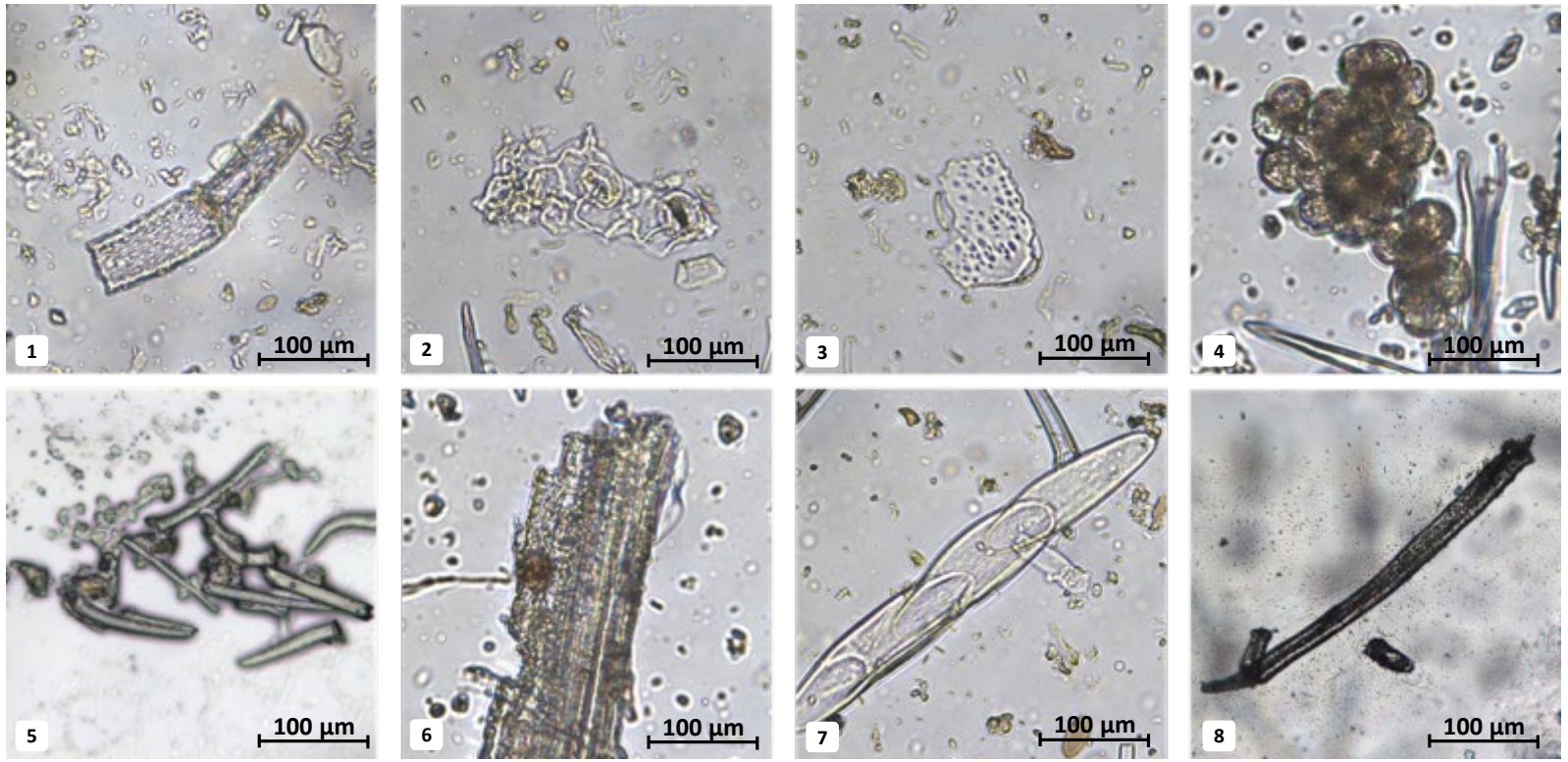
- **Taxonomy ID:** 41230 (for references in articles please use NCBI:txid41230)
- **Scientific name:** *Marrubium vulgare* L., 1753
- **Genbank common name:** white horehound
- **Inherited blast name:** eudicots
- **Rank:** species
- **Genetic code:** Translation table 1 (Standard)
- **Mitochondrial genetic code:** Translation table 1 (Standard)
- **Plastid genetic code:** Translation table 11 (Bacterial, Archaeal and Plant Plastid)
- **Lineage (full)**

cellular organisms; Eukaryota; Viridiplantae; Streptophyta; Streptophytina; Embryophyta; Tracheophyta; Euphyllophyta; Spermatophyta; Magnoliophyta; Mesangiospermae; eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Lamiales; Lamiaceae; Lamioideae; Marrubieae; Marrubium

- **English common name for *Marrubiium vulgare*:** Horehound
- **Part of the plant traditionally used:** Stems, leaves
- **Major reported Phytochemicals :** Marrubiin[2], flavonoids (apigenin, luteolin)[3]

Macroscopic and Microscopic Analyses (*Marrubium vulgare*)

- **Microscopic analyses[19]**



Marrubium vulgare
Commercial material

1. -
2. Paracytic stoma
3. -
4. Collenchyma
5. Trichome
6. Vessel
7. -
8. -

DNA-based Botanical Identification (*Marrubium vulgare*)

ITS-2 sequence: 290 pb

GCTCGCCGTTACTAGGGGAATCCTTGTAAGTTTCTTTTCCTCCGCTTATTGATATGCTTAAACTCAGCGGGTGATCCCGCCTGACCTGGGGTCGCGGTCGTGGGCACG
ATGCGTGCTCGCGCCGTTGGGTTGCGTTGCTGTTTCCGGACCGACGACGCCGTGGAGCGCGACAGCACGCGAGTTGATGGTTCAACCACCACTGGTCGCGACGCG
GGTCGACGGCGGATTCGCATTTGGGCCGGCCGCGCGGGCGTGCGCGCACGGGGGGCCAATCTCCGCCCCCCACCC

Representing only a clean portion of the DNA amplicon (really dirty sequence). Significant sequence alignment in GenBank with ***Marrubium supinum*** 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence, Sequence ID: AF335642.1

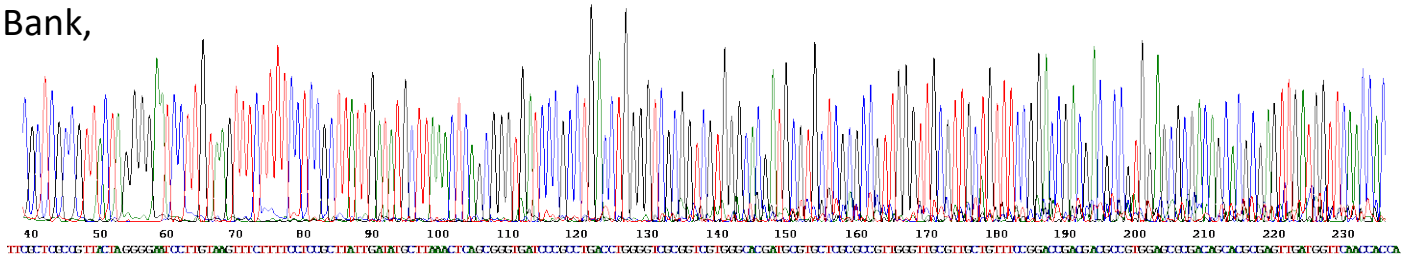
Score	Expect	Identities	Gaps	Stand
379 bits(205)	2e-101	217/223(97%)	0/223(0%)	Plus/Plus

Other significant sequence alignment with ***Phlomis mongolica*** voucher PS1732MT01 internal transcribed spacer 2 and 28S ribosomal RNA gene, partial sequence, Sequence ID: FJ546872.1

Score	Expect	Identities	Gaps	Stand
412 bits(223)	2e-111	242/251(99%)	2/251(0%)	Plus/Minus

As there is a lack of ITS-2 sequences for ***Marrubium vulgare*** in GenBank, the sequence was also compared to the reference Herbarium (#1663369) ITS-2 sequence (see slides after conclusion)

ITS-2 DNA chromatogram



DNA-based Botanical Identification (*Marrubium vulgare*)

psbA-trnH: 173 pb

GGATAAGACTTGGTCTTAGTGTATAGGAGTTTTTGAAAATAGAATAGATAAATATAAGGAGCAATAACCCCTCTTGATAAAACAAGAAAGAGTTTATTAGCTCCTTAAT
TTTCTTTTCAATTACTTTTTTCCTTTCCATTAAAGGATTCAGAAAATGAAAGAAGAAAAAAAAC
Representing only a clean portion of the DNA amplicon.

Alignment with ***Marrubium peregrinum*** voucher dimou1024TAU PsbA (psbA) gene, partial sequence; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast Sequence ID: EU627584.1

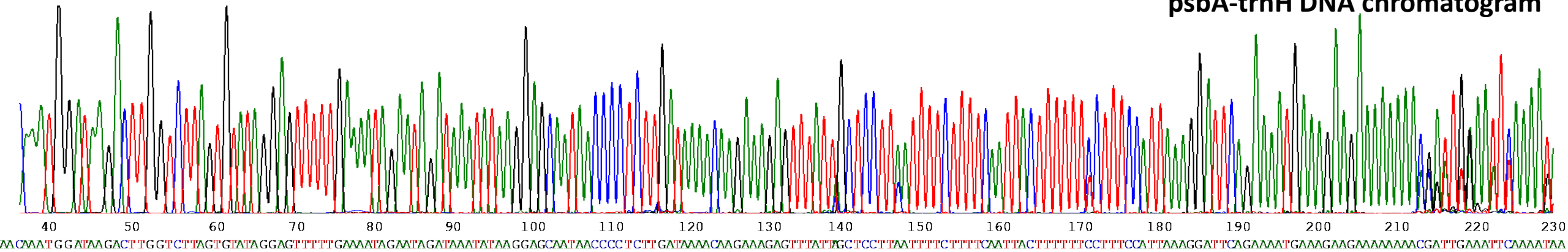
Score	Expect	Identities	Gaps	Stand
307 bits(166)	6e-80	171/173(99%)	1/173(0%)	Plus/Plus

and with ***Marrubium vulgare*** voucher SA1664 psbA-trnH intergenic spacer, partial sequence; chloroplast, Sequence ID: HQ902823.1

Score	Expect	Identities	Gaps	Stand
281 bits(152)	3e-72	157/159(99%)	1/159(0%)	Plus/Plus

The sequence was also compared to the reference Herbarium (#1663369)

psbA-trnH DNA chromatogram



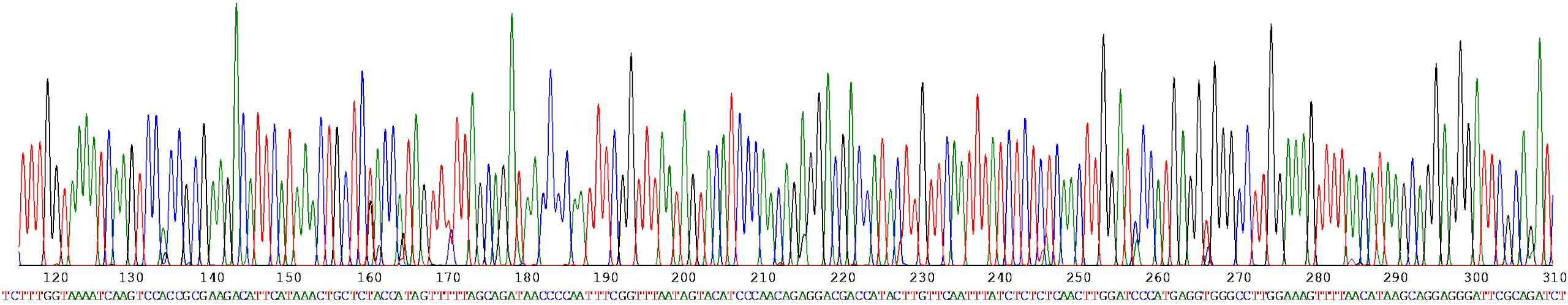
DNA-based Botanical Identification (*Marrubium vulgare*)

rbcL sequence: 649 pb

TATACCCCTGAATACGAAACCACAATGATACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCCGCCCGAAGAAGCAGGGGCCGCGGTAGCTGCC
GAATCTTCGACTGGTACATGGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGGCGATGCTACCACATCGAGCCTGTTCTTGGAGAAAAAG
ATCAATATATCTGTTATGTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTAGGAAATGTATTTGGATTCAAAGCCCTACGTG
CTCTACGTCTGGAAGATCTGCGAATCCCTCCTGCTTATGTTAAAACTTTCCAAGGCCCACCTCATGGGATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTC
CTCTGTTGGGATGTACTATTAAACCGAAATTGGGGTTATCTGCTAAAAACTATGGTAGAGCAGTTTATGAATGTCTTCGCGGTGGACTTGATTTTACCAAAGATGATGA
AAACGTGAACTCCCAGCCATTTATGCGTTGGAGAGATCGCTTCTTGTTTTGTGCCGAAGCAATTTATAAATCACAGGCTGAAACAGGTGAAATCAAAGGGCATTATTT

Significant alignment in Genbank with ***Marrubium vulgare*** (I) chloroplast rbcL gene for rubisco (large subunit) (partial), Sequence ID: Z37411.1

Score	Expect	Identities	Gaps	Stand
1186 bits(642)	0.0	647/649(99%)	2/649(0%)	Plus/Plus



DNA-based Botanical Identification (*Marrubium vulgare*)

matK sequence: 774pb

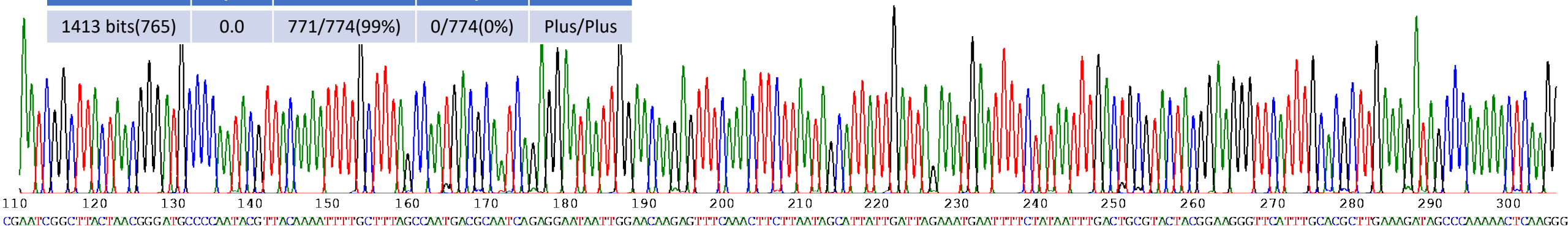
TGGTTCAAATCCTTCGCTATTGGGTAAAAGATGCTTCCTCCTTGCATTTATTACGAGTCTTTCTCAACGAATATTGTAGTTGGAATAGTCTTCTTATTCCAAAGAAAGCC
AGTTCCCCGTCTTTAAAAAAAAATCAAAGATTATTCTTATTCTTATATAATTCTCATGTATGCGAATATGAATCCATTTTCGTCTTTCTACGTAACCAATCTTTTCATTTACG
ATCAACATCTTCTGGAGTTTTTCTTGAACGAATATATTTCTATATAAAAATAGAACGTCTTGTGAACGTCTTTGTTAAGATTACGGGTTTGGGGGCAAACCTGCGGTTG
GTCAAGGAACCTTTCATGCATTATATTAGGTATCAAAAAAGATCCATTCTGGCTTCAAAAGGGACATTTCTTTTCATGAAGAAATGGAAATTTTACCTTGTCACCTTTTG
GCAATGGCATTTTTTGGTGTGGTTTCATTCAAGAAGCATTTATATAAACCAATTATCCAAGCATTCCCTTGAGTTTTTGGGCTATCTTTCAAGCGTGCAAATGAACCCTT
CCGTAGTACGCAGTCAAATTATAGAAAATTCATTTCTAATCAATAATGCTATTAAGAAGTTTGAAACTCTTGTTCCAATTATTCCTCTGATTGCGTCATTGGCTAAAGCAA
AATTTTGTAACGTATTGGGGCATCCCGTTAGTAAGCCGATTCGGACTGATTTATCAGATTCTAATATTATTGACCGATTTGGGCGTATATGCAGAAATATTTCTCGTTATC

Significant alignment in Genbank with ***Marrubium crassidens*** voucher SKU:outgroup maturase K (matK) gene, partial cds; chloroplast, Sequence ID: KP993196.1

Score	Expect	Identities	Gaps	Stand
1419 bits(768)	0.0	772/774(99%)	0/774(0%)	Plus/Plus

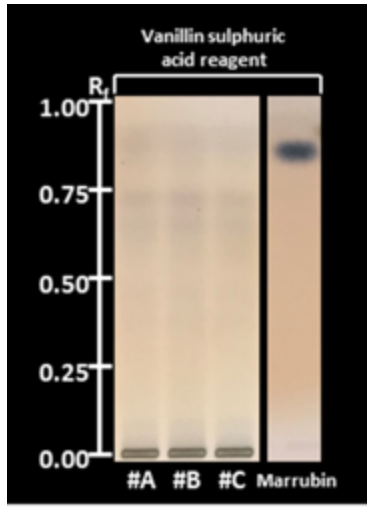
…and with ***Marrubium vulgare*** maturase K (matK) gene, partial cds; chloroplast, Sequence ID: HM850794.1

