Botanical Ingredient Characterization; a tale of more than one thousand and one compounds

Stefan Gafner, PhD
CSO, American Botanical Council

May 29, 2020

The American Botanical Council

- Non-profit educational organization
- Provides information on responsible and safe use of medicinal herbs
- Members include consumers, healthcare professionals, researchers, educators, industry
- Founded in 1988 with James A. Duke, Norman R. Farnsworth and Mark Blumenthal as first Trustees of ABC board

Chemical Analysis TWG

Chemical Analysis Mission: To develop a strategy and methodologies to characterize botanical ingredients for the purpose of enabling safety assessments.

Objectives:

- **Prioritize selected candidates for comprehensive chemical characterization** based on the needs of other Technical Working Groups;
- **Identify a strategy to compile existing literature on analytical methods used and chemical composition** of selected botanical ingredients;
- **Select resource-efficient analytical approaches, methods, and partners that can comprehensively characterize botanical ingredients with respect to safety**, including, but not limited to, identifying and quantifying constituents of botanicals to the degree required for material selection and safety assessment.

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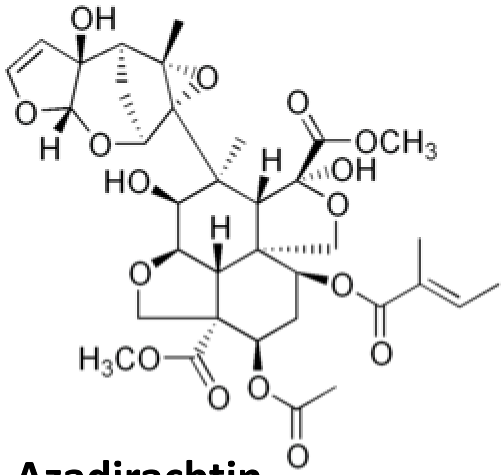
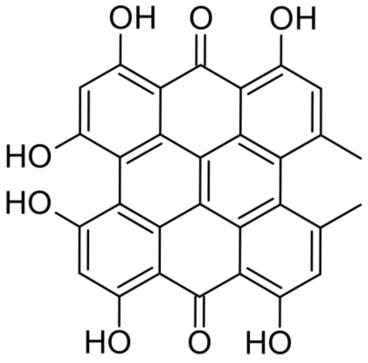
A Tale Of Thousand And One Nights

Scheherazade (painting by Hermann Emil Sprengel, 1881)

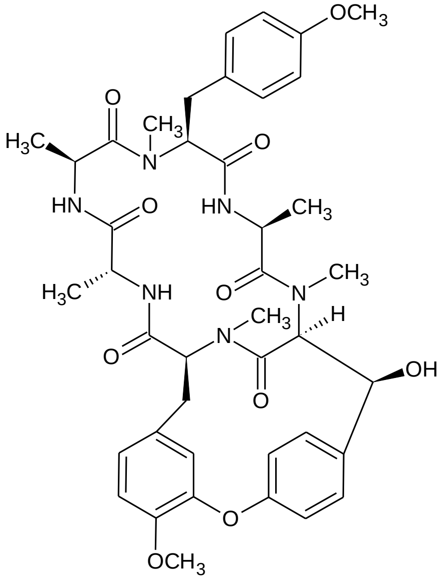
The Beauty of Plant Chemistry



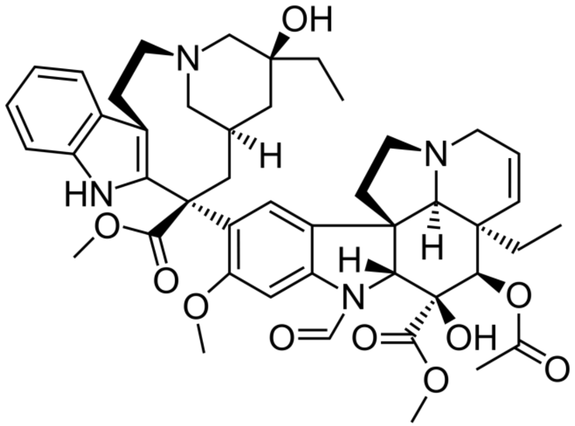
Azadirachtin



Hypericin



6-*O*-methylbouvardin



Vincristine

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The Challenges of Plant Chemistry