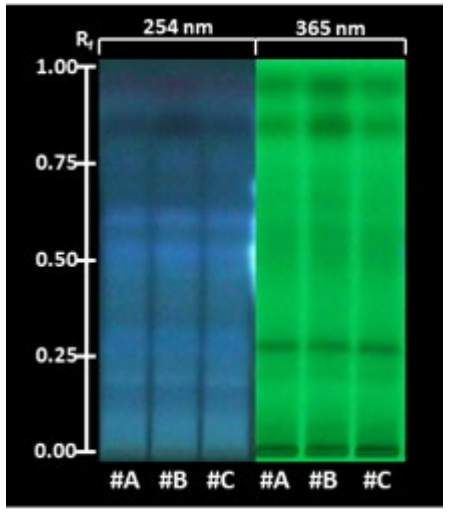
Score	Expect	Identities	Gaps	Stand
1413 bits(765)	0.0	771/774(99%)	0/774(0%)	Plus/Plus

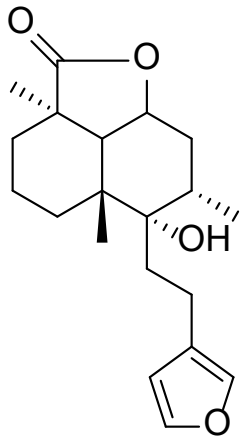


Phytochemical Fingerprinting by HPTLC (*Marrubium vulgare*)

[A] HPTLC







Marrubin

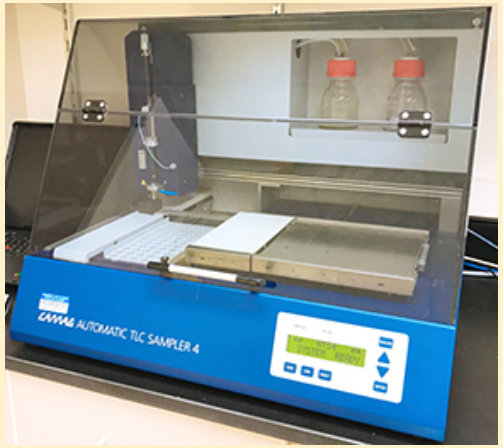
▸ **Sample Preparation**
· Crude extract: 10 mg/ml in the 50% CH_3CN/H_2O
· Reference compounds: 1 ml/ml in the 50% CH_3CN/H_2O

▸ **HPTLC Plate**
· HPLTC Silica gel F_{254} [EMD]

▸ **HPTLC Solvent System**
· Hexanes/acetone (50/50)
· EtOAc/acetic acid/formic acid/water (100/11/11/26)

▸ **HPTLC Image Capture**
· UVP MultiDoc-It Digital Imaging system (254 nm, 365nm)

▸ **TLC Visualization reagent**
· 5% Vanillin-sulphuric reagent

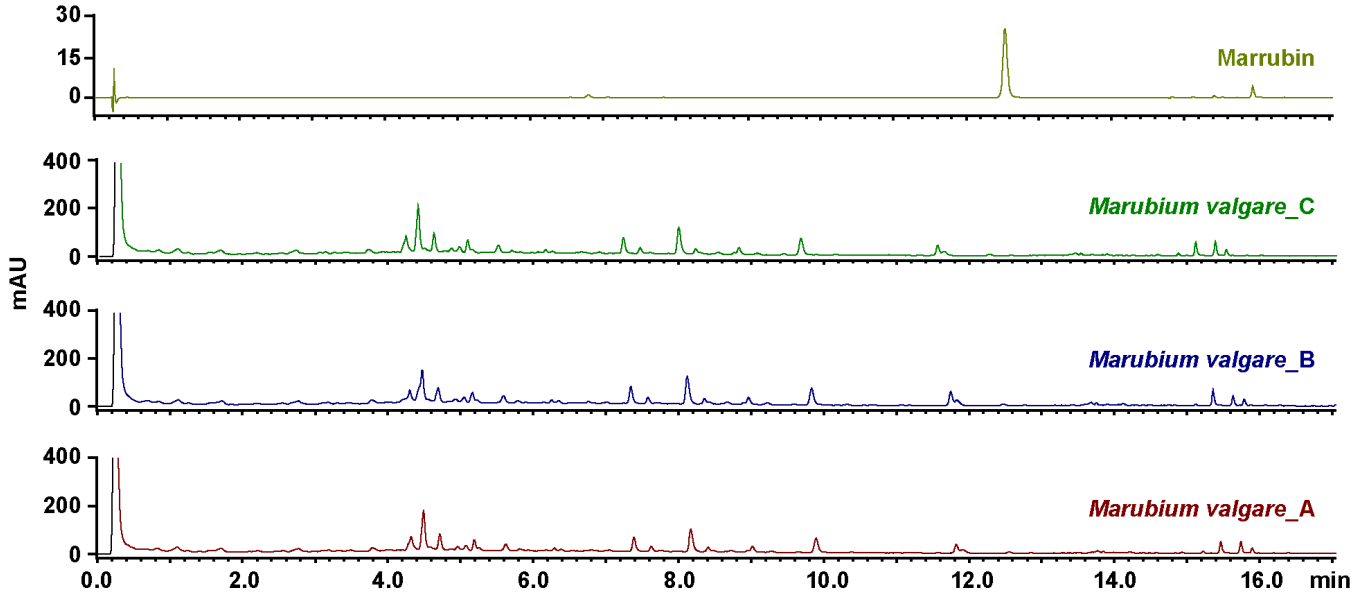


▸ **HPTLC condition**

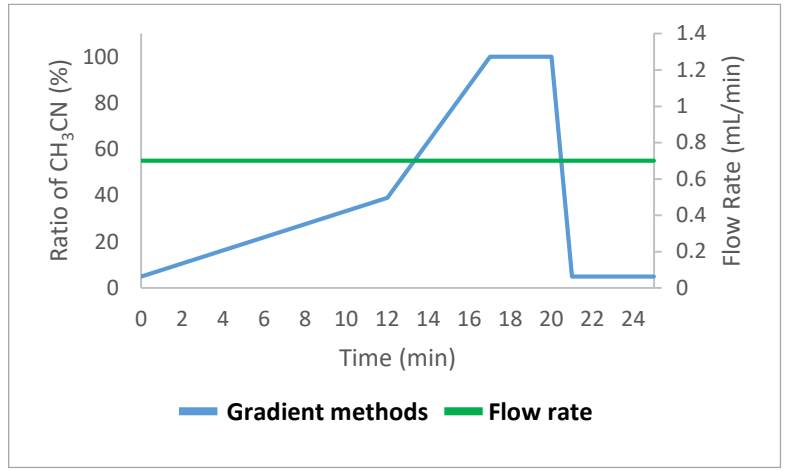
· **Instrument: CAMAG Automatic TLC Sampler 4**
· **Band Length (mm): 4.0**
· **Application volume (µl): 5.0**
· **Filling speed (µl/s): 11.0**
· **Predosage volume (nl): 200**
· **Retraction volume (nl): 200**
· **Dosage speed (nl/s): 100**
· **Rinsing vacuum time (s): 6**
· **Filling vacuum times (s): 0**
· **Gas: Air**
· **TLC size (cm): 5*10**

Phytochemical Fingerprinting by UHPLC-UV (*Marrubium vulgare*)

[B] UHPLC (190 nm)



Gradient method

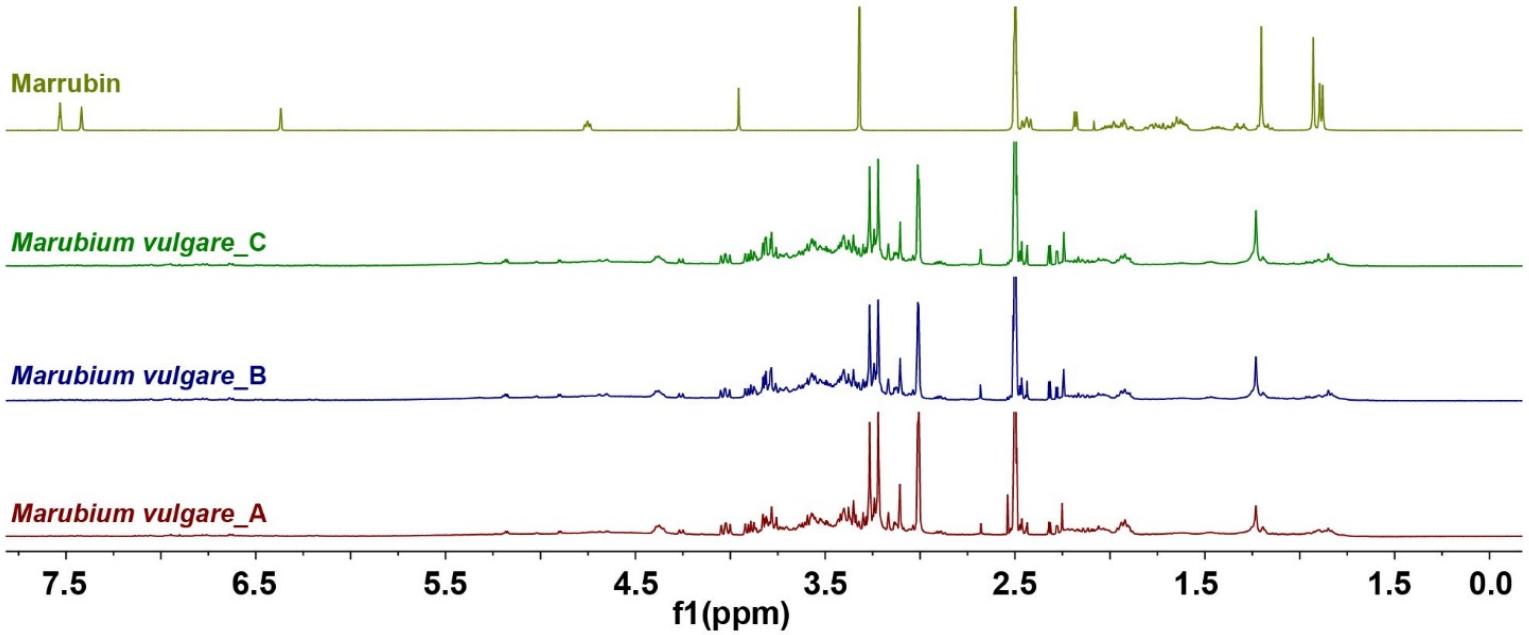


- **Instrument**
  - Shimadzu UFLC (Shimadzu Corp) with DAD and fluorescence detector
  - Column: Kinetex 1.7 µm XB-C18 100Å column (50.0 X 2.1 mm Phenomenex, USA)
  - Software: Shimadzu Labsolution software package
  - Solvent system: CH_3CN/Water gradient from 95% to 5% water with 0.1% formic acid

- **Sample preparation**
  - Crude extracts (10 mg/ml) and reference compound (1 mg/ml) prepared in 50% CH_3CN/water

Phytochemical Fingerprinting by ^{1}H-NMR (*Marrubium vulgare*)

[C] ^{1}H-NMR



► **Sample preparation**
- Crude extracts: 123#A: 5.13 mg/200 µl; 123#B: 5.08 mg/200 µl; 123#C: 5.59 mg/200 µl
- Marrubin: 0.91 mg/200 µl
- Solvent: DMSO-d_6 (99.9%), Cambridge Isotope Laboratories, Inc. (Cas #: 2206-27-1, Lot #: 12G-464)

► **Instrument**
- Jeol ECZ 400 MHz in 3 mm NMR tube under the Ultra COOL probe.

► **Parameter (qNMR)**
- Temperature 25°C
- 90° single-pulse (relaxation delay: 60sec, receiver gain: 46, number of scan: 64)

Conclusions:
Identification of a Commercial Plant Powder declared as *Marrubium vulgare*

- Microscopic analyses of **the commercial sample** showed paracytic stomata, collenchyma, trichomes, and vessels of plant aerial parts. However, some other unattributable fragments were also observed. Collectively, this data confirmed that the sample contains aerial parts.

- The DNA barcoding analysis led to the amplification of multiple ITS-2 and psbA-trnH sequences, possibly indicating a mixture of ***species***. However, rbcL and matK sequences obtained from the commercial sample perfectly aligned with those obtained from the Herbarium specimen, and with sequences referenced in Genbank. Therefore, the commercial sample contains ***M. vulgare.***

- Marrubiin defined as a specific marker of ***M. vulgare***, was not detected in the three crude extracts (A-C). LC-Ms analysis will be necessary to confirm the observations made by comparing the HPTLC, UHPLC-UV and ^{1}H-NMR fingerprints, and possibly detect marrubiin.

- On the basis of all these results the commercial sample could contain mixture of botanical species., including ***M. vulgare***.

Herbarium Specimen used a DNA Barcoding Reference

From the Field Museum of National History in Chicago
Thanks to Dr. Deol D. Soejarto and Dr. Bethany Elkington

DNA-based Botanical Identification (*Marrubium vulgare*)

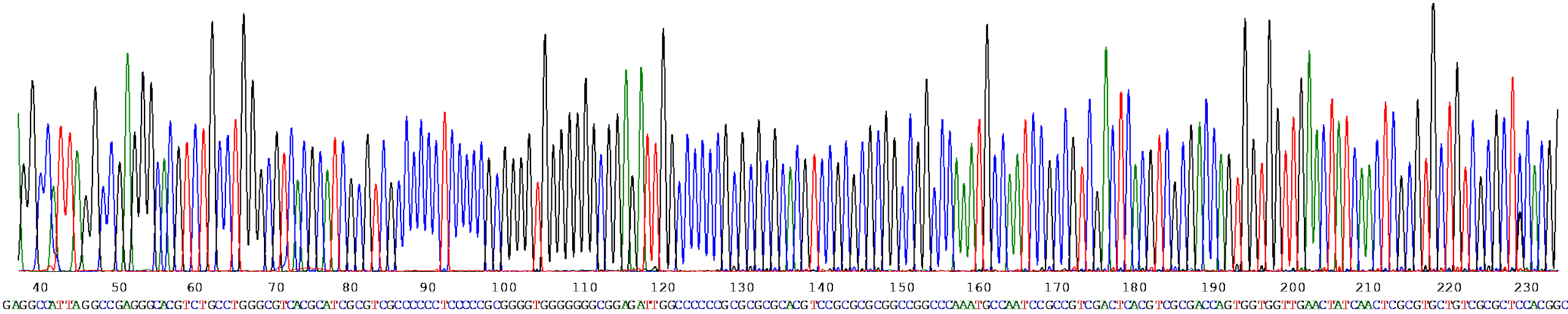
Herbarium specimen: Field Museum accession number: 1663369
Specimen collected in 1966, in Peru
Serving as reference sequence

ITS-2 sequence: 390 pb

CACGTCTGCCTGGGCGTCACGCATCGCGTCGCCCCCCTCCCCCGCGGGGTGGGGGGGCGGAGATTGGCCCCCCGCGCGCGCACGTCCGCGCGCGGCCGGCCCAA
ATGCCAATCCGCCGTCGACTCACGTCGCGACCAGTGGTGGTTGAACTATCAACTCGCGTGCTGTCGCGCTCCACGGCGTCGTCGGTCCGGAAACAGCAACGCAACC
CAACGGCGCGAGCACGCATCGTGCCCACGACCGCGACCCCAGGTCAGGCGGGATCACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAAACTTACAAG
GATTCCCCTAGTAACGGCGAGCGAACCGGGAATAGCCCAACTTGAGAATCGGGCGGCCACGCCGTCCGAATTGTA

Lack of M. vulgare ITS/ITS-2 DNA sequences in Genbank! Sequence alignments (97% identities) were obtained with ***Phlomis mongolica*** voucher PS1732MT01 internal transcribed spacer 2 and 28S ribosomal RNA gene, partial sequence, Sequence ID: FJ546872.1

Score	Expect	Identities	Gaps	Stand
499 bits(270)	2e-137	289/298(97%)	2/298(0%)	Plus/Plus



DNA-based Botanical Identification (*Marrubium vulgare*)

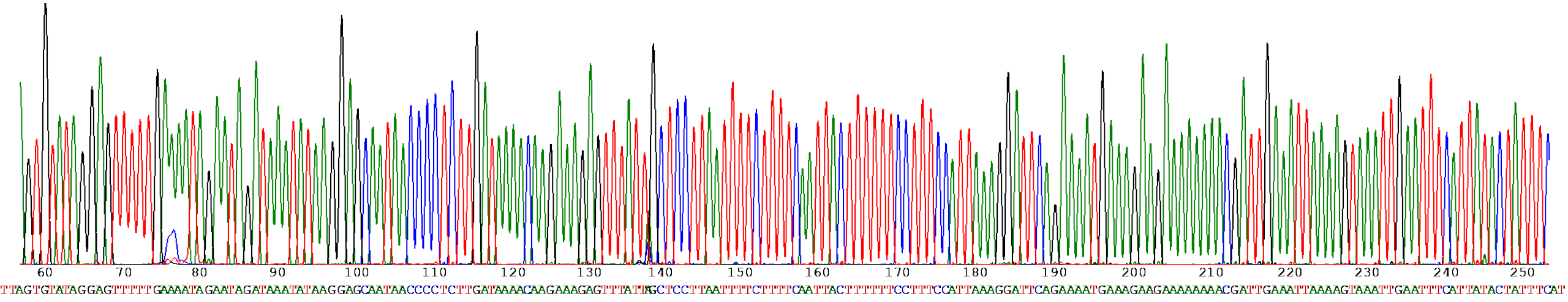
Herbarium specimen: Field Museum accession number: 1663369
Specimen collected in 1966, in Peru
Serving as reference sequence

psbA-trnH sequence:260 pb

TGGTCTTAGTGTATAGGAGTTTTTGAAAATAGAATAGATAAATATAAGGAGCAATAACCCCTCTTGATAAAACAAGAAAGAGTTTATTAGCTCCTTAATTTTCTTTTCAA
TTACTTTTTTCCTTTCCATTAAAGGATTCAGAAAATGAAAGAAGAAAAAAAACGATTGAAATTAAAAGTAAATTGAATTTCATTATACTATTTCATTATACTAATAGTTGA
GGGGCGGATGTAGCCAAGTGGATCAAGGCAGTGGATTGT

Sequence alignment in Genbank with ***Marrubium vulgare*** voucher SA1664 psbA-trnH intergenic spacer, partial sequence; chloroplast, Sequence ID: [HQ902823.1](HQ902823.1)

Score	Expect	Identities	Gaps	Stand
451 bits(244)	2e-129	251/254(99%)	2/254(0%)	Plus/Plus



DNA-based Botanical Identification (*Marrubium vulgare*)

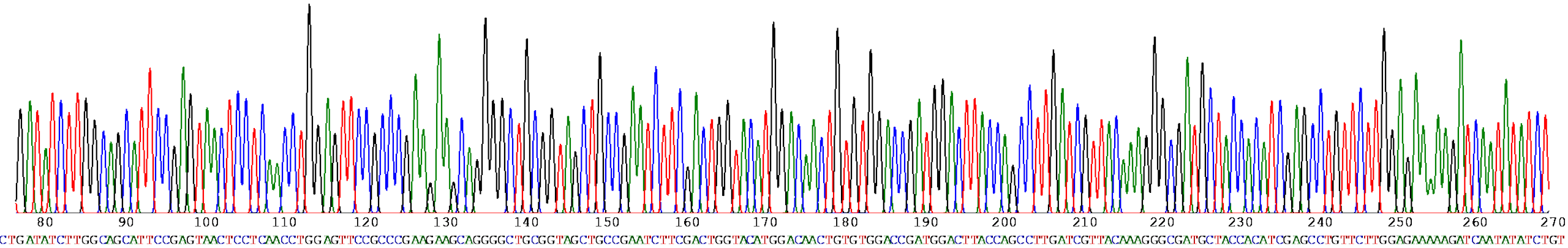
Herbarium specimen: Field Museum accession number: 1663369
Specimen collected in 1966, in Peru
Serving as reference sequence

rbcL sequence: 659 pb

CCCCTGAATACGAAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCCGCCCGAAGAAGCAGGGGCTGCGGTAGCTGCCGAATCT
TCGACTGGTACATGGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGGCGATGCTACCACATCGAGCCTGTTCTTGGAGAAAAAGATCAATA
TATCTGTTATGTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTAGGAAATGTATTTGGATTCAAAGCCCTACGTGCTCTACG
TCTGGAAGATCTGCGAATCCCTCCTGCTTATGTTAAAACTTTCCAAGGCCCACCTCATGGGATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCTCTGTT
GGGATGTACTATTAAACCGAAATTGGGGTTATCTGCTAAAAACTATGGTAGAGCAGTTTATGAATGTCTTCGCGGTGGACTTGATTTTACCAAAGATGATGAAAACGT
GAACTCCCAGCCATTTATGCGTTGGAGAGATCGCTTCTTGTTTTGTGCCGAAGCAATTTATAAATCACAGGCTGAAACAGGTGAAATCAAAGGGCATTATTTGAATGC
TACTGCAGGT

Significant alignment in Genbank with ***Marrubium vulgare*** (I) chloroplast rbcL gene for rubisco (large subunit) (partial), Sequence ID: Z37411.1

Score	Expect	Identities	Gaps	Stand
1206 bits(653)	0.0	657/659(99%)	0/659(0%)	Plus/Plus



DNA-based Botanical Identification (*Marrubium vulgare*)

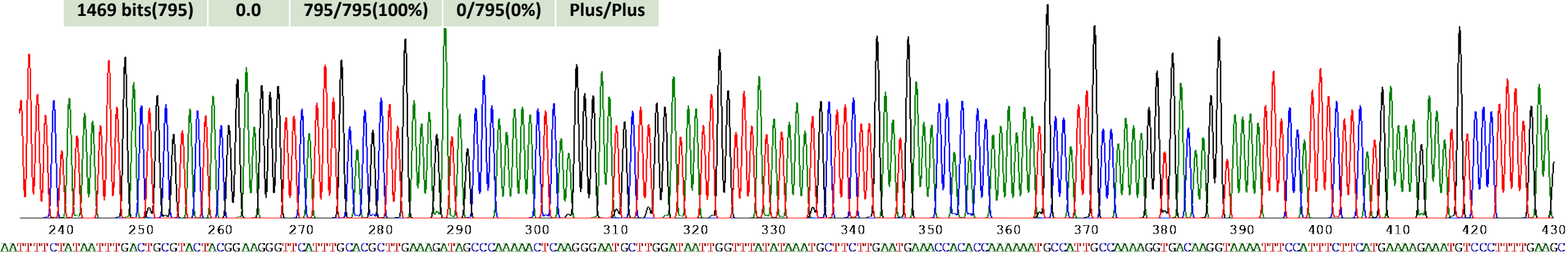
Herbarium specimen: Field Museum accession number: 1663369
Specimen collected in 1966, in Peru
Serving as reference sequence

matK sequence:850 pb

ATGGTAATACCTCGCCCTGTTCATGCGGAAATCTTGGTTCAAATCCTTCGCTATTGGGTAAAAGATGCTTCCTCCTTGCATTTATTACGAGTCTTTCTCAACGAATATTGT
AGTTGGAATAGTCTTCTTATTCCAAAGAAAGCCAGTTCCCCGTCTTTAAAAAAAAATCAAAGATTATTCTTATTCTTATATAATTCTCATGTATGCGAATATGAATCCATTT
TCGTCTTTCTACGTAACCAATCTTTTCATTTACGATCAACATCTTCTGGAGTTTTTCTTGAACGAATATATTTCTATATAAAAATAGAACGTCTTGTGAACGTCTTTGTTAA
GATTACGGATTTGGGGGCAAACCTGCGGTTGGTCAAGGAACCTTTCATGCATTATATTAGGTATCAAAAAAGATCCATTCTGGCTTCAAAAGGGACATTTCTTTTCAT
GAAGAAATGGAAATTTTACCTTGTCACCTTTTGGCAATGGCATTTTTTGGTGTGGTTTCATTCAAGAAGCATTTATATAAACCAATTATCCAAGCATTCCCTTGAGTTTT
TGGGCTATCTTTCAAGCGTGCAAATGAACCCTTCCGTAGTACGCAGTCAAATTATAGAAAATTCATTTCTAATCAATAATGCTATTAAGAAGTTTGAAACTCTTGTTCCA
ATTATTCCTCTGATTGCGTCATTGGCTAAAGCAAAATTTTGTAACGTATTGGGGCATCCCGTTAGTAAGCCGATTCGGACTGATTTATCAGATTCTAATATTATTGACCGA
TTTGGGCGTATATGCAGAAATATTTCTCGTTATCATAGTGGATCTTCAAAAAAAAAGAGTTTGTCTCGAATAAAGT

Significant alignment in Genbank with ***Marrubium vulgare*** isolate NMW529 maturase K (matK) gene, partial cds; chloroplast, Sequence ID: JN895787.1

Score	Expect	Identities	Gaps	Stand
1469 bits(795)	0.0	795/795(100%)	0/795(0%)	Plus/Plus



Botanical Information (*Pausinystalia johimbe*)

https://www.ncbi.nlm.nih.gov/Taxonomy/

Tropicos.org. Missouri Botanical Garden. 29 Nov 2018
https://nccih.nih.gov/health/yohimbe

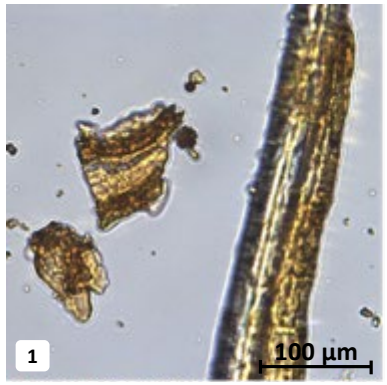
- **Taxonomy ID:** 170026 (for references in articles please use NCBI:txid170026)
- **Scientific name:** *Pausinystalia johimbe*
- **Inherited blast name:** eudicots
- **Rank:** species
- **Genetic code:** Translation table 1 (Standard)
- **Mitochondrial genetic code:** Translation table 1 (Standard)
- **Plastid genetic code:** Translation table 11 (Bacterial, Archaeal and Plant Plastid)
- **Lineage (full)**

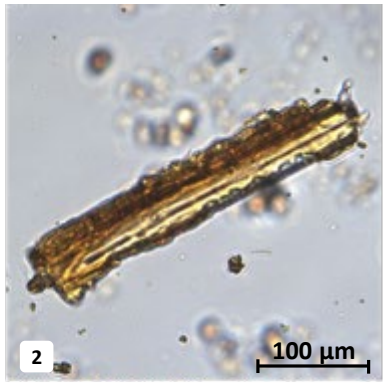
Cellular organisms; Eukaryota; Viridiplantae; Streptophyta; Streptophytina; Embryophyta; Tracheophyta; Euphyllophyta; Spermatophyta; Magnoliophyta; Mesangiospermae; eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Gentianales; Rubiaceae; Cinchonoideae; Naucleeae; Pausinystalia

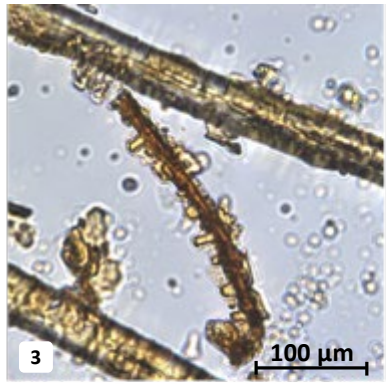
- **English common name for *Pausinystalia johimbe*:** Yohimbe
- **Part of the plant traditionally used:** Bark
- **Major Phytochemicals :** Alkaloids (corynanthine, yohimbine)[5, 6]

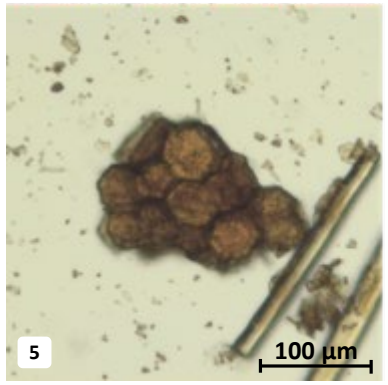
Macroscopic and Microscopic Analyses (*Pausinystalia johimbe*)

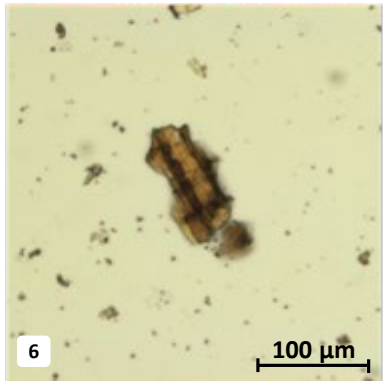
- **Microscopic analyses**[19]











1. **Reticulated vessel**
2. **-**
3. **-**
4. **Long thin fiber**
5. **Medullary ray**
6. **-**

***Pausinystalia johimbe* *Commercial* powder**

DNA-based Botanical Identification (*Pausinystalia johimbe*)

rbcL sequence: 658 pb

TTGACTTATTATACTCCCGAATATCAACCCCAGGATACCGATATCTTGGCAGCATTCCGAGTAACCCCTCAACCTGGAGTTCCGTCAGAAGAAGCAGGAGCCGCAGTAGCTGCCG
AATCTTCTACTGGTACATGGACAACTGTATGGACCGACGGACTTACCAGTCTTGATCGTTACAAAGGACGATGCTACCACATCGATGCCGTTCCTGGAGAAGACAATCAATATATT
TGTTATGTAGCTTACCCATTAGACCTTTTTGAAGAAGGTTCTGTTACTAATATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAGGCCCTGCGTGCTCTACGTTTGGAGGAT
TTGCGAATCCCTGTTGCTTATATAAAAACTTTCCAAGGCCCGCCTCACGGTATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGCCGTCCTCTACTGGGATGCACTATTAAGC
CGAAATTGGGGTTATCCGCTAAAAACTATGGTCGAGCAGTTTATGAATGTCTTCGCGGTGGACTTGATTTTACCAAAGATGACGAAAACGTGAACTCCCAGCCATTTATGCGTTG
GAGAGACCGTTTCTTATTTTGTGCCGAAGCAATTTATAAAGCACAGGCCGAAACAGGTGAAATCAAAGGGCATTACTTG

Significant sequence alignment with ***Opuntia maxima*** (!) ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast, Sequence ID: HM850212.1

Score	Expect	Identities	Gaps	Stand
1214 bits(657)	**0.0**	**657/657(100%)**	**0/657(0%)**	**Plus/Plus**

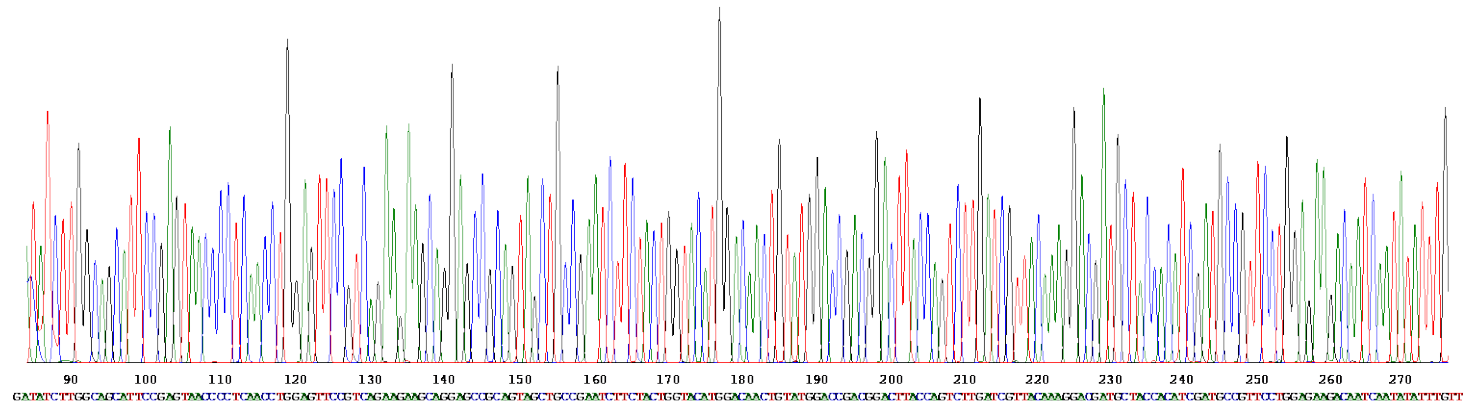
This alignment does not make sense
in light of the powder (macro)/microscopic characteristics and its chemistry.