Between 500 – 10000 different molecules estimated in single plant species

Primary metabolism	Secondary metabolism
• Essential to survival of plant • Directly involved in growth and development Examples: - DNA - Proteins - Starch - Cellulose - Fats/Waxes - Chlorophyll	• Facilitates primary metabolism • Allows adaptation to, and survival in natural environment • Involved in defense mechanisms • Plays a role in reproduction Examples: - Terpenes - Alkaloids - Flavonoids - Tannins

Villas-Boas SG, et al. *Metabolome Analysis: An Introduction*. 2007

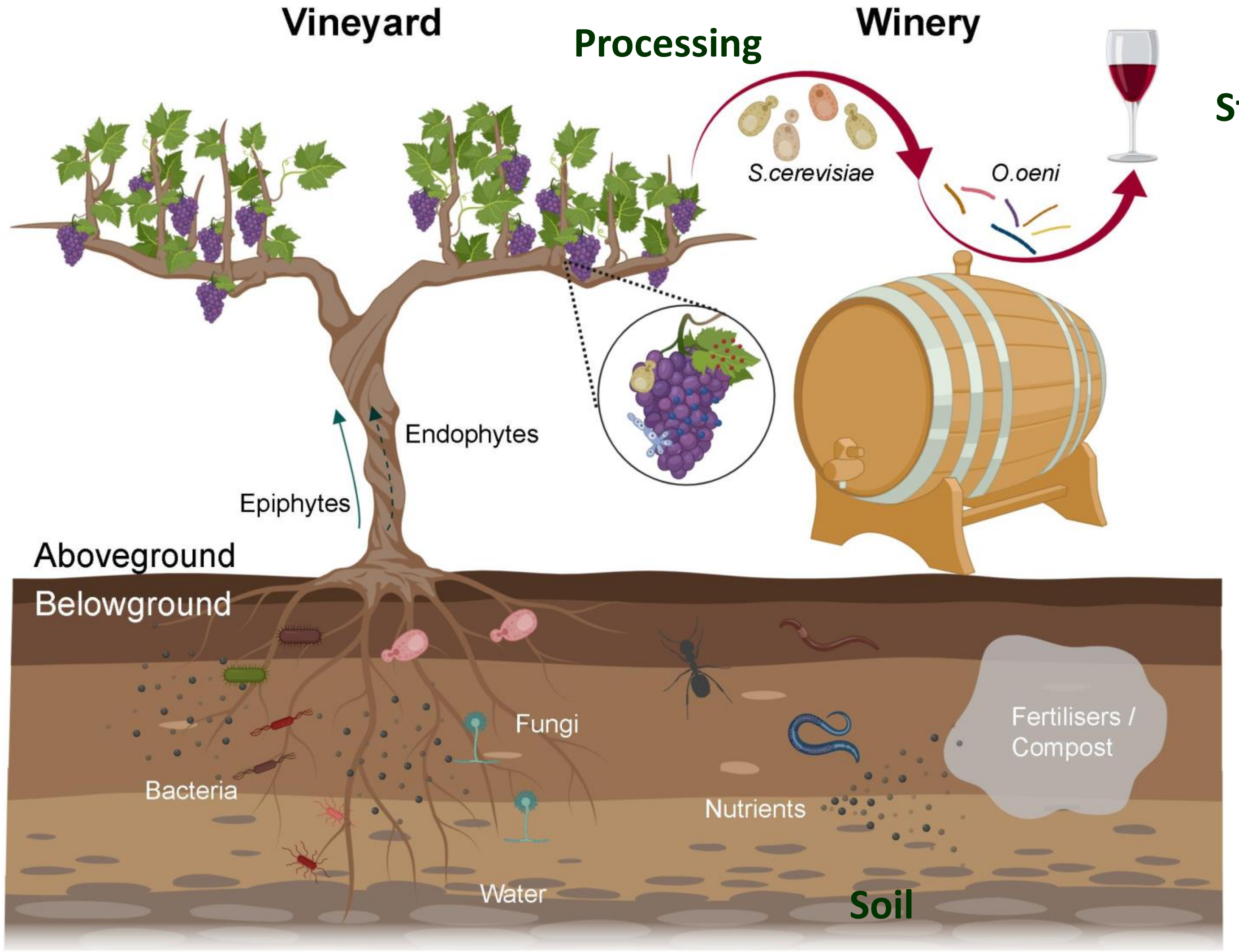
Miller JS. The discovery of medicines from plants: A current biological perspective. *Econ Bot*. 2011;65:396-407.