The alignment with ***Pausynistalia*** species gave less homologies (identity percentage 91%):
Alignment with ***Pausinystalia*** lane-poolei subsp. ituriense chloroplast partial rbcL gene for ribulose-1, 5-bisphosphate carboxylase, specimen voucher Hart 1350 (BR), Sequence ID: AJ346999.1

Score	Expect	Identities	Gaps	Stand
893 bits(483)	**0.0**	**600/658(91%)**	**2/658(0%)**	**Plus/Plus**

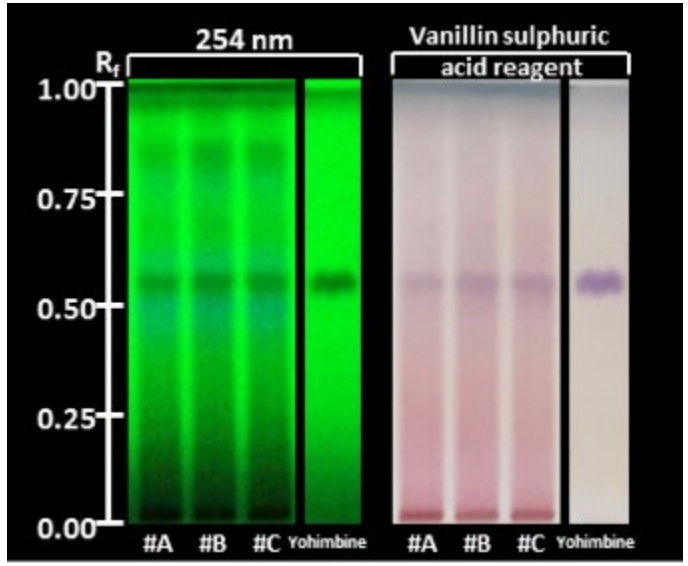
Alignment with ***Pausinystalia johimbe*** chloroplast partial rbcL gene
Sequence ID: AJ346998.1

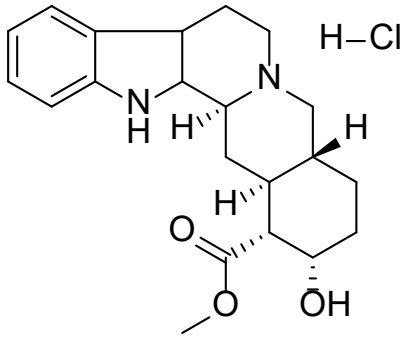
Score	Expect	Identities	Gaps	Stand
893 bits(483)	**0.0**	**600/658(91%)**	**2/658(0%)**	**Plus/Plus**



Phytochemical Fingerprinting by HPTLC (*Pausinystalia johimbe*)

[A] HPTLC





Yohimbine HCl

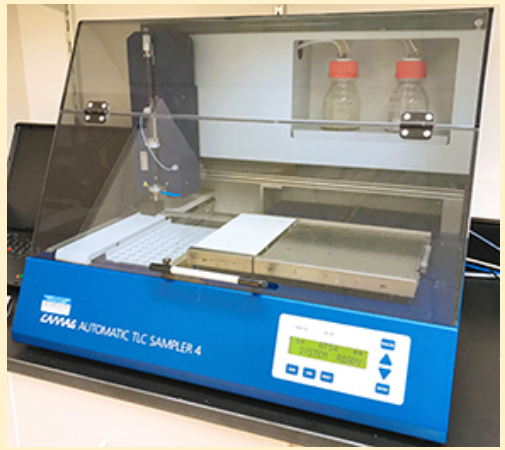
▸ **Sample Preparation**
· Crude extract: 10 mg/ml in the 50% CH_3CN/H_2O
· Reference compounds: 1 ml/ml in the 50% CH_3CN/H_2O

▸ **HPTLC Plate**
· HPLTC Silica gel F_{254} [EMD]

▸ **HPTLC Solvent System**
· EtOAc/acetic acid/formic acid/water (100/11/11/26)

▸ **HPTLC Image Capture**
· UVP MultiDoc-It Digital Imaging system (254 nm, 365nm)

▸ **TLC Visualization reagent**
· 5% Vanillin-sulphuric reagent

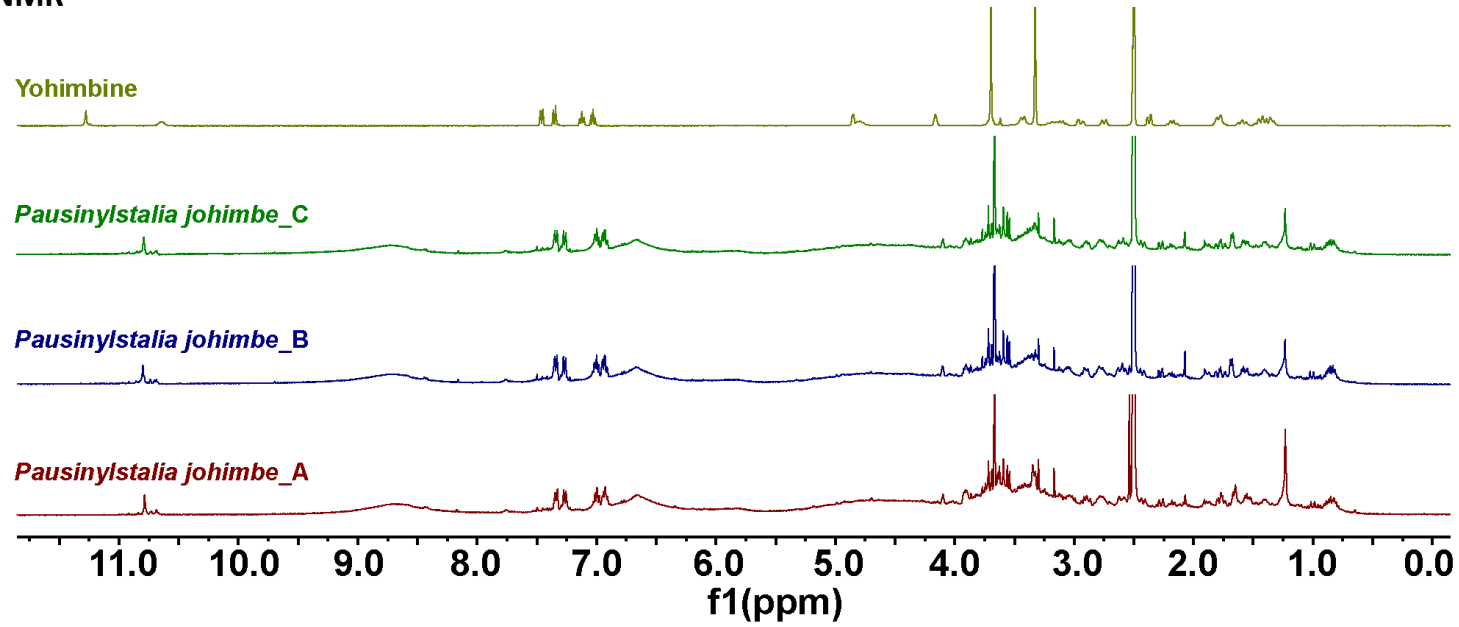


▸ **HPTLC condition**

Instrument: CAMAG Automatic TLC Sampler 4
Band Length (mm): 4.0
Application volume (µl): 5.0
Filling speed (ul/s): 11.0
Predosage volume (nl): 200
Retraction volume (nl): 200
Dosage speed (nl/s): 100
Rinsing vacuum time (s): 6
Filling vacuum times (s): 0
Gas: Air
TLC size (cm): 5*10

Phytochemical Fingerprinting by ^{1}H-NMR (*Pausinystalia johimbe*)

[B] ^{1}H-NMR



► **Sample preparation**
- Crude extracts 123#A: 6.78 mg/200 µl; 123#B: 5.84 mg/200 µl; 123#C: 5.03 mg/200 µl
- Yohimbine: 0.93 mg/200 µl
- Solvent: DMSO-d_6 (99.9%), Cambridge Isotope Laboratories, Inc. (Cas #: 2206-27-1, Lot #: 12G-464)

► **Instrument**
- Jeol ECZ 400 MHz in 3 mm NMR tube under the Ultra COOL probe

► **Parameter (qNMR)**
- Temperature 25℃
- 90° single-pulse (relaxation delay: 60sec, receiver gain: 46, number of scan: 64)

Conclusions:
Identification of a Commercial Plant Powder declared as *Pausinystalia johimbe*

- Microscopic analysis of the commercial plant powder showed reticulated vessels, long thin fibers and medullary ray. Other unknown fragments were also observed. The commercial sample may contain bark powder and has the same color than referenced ***P. johimbe*** bark in monographs.

- The DNA sequences obtained for ITS-2 were dirty, showing overlapping of multiple amplified sequences. The clean portion of the rbcL sequence displayed 90% of homology with a referenced sequence of ***Pausinystalia* species** from GenBank. **There is a lack of referenced DNA sequences of *P. johimbe* in Genbank**. The universal primers used for psbA-trnH and matK could not lead to any PCR amplification for this species.

- HPTLC and ^{1}H-NMR analyses confirmed the presence of yohimbine and/or analogues in all extract replicates.

- All the data gathered herein demonstrated that the commercial powder contains ***Pausinystalia johimbe***, DNA based identification of this type of samples appeared to be challenging due to the presence of PCR inhibitors (e.g. tannins), the low yield of extracted DNA, and the lack of referenced sequences.

Botanical Information (*Senna alexandrina*)

https://www.ncbi.nlm.nih.gov/Taxonomy/

- **Taxonomy ID:** 72402 (for references in articles please use NCBI:txid72402)
- **Scientific name:** *Senna alexandrina* Mill.

Other names:
synonym: *Cassia acutifolia* Delile
synonym: *Cassia angustifolia* Vahl
synonym: *Cassia senna*

Lalithamba from India
https://www.diark.org/diark/species_list/Senna_alexandrina

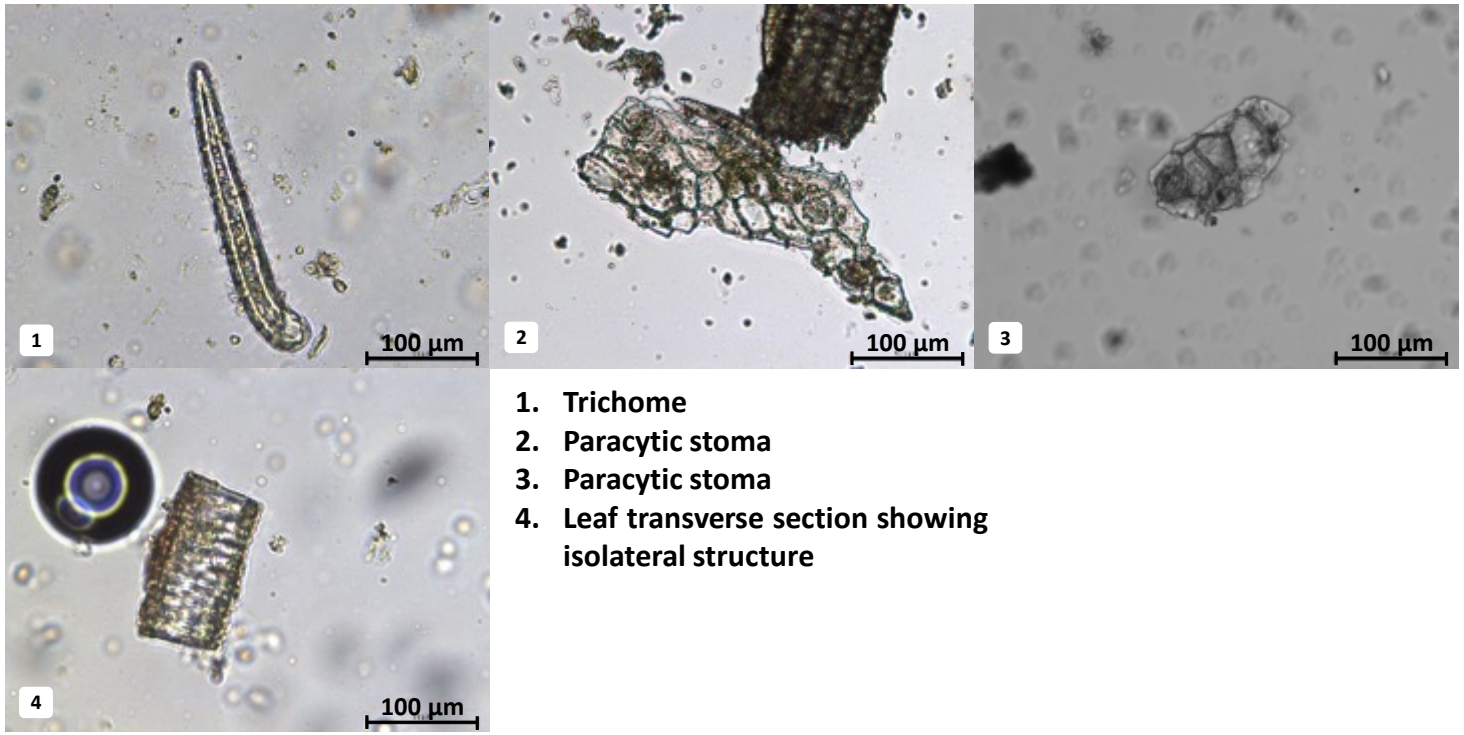
- **Inherited blast name:** eudicots
- **Rank:** species
- **Genetic code:** Translation table 1 (Standard)
- **Mitochondrial genetic code:** Translation table 1 (Standard)
- **Plastid genetic code:** Translation table 11 (Bacterial, Archaeal and Plant Plastid)
- **Lineage (full)**

Cellular organisms; Eukaryota; Viridiplantae; Streptophyta; Streptophytina; Embryophyta; Tracheophyta; Euphyllophyta; Spermatophyta; Magnoliophyta; Mesangiospermae; eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Fabales; Fabaceae; Caesalpinioideae; Cassia clade; Senna

- **English common name for *Senna alexandrina*:** Senna, Indian senna, Casse
- **Part of the plant traditionally used:** Leaves
- **Major Phytochemical markers:** Anthraquinones (Sennoside A and B)[17, 18, 19]

Macroscopic and Microscopic Analyses (*Senna alexandrina*)

- **Microscopic analyses[19]**



1. **Trichome**
2. **Paracytic stoma**
3. **Paracytic stoma**
4. **Leaf transverse section showing isolateral structure**

Senna alexandrina
Commercial powder

DNA-based Botanical Identification (*Senna alexandrina*)

ITS-2 sequence: 399 pb

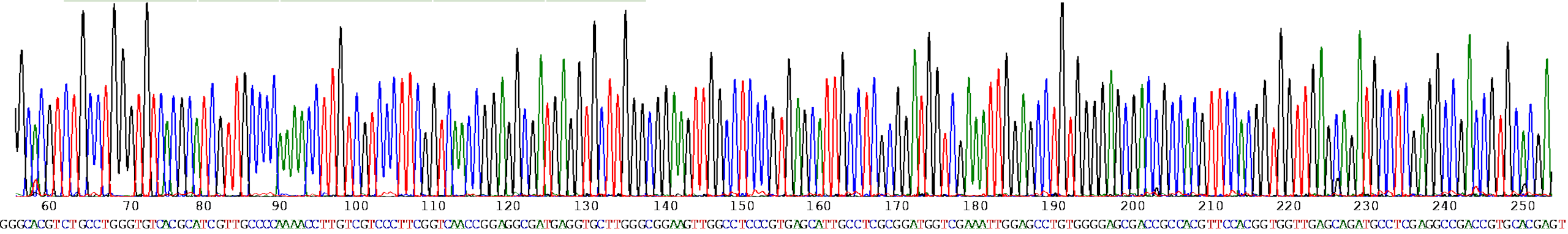
TCTGCCTGGGTGTCACGCATCGTTGCCCCAAAACCTTGTCGTCCCTTCGGTCAACCGGAGGCGATGAGGTGCTTGGGCGGAAGTTGGCCTCCCGTGAGCATTGCCT
CGCGGATGGTCGAAATTGGAGCCTGTGGGGAGCGACCGCCACGTTCCACGGTGGTTGAGCAGATGCCTCGAGGCCGACCGTGCACGAGTTGTCCCCACGTCAAA
GGCTGCGAGACCCTTGCGAGCAAGAAAGTGCTCCCAACGCGACCCCAGGTCAGGCGGGGCCACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAAAGAAAC
TAACAAGGATTCCCCTAGTAACGGCGAGCGAACCGGGAAAAGCCCACCATGAGAATCGGTCGTCCTCGGCGTCCGAATTGTAGTC

Significant alignment in Genbank ***Senna alexandrina*** isolate CIMAP-C039 clone Senna species 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence Sequence ID: KY492293.1,

Score	Expect	Identities	Gaps	Stand
737 bits(399)	**0.0**	**399/399(100%)**	**0/399(0%)**	**Plus/Plus**

And with ***Senna alexandrina*** internal transcribed spacer 2, complete sequence, Sequence ID: JQ301846.1

Score	Expect	Identities	Gaps	Stand
721 bits(390)	**0.0**	**390/390(100%)**	**0/390(0%)**	**Plus/Plus**



DNA-based Botanical Identification (*Senna alexandrina*)

psbA-trnH sequence: 190pb

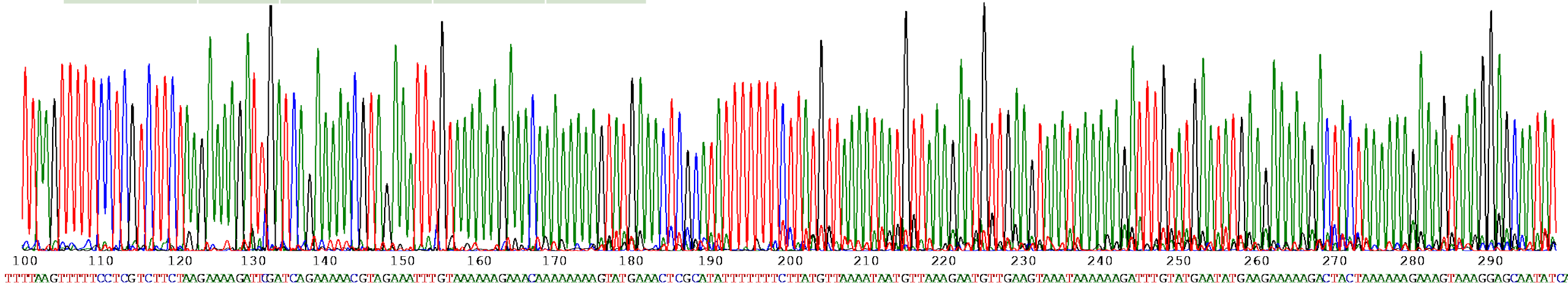
AAATTGTGGTCTTAATATATATGAGTTTTTGAACGTAAAGGAGCAATATCAAGAGGGTTGATATTGCTCCTTTACTTTCTTTTTTAGTAGTCTTTTTCTTCATATTCATACA
AATCTTTTTTATTTACTTCAACATTCTTTAACATTATTTTAACATAAGAAAAAAATATGCGAGTTTCATACTTTTTTT

Representing only a clean portion of the amplified DNA
Significant alignment in Genbank with ***Senna alexandrina*** isolate KMS0206A01 psbA-trnH intergenic spacer region, partial sequence; chloroplast Sequence ID: MF097036.1

Score	Expect	Identities	Gaps	Stand
350 bits(189)	1e-92	189/189(100%)	0/189(0%)	Plus/Plus

But also with ***Chamaecrista nigricans*** psbA-trnH intergenic spacer, partial sequence; chloroplast, Sequence ID: HQ161770.1

Score	Expect	Identities	Gaps	Stand
351 bits(390)	3e-93	190/190(100%)	0/190(0%)	Plus/Plus



DNA-based Botanical Identification (*Senna alexandrina*)

rbcL sequence: 719 pb

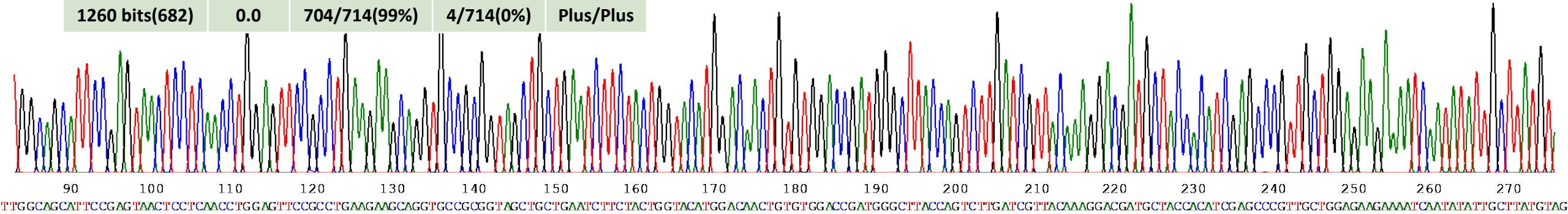
GTGAGGTTTGGGTCAAGCTGGTGTTAAGATTATAAATTGACTTATTATACTCCTGAATATGAAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAACC
TGGAGTTCCGCCTGAAGAAGCAGGTGCCGCGGTAGCTGCTGAATCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGTCTTGATCGTTACAAA
GGACGATGCTACCACATCGAGCCCGTTGCTGGAGAAGAAAATCAATATATTGCTTATGTAGCTTATCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTA
CTTCCATTGTGGGTAATGTATTTGGATTCAAGGCCCTGCGCGCTCTACGTCTGGAGGATTTGCGAATCCCTACTTCTTATATTAAAACTTTCCAAGGTCCGCCTCACGG
CATCCAAGTTGAGAGAGATAAATTGAACAAGTACGGCCGTCCCCTATTGGGATGTACTATTAAACCTAAATTGGGGTTATCCGCTAAGAATTACGGTAGAGCAGTTTA
TGAATGTCTCCGCGGTGGACTTGATTTTACCAAAGATGATGAGAATGTGAATTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTATTTTGTGCCGAAGCAATTTTT
AAAGCACAGGCCGAAACTGGTGAAATCAAAGGGCATTACTTGAATGCTACTGCAGGTAACATGCGAAA

Significant alignment in Genbank with ***Cassia didymobotrya*** **chloroplast rbcL gene Sequence ID: Z70154.1,**

Score	Expect	Identities	Gaps	Stand
1271 bits(688)	**0.0**	**706/714(99%)**	**4/714(0%)**	**Plus/Plus**

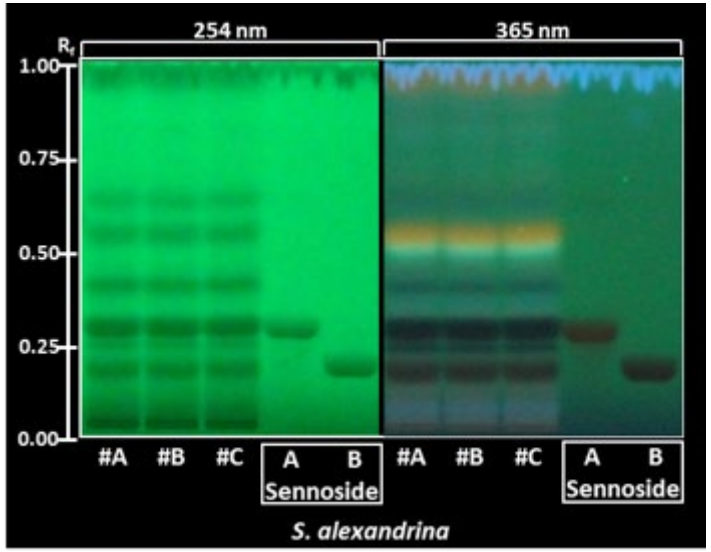
also with ***Cassia senna*** chloroplast rbcL gene, Sequence ID: Z70155.1

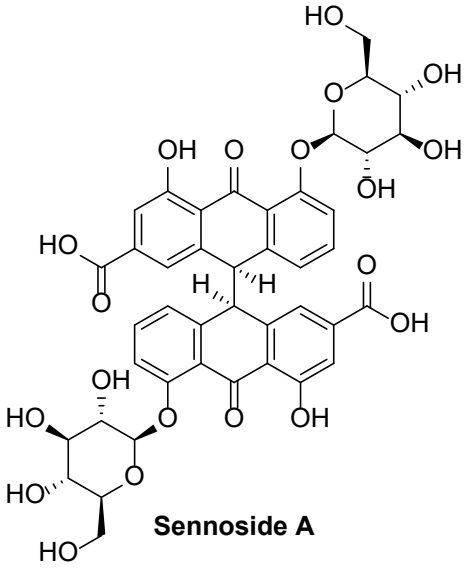
Score	Expect	Identities	Gaps	Stand
1260 bits(682)	**0.0**	**704/714(99%)**	**4/714(0%)**	**Plus/Plus**

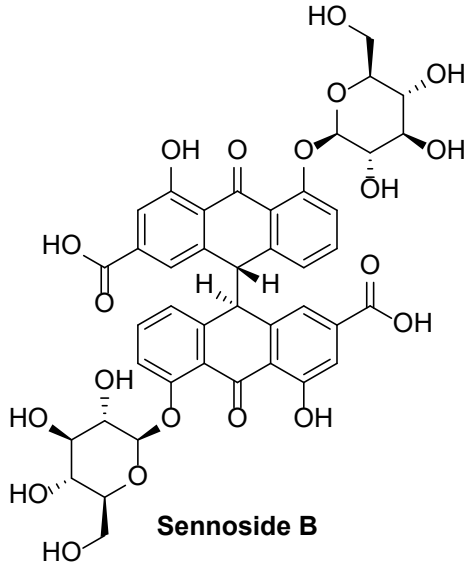


Phytochemical Fingerprinting by HPTLC (*Senna alexandrina*)

[A] HPTLC





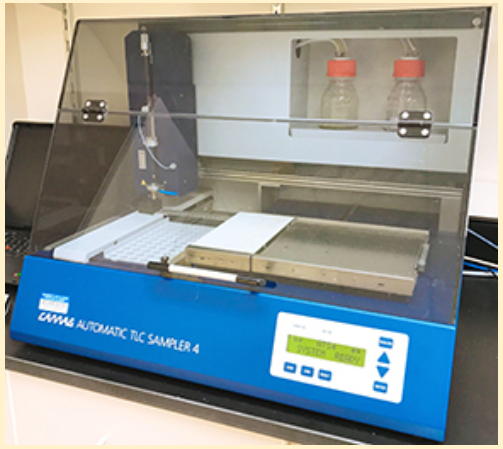


▸ **Sample Preparation**
· Crude extracts: 10 mg/ml in the 50% CH_3CN/H_2O
· Reference compounds: 1 mg/ml in the 50% CH_3CN/H_2O

▸ **HPTLC Plate**
· HPTLC Plates Nano-SIL HD/UV_{254} [MN]

▸ **HPTLC Solvent System**
· EtOAc/acetic acid/formic acid/water (100/11/11/26)

▸ **HPTLC Image Capture**
· UVP MultiDoc-It Digital Imaging system (254 nm, 365 nm)

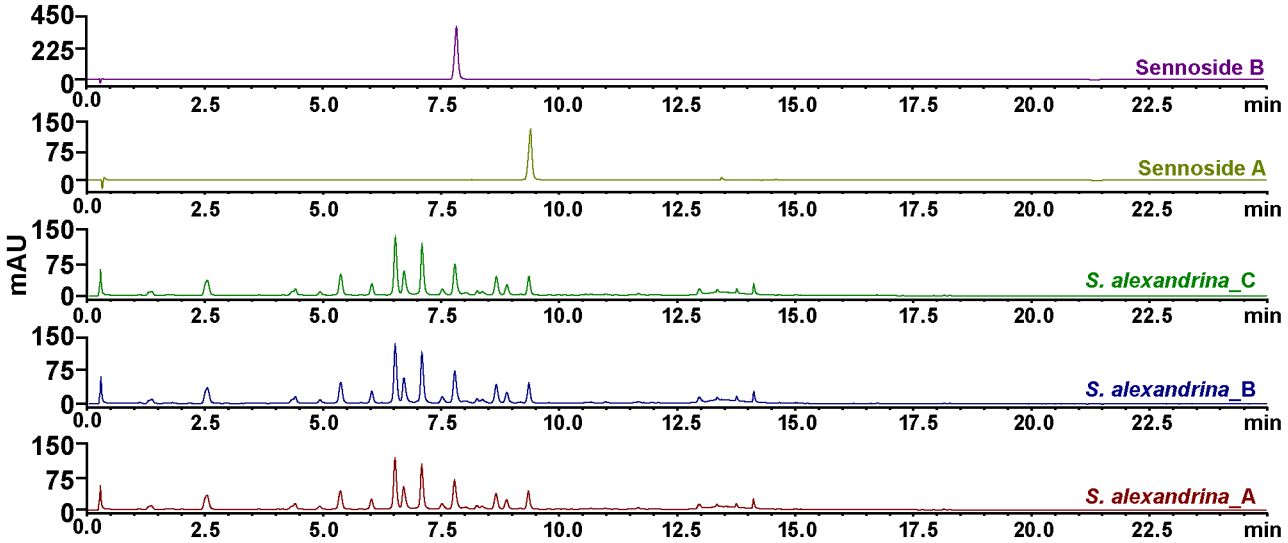


▸ **HPTLC condition**

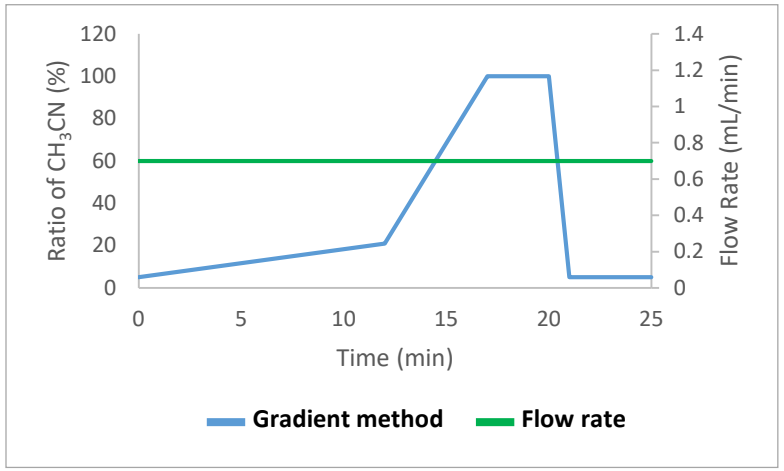
· **Instrument: CAMAG Automatic TLC Sampler 4**
· **Band Length (mm): 4.0**
· **Application volume (µl): 5.0**
· **Filling speed (µl/s): 11.0**
· **Predosage volume (nl): 200**
· **Retraction volume (nl): 200**
· **Dosage speed (nl/s): 100**
· **Rinsing vacuum time (s): 6**
· **Filling vacuum times (s): 0**
· **Gas: Air**
· **TLC size (cm): 5*10**

Phytochemical Fingerprinting by UHPLC (*Senna alexandrina*)

[B] UHPLC (254 nm)



Gradient method

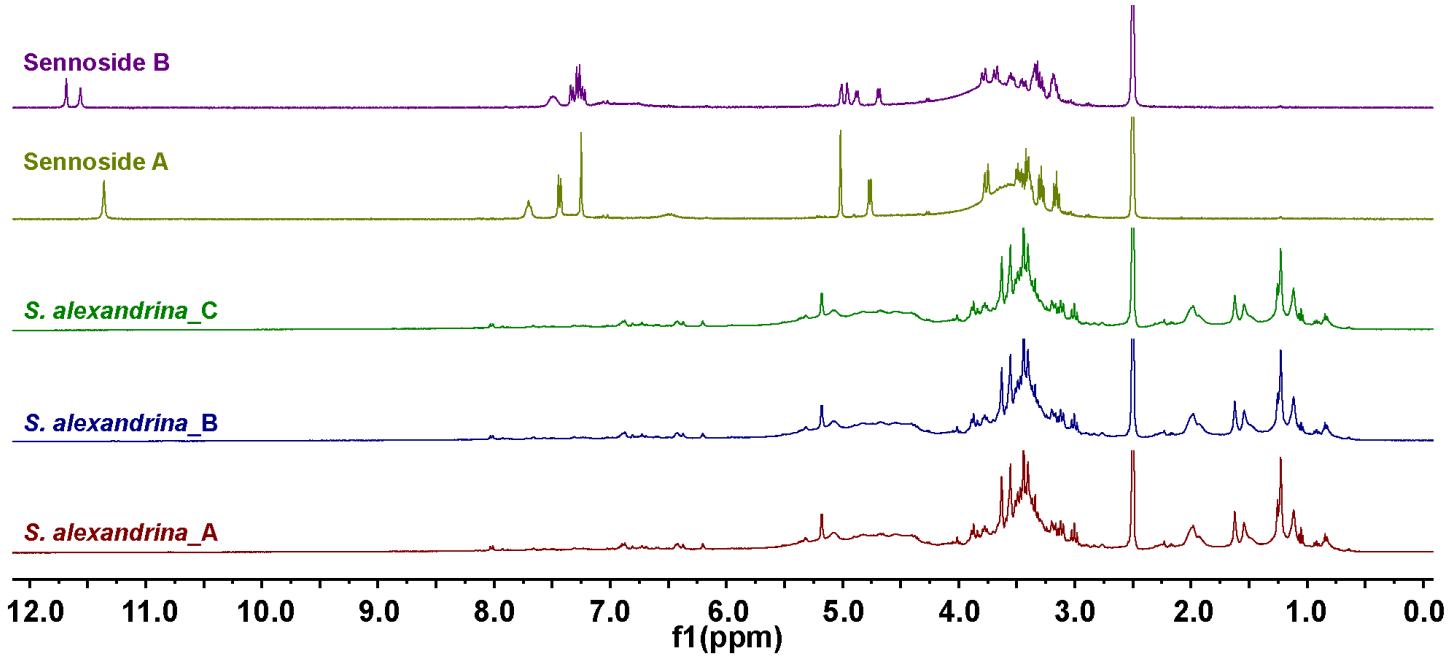


- **Instrument**
  - Shimadzu UFLC (Shimadzu Corp) with DAD and fluorescence detector
  - Column: Kinetex 1.7 µm XB-C18 100Å column (50.0 X 2.1 mm Phenomenex, USA)
  - Software: Shimadzu Labsolution software package
  - Solvent system: CH_3CN/Water gradient from 95% to 5% water with 0.1% formic acid

- **Sample preparation**
  - Crude extracts (10 mg/ml) and reference compound (1 mg/ml) prepared in 50% CH_3CN/water

Phytochemical Fingerprinting by ^{1}H-NMR (*Senna alexandrina*)

[C] ^{1}H-NMR



► **Sample Preparation**
· Crude extracts: 123#A: 7.30 mg/200 µl; 123#B: 7.42 mg/200 µl; 123#C: 7.14 mg/200 µl
· Sennoside A: 0.94 mg/ 200 µl
· Sennoside B: 0.96 mg/ 200 µl
· Solvent: DMSO-d_6 (99.9%), Cambridge Isotope Laboratories, Inc. (Cas #: 2206-27-1, Lot #: 12G-464)
► **Instrument**
· Jeol ECZ 400 MHz in 3 mm NMR tube under the Ultra COOL probe.
► **Parameter (qNMR)**
· Temperature 25℃
· 90° single-pulse (relaxation delay: 60sec, receiver gain: 46, number of scan: 64)

Conclusions:
Identification of a Commercial Plant Powder Declared as *Senna alexandrina*

- The microscopic analysis of the commercial powder revealed the presence of trichomes, paracytic stoma, leaf transverse sections with stomata, thereby suggesting that the powder contains grinded leaves.

- All the amplified DNA sequences (MatK, psbA-trnH and rbcL) confirm that this commercial sample contains ***Cassia senna* L.** (***Senna alexandrina* Mill.**).

- The HPTLC and UHPLC-UV analyses confirmed the presence of both sennosides A and B in all three extract replicates. The chemical complexity of the produced extracts did not favor the detection of such specific compounds using ^{1}H-NMR analysis.

- All the gathered results, herein presented, confirmed the botanical identity of the commercial powder as leaves of ***Senna alexandrina* Mill.** or ***Cassia senna* L.**

Botanical Information (*Trigonella foenum-graecum*)

https://www.ncbi.nlm.nih.gov/Taxonomy/

- **Taxonomy ID:** 78534 (for references in articles please use NCBI:txid78534)
- **Scientific name:** *Trigonella foenum-graecum*
- **Inherited blast name:** eudicots
- **Rank:** species
- **Genetic code:** Translation table 1 (Standard)
- **Mitochondrial genetic code:** Translation table 1 (Standard)
- **Plastid genetic code:** Translation table 11 (Bacterial, Archaeal and Plant Plastid)
- **Lineage (full)**

Cellular organisms; Eukaryota; Viridiplantae; Streptophyta; Streptophytina; Embryophyta; Tracheophyta; Euphyllophyta; Spermatophyta; Magnoliophyta; Mesangiospermae; eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Fabales; Fabaceae; Papilionoideae; 50 kb inversion clade; NPAAA clade; Hologalegina; IRL clade; Trifolieae; Trigonella

***Trigonella foenum-graecum*, Jan 2017**
https://en.wikipedia.org/wiki/index.html?curid=289834#/media/File:2017_0102_fenugreek_seeds.jpg

► **English common name for *Trigonella foenum-graecum*:** Fenugreek

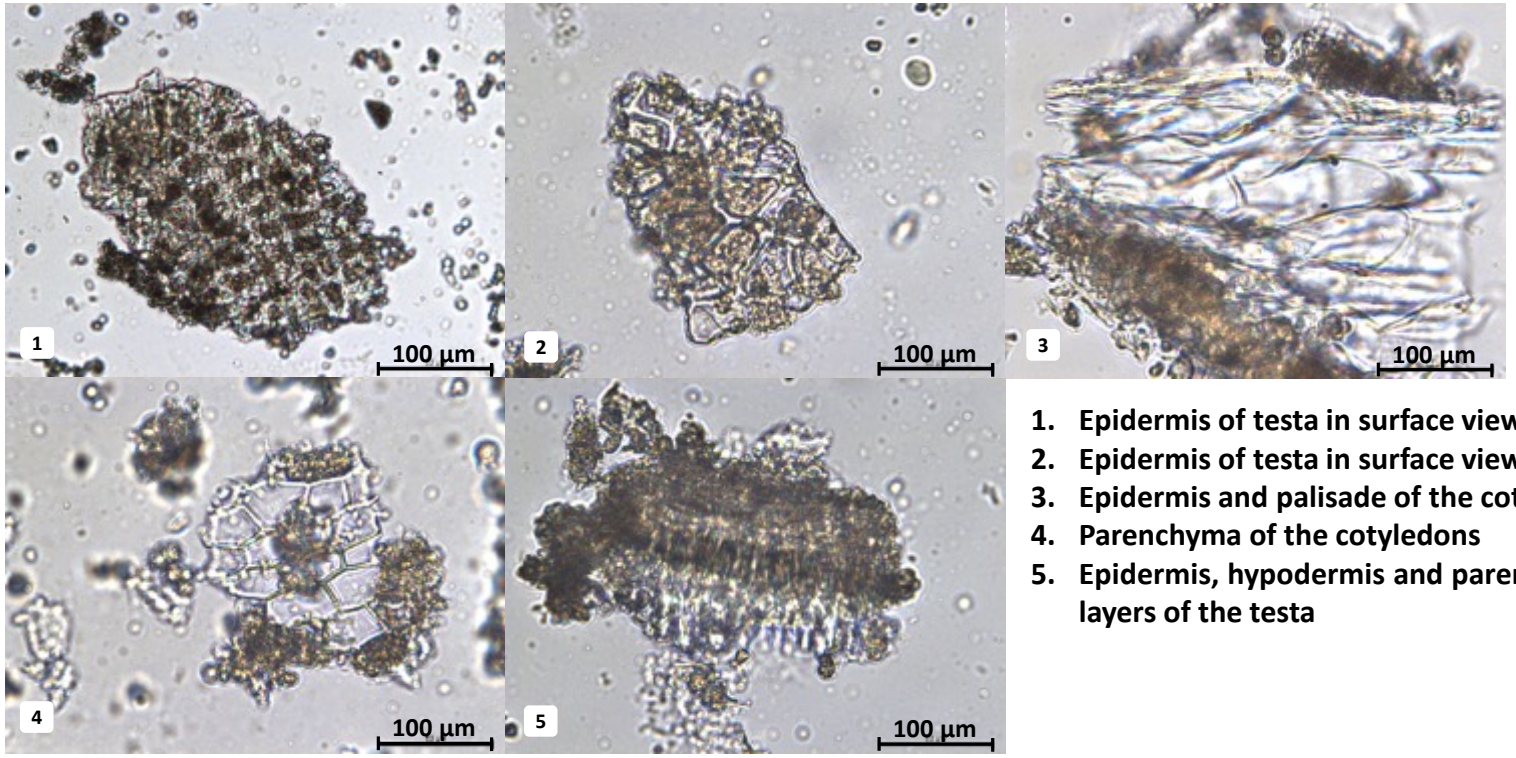
► **Part of the plant traditionally used:** Seeds

► **Major Phytochemicals**

Saponins (diosgenin, yamogenin)[7], flavonoids (apigenin, luteolin)[8, 9], Pyridine-type alkaloids (trigonelline, gentianin)[10], fatty acids[9, 11]

Macroscopic and Microscopic Analyses (*Trigonella foenum-graecum*)

- **Microscopic analyses[19]**



1. **Epidermis of testa in surface view**
2. **Epidermis of testa in surface view**
3. **Epidermis and palisade of the cotyledons**
4. **Parenchyma of the cotyledons**
5. **Epidermis, hypodermis and parenchymatous layers of the testa**

***Trigonella foenum-graecum* Commercial powder**

DNA-based Botanical Identification (*Trigonella foenum-graecum*)

ITS-2 sequence: 398 pb

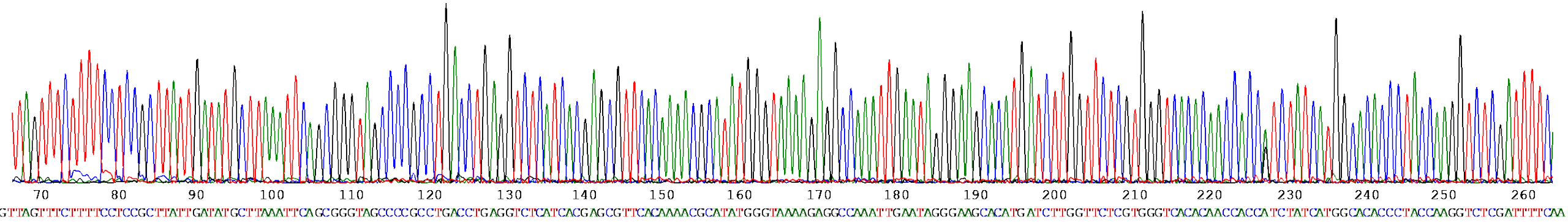
CACGTCTGCCTGGGTGTCACATATCGAAGCCTCATGCCAATTTCCTTTTTTAGTAGGTATTGTGCATGCTGGTGAATGTTGGCCTCCCGTGAGCTCTATTGTCTCATGGT
TGGTTGAAAATCGAGACCTTGGTAGGGTGTGCCATGATAGATGGTGGTTGTGTGACCCACGAGAACCAAGATCATGTGCTTCCCTATTCAATTTGGCCTCTTTTACCC
ATATGCGTTTTGTGAACGCTCGTGATGAGACCTCAGGTCAGGCGGGGCTACCCGCTGAATTTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCT
TAGTAACGGCGAGCGAACCGGGATAAGCCCACCATGAAAATCGGTCGCCTTCGGCGTTCGAATTGTAGTCTGG

Significant alignment in Genbank with *Trigonella foenum-graecum* voucher 65230283 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence, Sequence ID: KF454107.1,

Score	Expect	Identities	Gaps	Stand
713 bits(386)	0.0	386/386(100%)	0/386(0%)	Plus/Plus

as well as voucher 200505082 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence, Sequence ID: MH688883.1

Score	Expect	Identities	Gaps	Stand
710 bits(384)	**0.0**	**385/386(99%)**	**0/386(0%)**	**Plus/Plus**



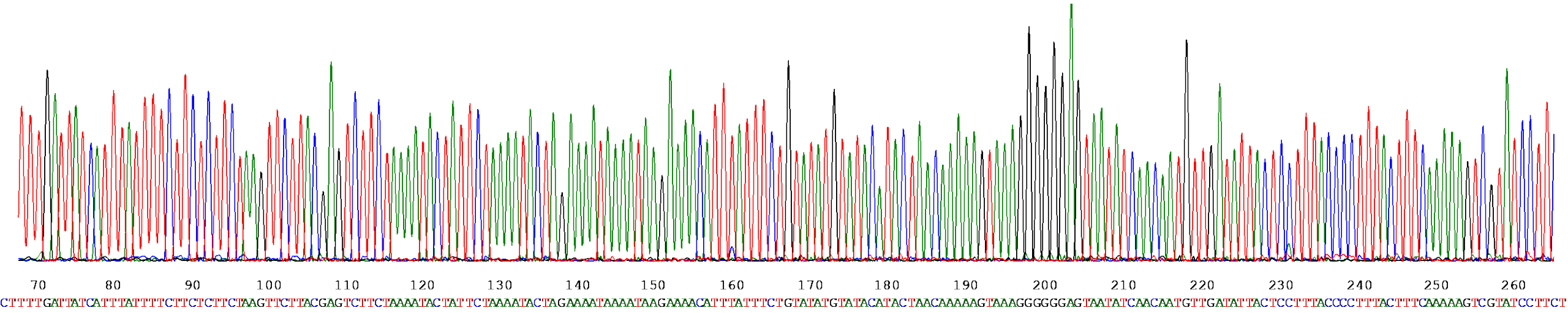
DNA-based Botanical Identification (*Trigonella foenum-graecum*)

psbA-trnH sequence: 358 pb

TAGGGGTACTTCAACGACATGGCTCTCCGCCCTATACTATGTCTAAATTACAGAACTTTTTATACCTTTTGATTATCATTTATTTTCTTCTCTTCTAAGTTCTTACGAGTCTT
CTAAAATACTATTCTAAAATACTAGAAAATAAAATAAGAAAACATTTATTTCTGTATATGTATACATACTAACAAAAAGTAAAGGGGGGAGTAATATCAACAATGTTGATA
TTACTCCTTTACCCCTTTACTTTCAAAAAGTCGTATCCTTCTTTAAAACAAAAATATTATCCATTTATAGATGGAGCCTCGACCGCAGCTAGGTCTAGAGGGAAATTATG
AGCATTACGTTCATGCATAACAAA

Significant alignment *Trigonella foenum-graecum* PsbA (psbA) gene, partial cds; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast, Sequence ID: MG947135.1

Score	Expect	Identities	Gaps	Stand
604 bits(327)	4e-169	327/327(100%)	0/327(0%)	Plus/Minus



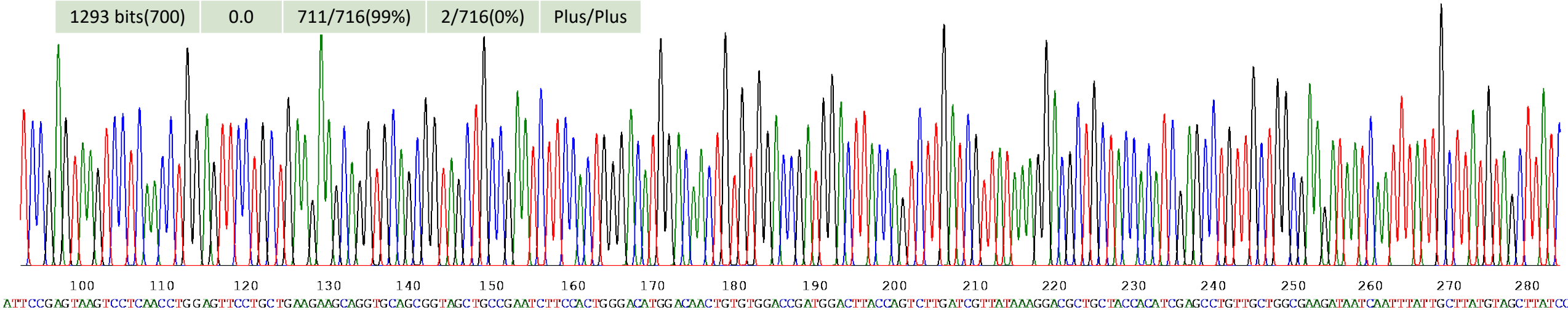
DNA-based Botanical Identification (*Trigonella foenum-graecum*)

rbcL sequence: 719 pb

CGAGAGGTGGGGTTCAAGCTGGTGTTAAGATTATAAATTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAAGTCCTCAA
CCTGGAGTTCCTGCTGAAGAAGCAGGTGCAGCGGTAGCTGCCGAATCTTCCACTGGGACATGGACAACTGTGTGGACCGATGGACTTACCAGTCTTGATCGTTATA
AAGGACGCTGCTACCACATCGAGCCTGTTGCTGGCGAAGATAATCAATTTATTGCTTATGTAGCTTATCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTT
ACCTCCATTGTAGGTAATGTATTTGGGTTCAAGGCCTTGCGCGCTCTACGTCTGGAAGATTTGCGAATCCCAGTTGCTTATGTTAAAACTTTCCAAGGCCCTCCTCACG
GAATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGACGTCCCTTATTGGGATGTACTATTAAACCTAAATTGGGGTTATCCGCTAAGAATTACGGTAGAGCAGTTT
ATGAATGTCTACGCGGTGGACTTGATTTTACCAAAGATGATGAAAATGTGAACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTATTTTGTGCCGAAGCTATTTA
TAAATCACAGGCCGAAACAGGTGAAATCAAAGGACATTATTTGAATGCTACTGCAGGTACATGCGAAA

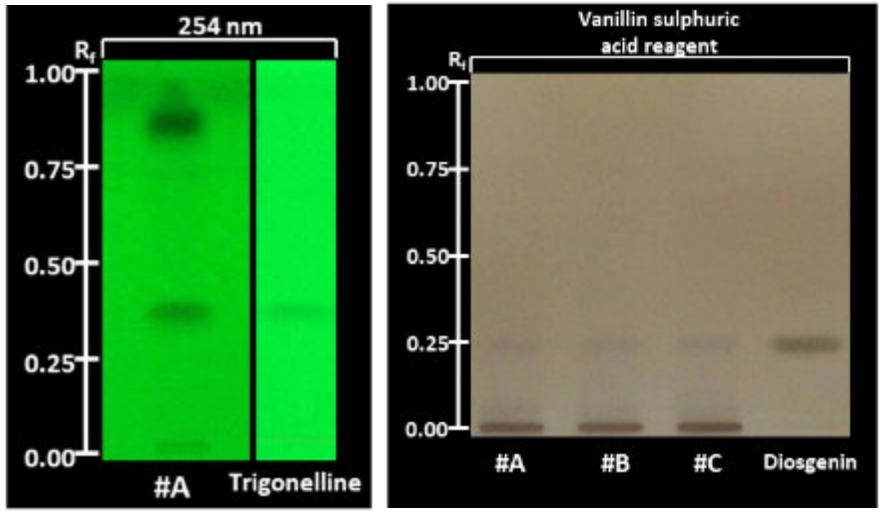
Significant alignment in Genbank with *Trigonella foenum-graecum* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene, partial cds; chloroplast, Sequence ID: MG946901.1

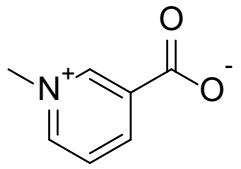
Score	Expect	Identities	Gaps	Stand
1293 bits(700)	0.0	711/716(99%)	2/716(0%)	Plus/Plus



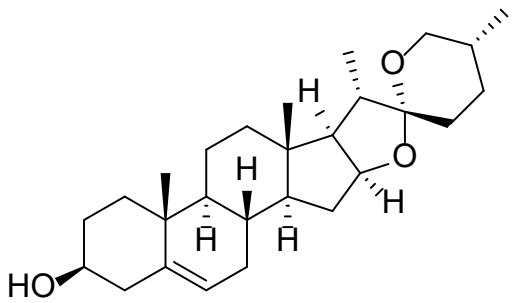
Phytochemical Fingerprinting by HPTLC (*Trigonella foenum-graecum*)

[A] HPTLC





Trigonelline



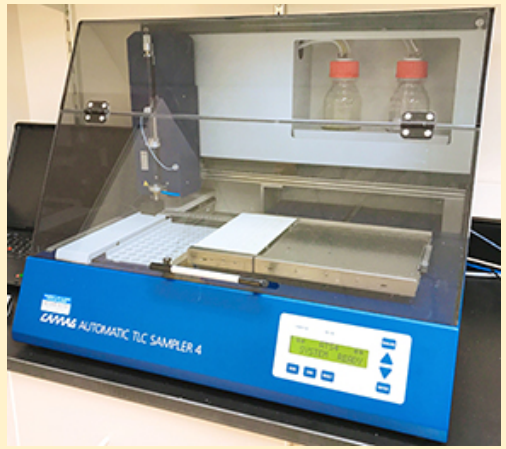
Diosgenin

▸ **Sample Preparation**
· Crude extract: 40 mg/ml in the 100% $CHCl_3$
· Reference compounds: 1 mg/ml in the 100% $CHCl_3$

▸ **HPTLC Plate**
· HPTLC silica gel F_{254} [EMD]

▸ **HPTLC Solvent System**
· Isopropyl alcohol/CH_3OH/water (4/1/4, Trigonelline)[20]
· Hexanes/EtOAc (70/30, Diosgenin)

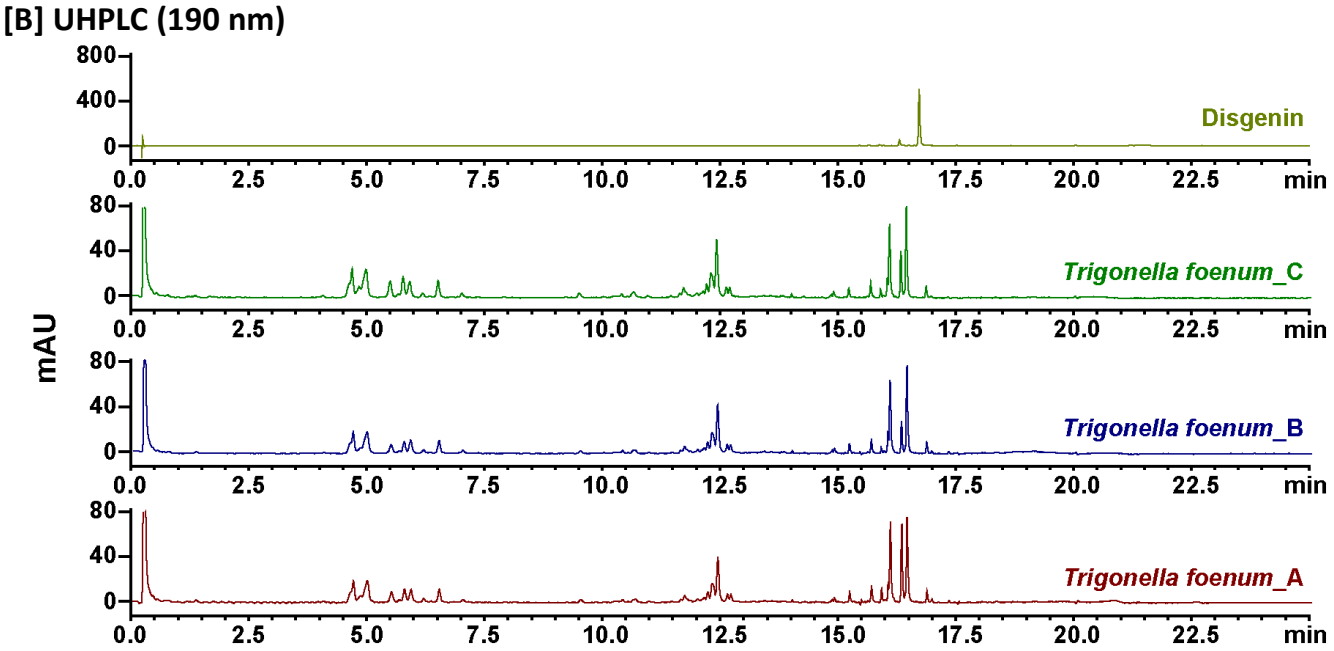
▸ **HPTLC Image Capture**
· UVP MultiDoc-It Digital Imaging system (254 nm, 365nm)



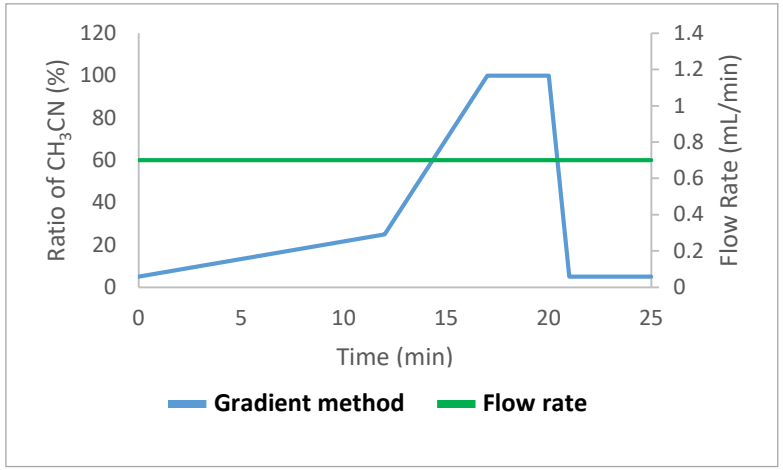
▸ **HPTLC condition**

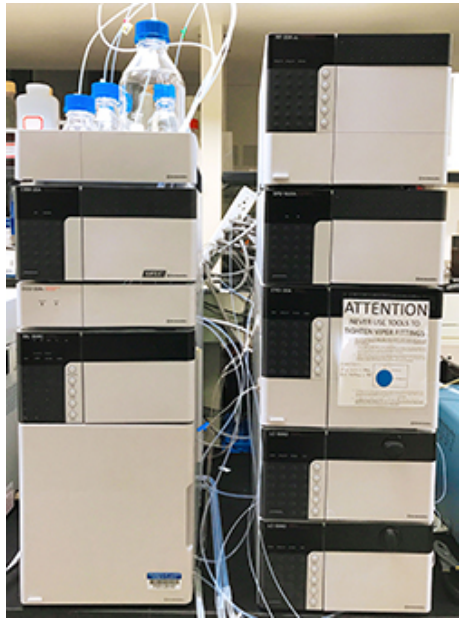
· **Instrument: CAMAG Automatic TLC Sampler 4**
· **Band Length (mm): 4.0**
· **Application volume (µl): 5.0**
· **Filling speed (µl/s): 11.0**
· **Predosage volume (nl): 200**
· **Retraction volume (nl): 200**
· **Dosage speed (nl/s): 100**
· **Rinsing vacuum time (s): 6**
· **Filling vacuum times (s): 0**
· **Gas: Air**
· **TLC size (cm): 5*10**

Phytochemical Fingerprinting by UHPLC (*Trigonella foenum-graecum*)



Gradient method

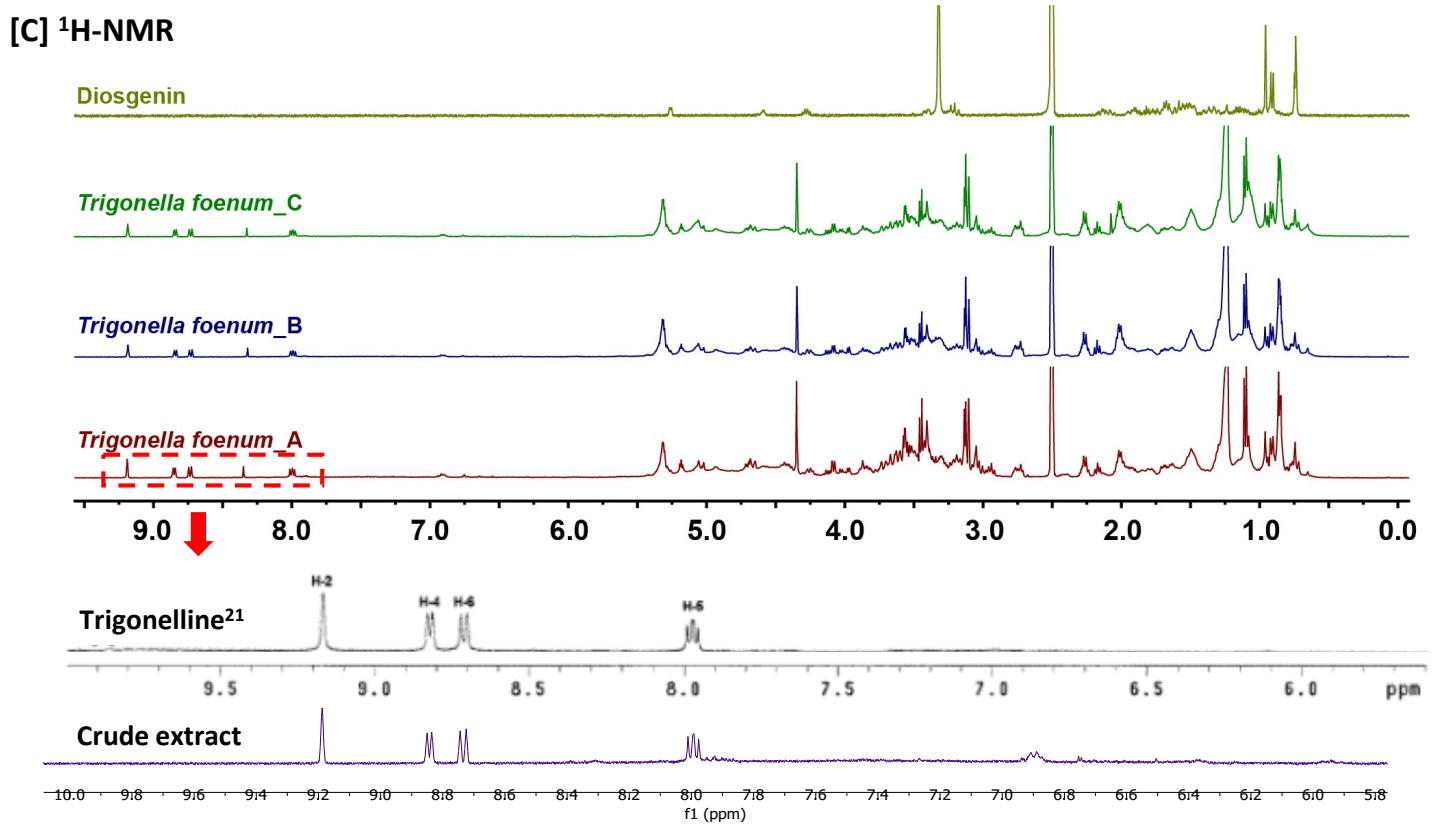




► **Instrument**
- Shimadzu UFLC (Shimadzu Corp) with DAD and fluorescence detector
- Column: Kinetex 1.7 µm XB-C18 100Å column (50.0 X 2.1 mm Phenomenex, USA)
- Software: Shimadzu Labsolution software package
- Solvent system: CH_3CN/Water gradient from 95% to 5% water with 0.1% formic acid

► **Sample preparation**
- Crude extracts (10 mg/ml) and reference compound (1 mg/ml) prepared in 50% CH_3CN/water

Phytochemical Fingerprint by ^{1}H-NMR (*Trigonella foenum-graecum*)



► **Sample preparation**
- · Crude extracts: 123#A: mg/200 µl; 123#B: mg/200 µl; 123#C: mg/200 µl
- · Diosgenin: 2.32 mg/200 µl
- · Solvent: DMSO-d_6 (99.9%), Cambridge Isotope Laboratories, Inc. (Cas #: 2206-27-1, Lot #: 12G-464)

► **Trigonelline[21]: 3.0 mg in DMSO-d_6**
- · Varian UNITY plus 400 MHz spectrometer.
- · Number of scan: 100 scans; spectra width: 0.187 Hz/point; spectra width 14400 Hz; pulse width 4.0 µs, relaxation delay 1s acquiring time 2.67 µs; temperature 25℃

► **Instrument**
- · Jeol ECZ 400 MHz in 3 mm NMR tube under the Ultra COOL probe.

► **Parameter (qNMR)**
- · Temperature 25℃, 90° single-pulse (relaxation delay: 60sec, receiver gain: 46, number of scan: 64)

Conclusions:
Identification of a Commercial Plant Powder: *Trigonella foenum-graecum*

- The microscopic analyses of the commercial powder revealed the presence of epidermis and parenchyma. The coloration and smell of the commercial powder also clearly suggested that the commercial sample contains Fenugreek seeds.

- All the obtained DNA barcode sequences matched with referenced *T. foenum graecum* DNA sequences in GenBank, thus, confirming the identity of the plant species as being *T. foenum-graecum.* The universal MatK primers used in this study could not amplify any sequence for this species.

- HPTLC and ^{1}H NMR analyses suggested the presence of trigonelline in the prepared crude extracts. However, the presence of diosgenin could not be confirmed by any analytical methods used here. ^{1}H NMR fingerprints revealed the presence of diverse aliphatic chains (possibly fatty acids) together with saponin/steroid derivatives.

- According to our macroscopic/microscopic and DNA barcoding analyses, the commercial sample contains *T. feonum-graecum* seeds. However, our phytochemical fingerprinting methods need to be optimized to better target the detection of amino acids, fatty acids and saponin constituents.

Identification of *T. pratense* plant material

DNA barcodes, and Phytochemical Profiling

Tropicos.org. Missouri Botanical Garden. 09 Jan 2019 <http://www.tropicos.org/Image/82799>

Seon Beom Kim, Shengnan Jin,
Charlotte Simmler, Guido F. Pauli

Tropicos.org. Missouri Botanical Garden. 09 Jan 2019 <http://www.tropicos.org/Image/82796>

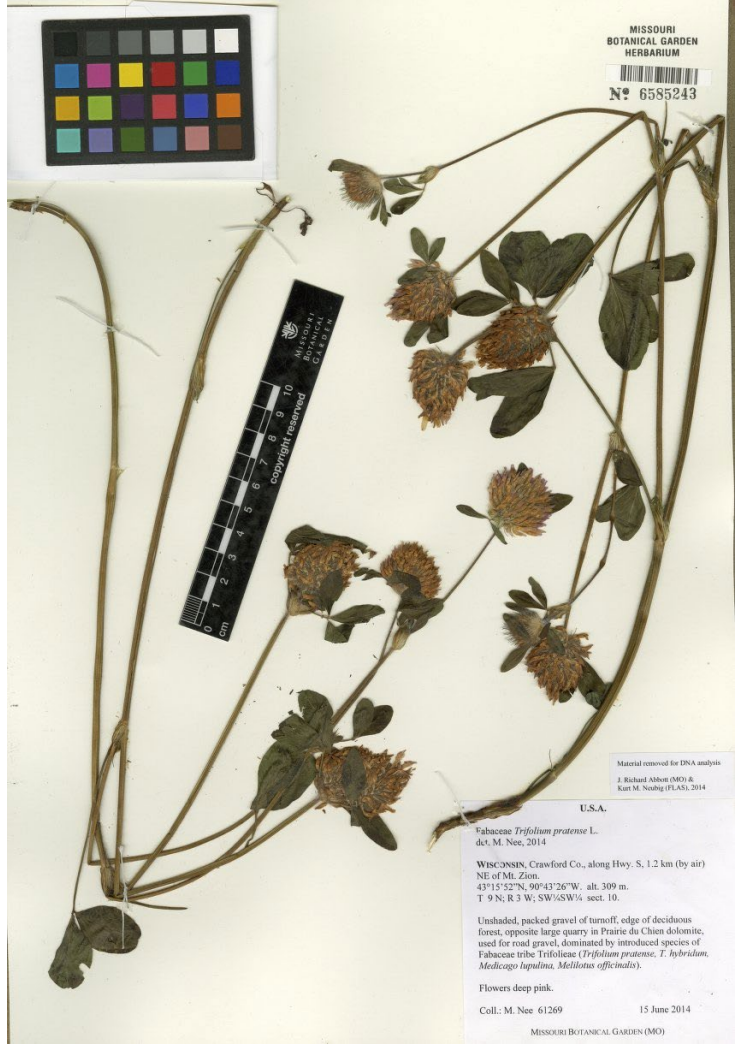
Botanical Information (*Trifolium pratense* L.)

Taxonomy ID: 57577 (for references in articles please use NCBI:txid57577)

- Scientific name: ***Trifolium pratense*** L.
- Inherited blast name: **eudicots**
- Rank: **species**
- Genetic code: Translation table 1 (Standard)
- Mitochondrial genetic code: Translation table 1 (Standard)
- Plastid genetic code: Translation table 11 (Bacterial, Archaeal and Plant Plastid)

Lineage*(full)*

cellular organisms; Eukaryota; Viridiplantae; Streptophyta; Streptophytina; Embryophyta; Tracheophyta; Euphyllophyta; Spermatophyta; Magnoliophyta; Mesangiospermae; eudicotyledons; Gunneridae; Pentapetalae; rosids; fabids; Fabales; Fabaceae; Papilionoideae; 50 kb inversion clade; NPAAA clade; Hologalegina; IRL clade; Trifolieae; Trifolium



Tropicos.org. Missouri Botanical Garden. 09 Jan 2019 <http://www.tropicos.org/Image/100374415>

- **English common name for *T. pratense* :** red clover, purple clover, peavine clover
- **Part of the plant traditionally used:** aerial parts
- **Major Phytochemicals :** isoflavonoids (e.g., genistein, biochanin A)

https://www.ncbi.nlm.nih.gov/Taxonomy/

DNA-based Botanical Identification (*Trifolium pratense*)

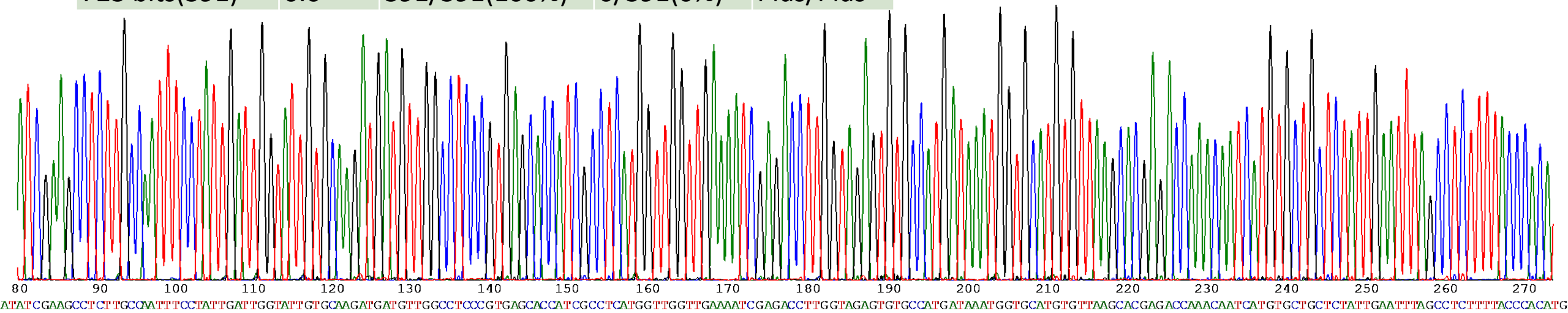
Trifolium pratense commercial powder

ITS-2: 391pb (forward sequencing)

AAGGGCACGTCTGCCTGGGCGTCACATATCGAAGCCTCTTGCCAATTTCCTATTGATTGGTATTGTGCAAGATGATGT
TGGCCTCCCGTGAGCACCATCGCCTCATGGTTGGTTGAAAATCGAGACCTTGGTAGAGTGTGCCATGATAAATGGTG
CATGTGTTAAGCACGAGACCAAACAATCATGTGCTGCTCTATTGAATTTAGCCTCTTTTACCCACATGCGTGTCTAAA
CGCTCGTGATGAGACCTCAGGTCAGGCGGGGCTACCCGCTGAATTTAAGCATATCAATAAGCGGAGGAAAAGAAA
CTAACAAGGATTCCCTTAGTAACGGCGAGCGAACCGGGATAAGCCCACCATGAGAATCGGTCGCCCTCGGCGTTCG
AATTGTAG

Trifolium pratense small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence Sequence ID: KY860927.1 Length: 3670 Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
723 bits(391)	0.0	391/391(100%)	0/391(0%)	Plus/Plus



DNA-based Botanical Identification (*Trifolium pratense*)

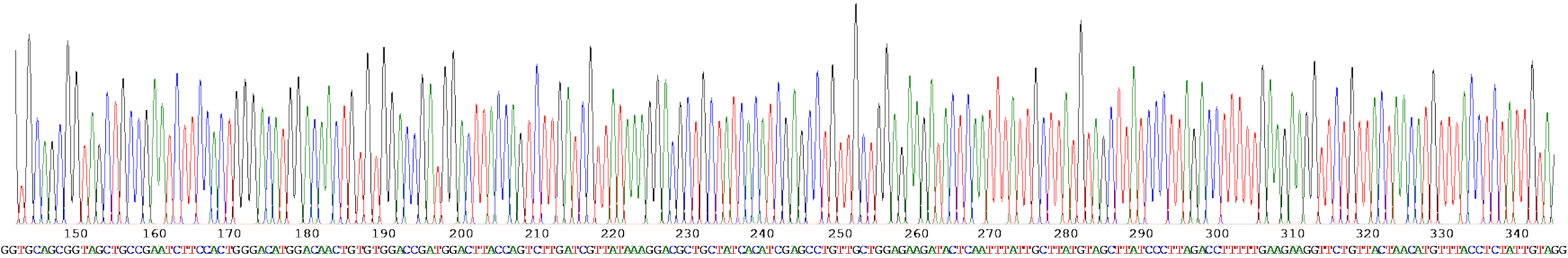
Trifolium pratense commercial powder

rbcL sequence: 680 pb (forward sequencing)

GATTATAGGTTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACTC
CTCAACCCGGAGTTCCGCCCGAAGAAGCAGGTGCAGCGGTAGCTGCCGAATCTTCCACTGGGACATGGACAA
CTGTGTGGACCGATGGACTTACCAGTCTTGATCGTTATAAAGGACGCTGCTATCACATCGAGCCTGTTGCTGGA
GAAGATACTCAATTTATTGCTTATGTAGCTTATCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTA
CCTCTATTGTAGGTAATGTATTTGGGTTCAAGGCCTTGCGTGCTCTACGTCTGGAAGATTTGCGAATCCCCGTTG
CTTATGTTAAAACTTTCCAAGGTCCTCCTCACGGAATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGACG
TCCCCTATTGGGATGTACTATTAAACCTAAATTGGGTTTATCCGCTAAGAATTACGGTAGAGCAGTTTATGAATG
TCTACGCGGTGGACTTGATTTTACAAAAGATGATGAAAATGTGAACTCCCAACCATTTATGCGTTGGAGAGAC
CGTTTCTTATTTTGTGCCGAAGCTATTTATAAATCACAGGCCGAAACAGGTGAAATCAAAGGACATTATTTGAA
TGCTACTGCAGGA

Trifolium pratense ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast Sequence ID: HM850419.1 Length: 1363 Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1256 bits(680)	0.0	680/680(100%)	0/680(0%)	Plus/Plus



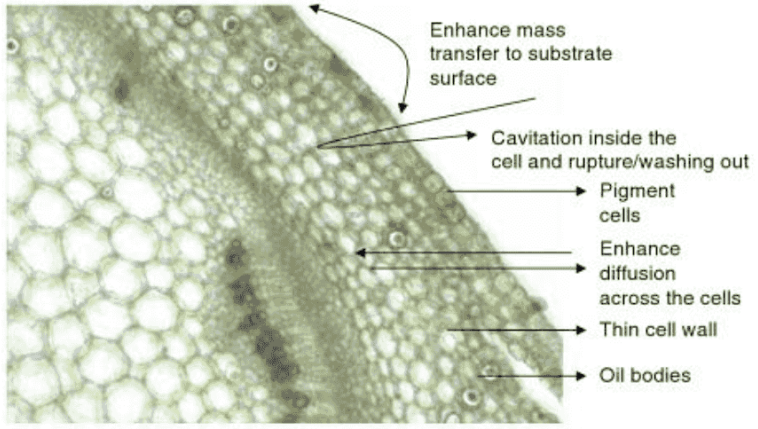
Extraction Method Leading to the Chemical Profiles as Presented in The Following Slides

Two types of extracts were prepared:

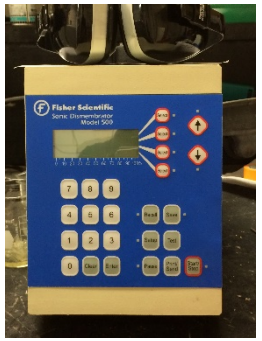
- Solvent A: Dichloromethane/Methanol 1/1, v/v
- Solvent B: Ethanol/ Water 8/2 v/v
  - Plant/ solvent ratio: 50 g/ 700 mL = 1/14 (g/mL)
  - Rinsing after extraction: 300 mL

- Extraction methods:
  - **Sonication** 30 min + maceration overnight

Ultrasonic extraction of cellular matter

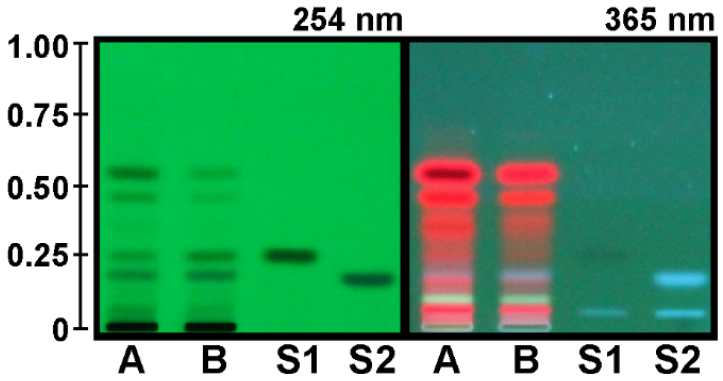


Ultrasonic extraction from cells: the microscopic transverse section (TS) of apical stem of mint (Mentha piperita) shows the mechanism of actions during ultrasonic extraction from cells (magnification 2000x) [resource: Vilkhu et al. 2011]

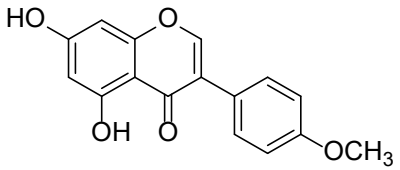


<u>Method used</u>:
Duration 30 min
Amplitude: 80%
Pulse on: 20 sec
Pulse off: 5 sec
No Heat

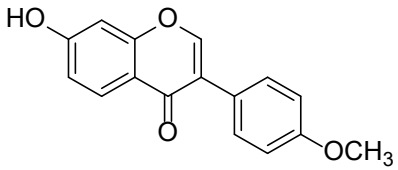
Phytochemical Fingerprinting by HPTLC (*Trifolium pratense*)



A: *T. pratense*_CH_2Cl_2/CH_3OH (50/50, v/v)
B: *T. pratense*_$EtOH/H_2O$ (80/20. v/v)
S1: Biochanin A
S2: Formononetin



Biochanin A



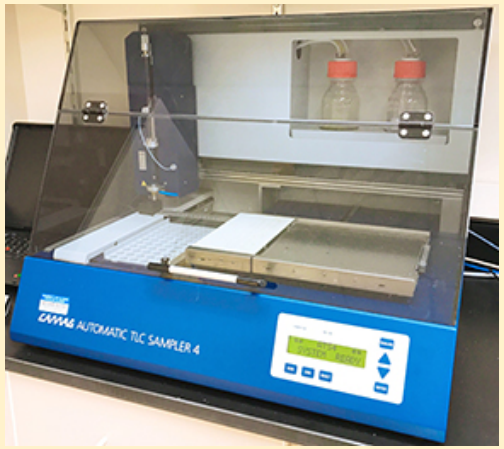
Formononetin

► **Sample Preparation**
· Reference compounds: 1 mg/ml in the 100% $CHCl_3$

► **HPTLC Plate**
· HPTLC silica gel F_{254} [EMD]

► **HPTLC Solvent System**
· Hexanes/EtOAc/acetone/MeoH (6/1/1/0.4)

► **HPTLC Image Capture**
· UVP MultiDoc-It Digital Imaging system (254 nm, 365nm)

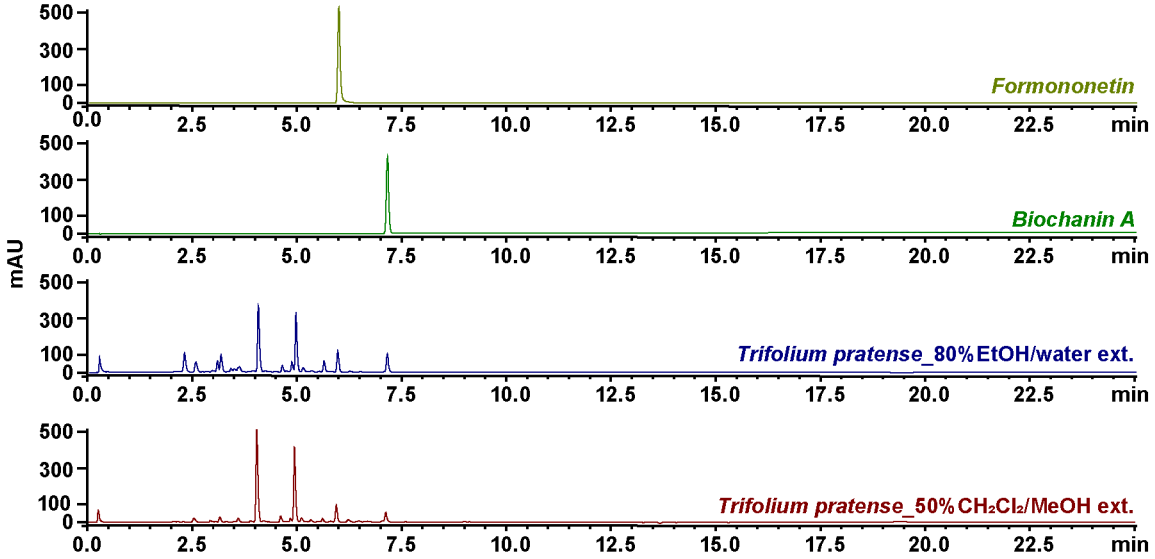


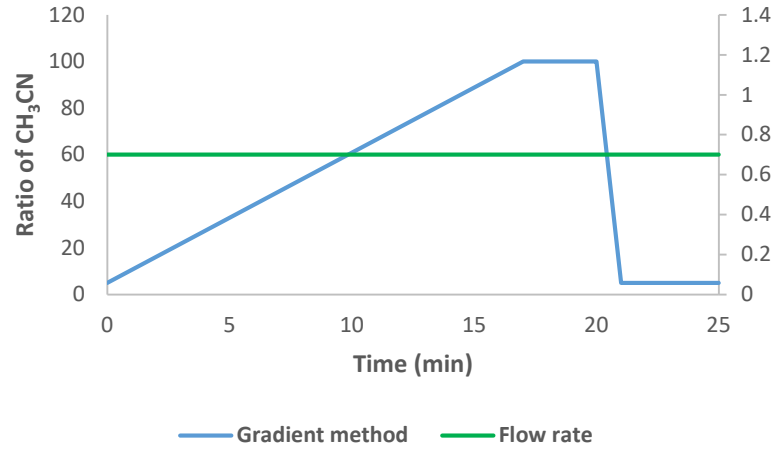
► **HPTLC condition**

· **Instrument: CAMAG Automatic TLC Sampler 4**
· **Band Length (mm): 4.0**
· **Application volume (µl): 5.0**
· **Filling speed (µl/s): 11.0**
· **Predosage volume (nl): 200**
· **Retraction volume (nl): 200**
· **Dosage speed (nl/s): 100**
· **Rinsing vacuum time (s): 6**
· **Filling vacuum times (s): 0**
· **Gas: Air**
· **TLC size (cm): 5*10**

Phytochemical Fingerprinting by UHPLC (*Trifolium pratense*)

[B] UHPLC (254 nm)



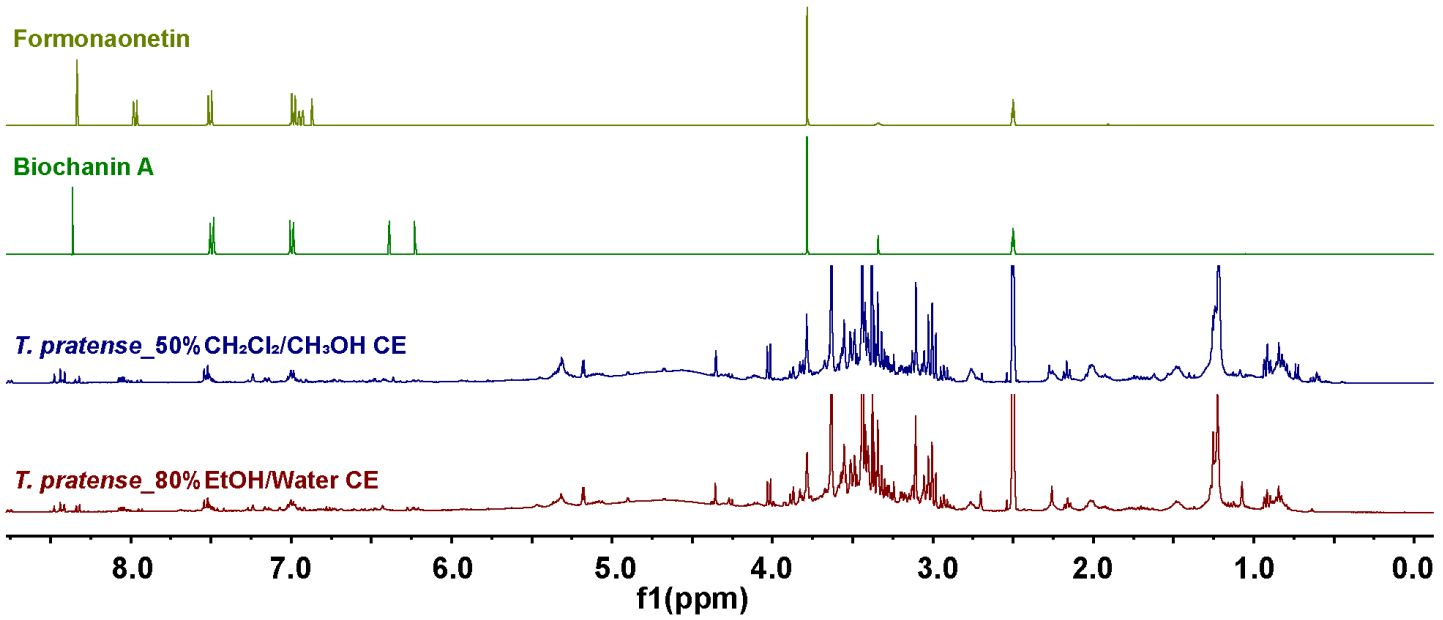


- **Instrument**
  - Shimadzu UFLC (Shimadzu Corp) with DAD and fluorescence detector
  - Column: Kinetex 1.7 µm XB-C18 100Å column (50.0 X 2.1 mm Phenomenex, USA)
  - Software: Shimadzu Labsolution software package
  - Solvent system: CH_3CN/Water gradient from 95% to 5% water with 0.1% formic acid

- **Sample preparation**
  - Crude extracts (10 mg/ml) and reference compound (1 mg/ml) prepared in 100% MeOH

Phytochemical Fingerprinting by ^{1}H-NMR (*Trifolium pratense*)

[C] ^{1}H-NMR



► Sample preparation
- Crude extracts: 80% EtOH/water CE (8.03 mg/200 µl), 50% CH_2Cl_2/CH_3OH CE (6.85 mg/200 µl)
- Biochanin A: 3.87 mg/200 µl, Formononetin: 3.15 mg/200 µl
- Solvent: DMSO-d_6 (99.9%), Cambridge Isotope Laboratories, Inc. (Cas #: 2206-27-1, Lot #: 12G-464)
- Number of scan: 100 scans; spectra width: 0.187 Hz/point; spectra width 14400 Hz; pulse width 4.0 µs, relaxation delay 1s acquiring time 2.67 µs; temperature 25℃

► Instrument
- Jeol ECZ 400 MHz in 3 mm NMR tube under the Ultra COOL probe.

► Parameter (qNMR)
- Temperature 25℃, 90° single-pulse (relaxation delay: 60sec, receiver gain: 46, number of scan: 64)

Conclusions:
Identification of a Commercial Plant Powder: *Trifolium pratense*

- All the obtained ITS-2 and rbcL DNA barcode sequences matched with referenced *T. pratense* DNA sequences in GenBank, thereby confirming that the commercial plant material contains *T. pratense.* The universal MatK and psbA-trnH primers used could not amplify any sequence for this species. More specific primers should be designed for that purpose.

- All HPTLC, UHPLC-UV and ^{1}H NMR analyses confirmed the presences of major characterstic *T. pratense* isoflavonoids such as formononetin and biochanin A in both extracts. HPTLC and ^{1}H NMR analyses indicated that these extracts contained a relatively high proportion of chlorophylls, fatty acids and glucosidic derivatives.

- Both DNA barcoding and phytochemical analyses confirm that the investigated commercial sample contains *T. pratense* crushed leaves.