The Challenges of Plant Chemistry

Factors that impact the ingredient metabolome

Climate

Cultivar



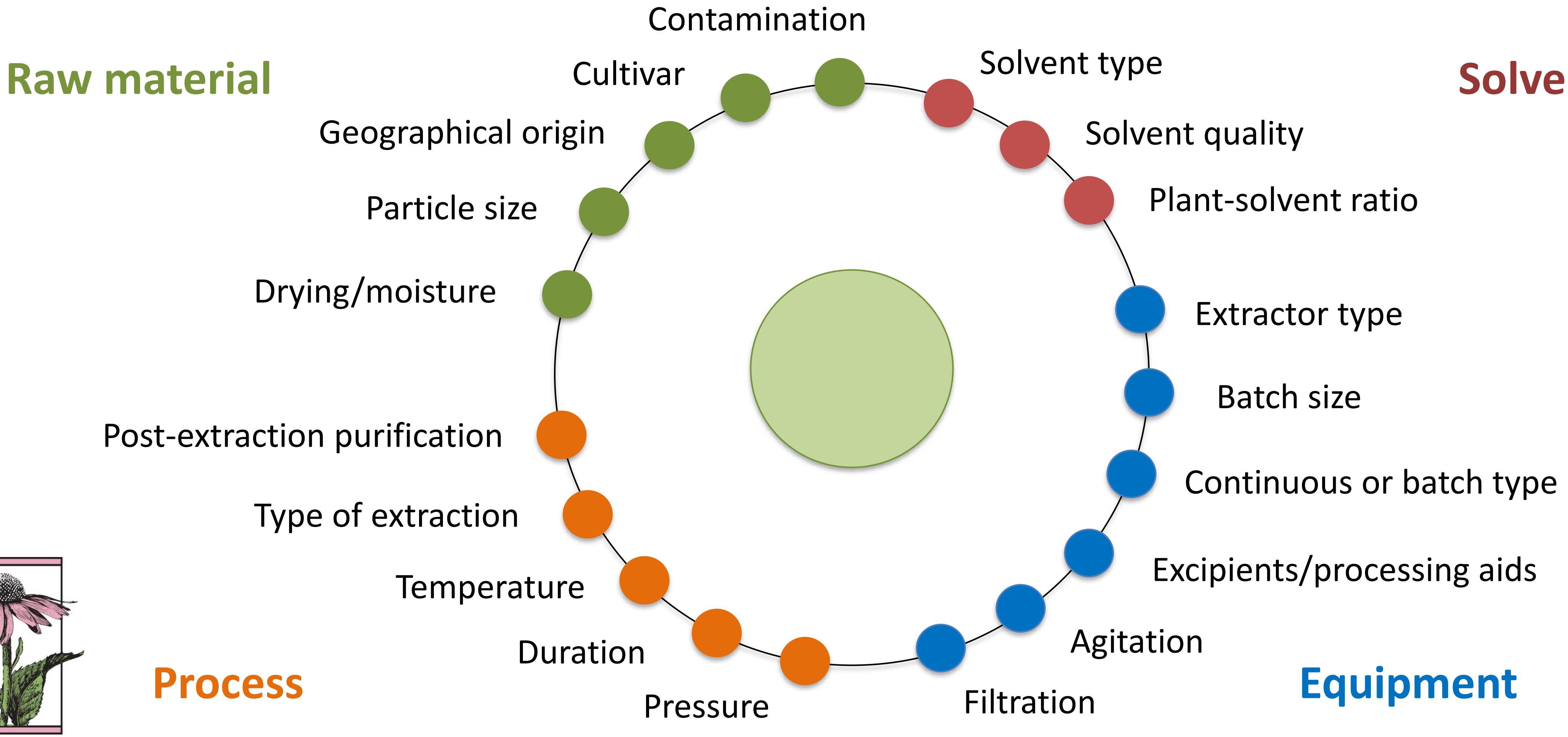
Stability

Agricultural conditions

Liu G, et al. From the vineyard to the winery: How microbial ecology drives regional distinctiveness of wine. *Front Microbiol*, 2019.

Ingredient Chemistry

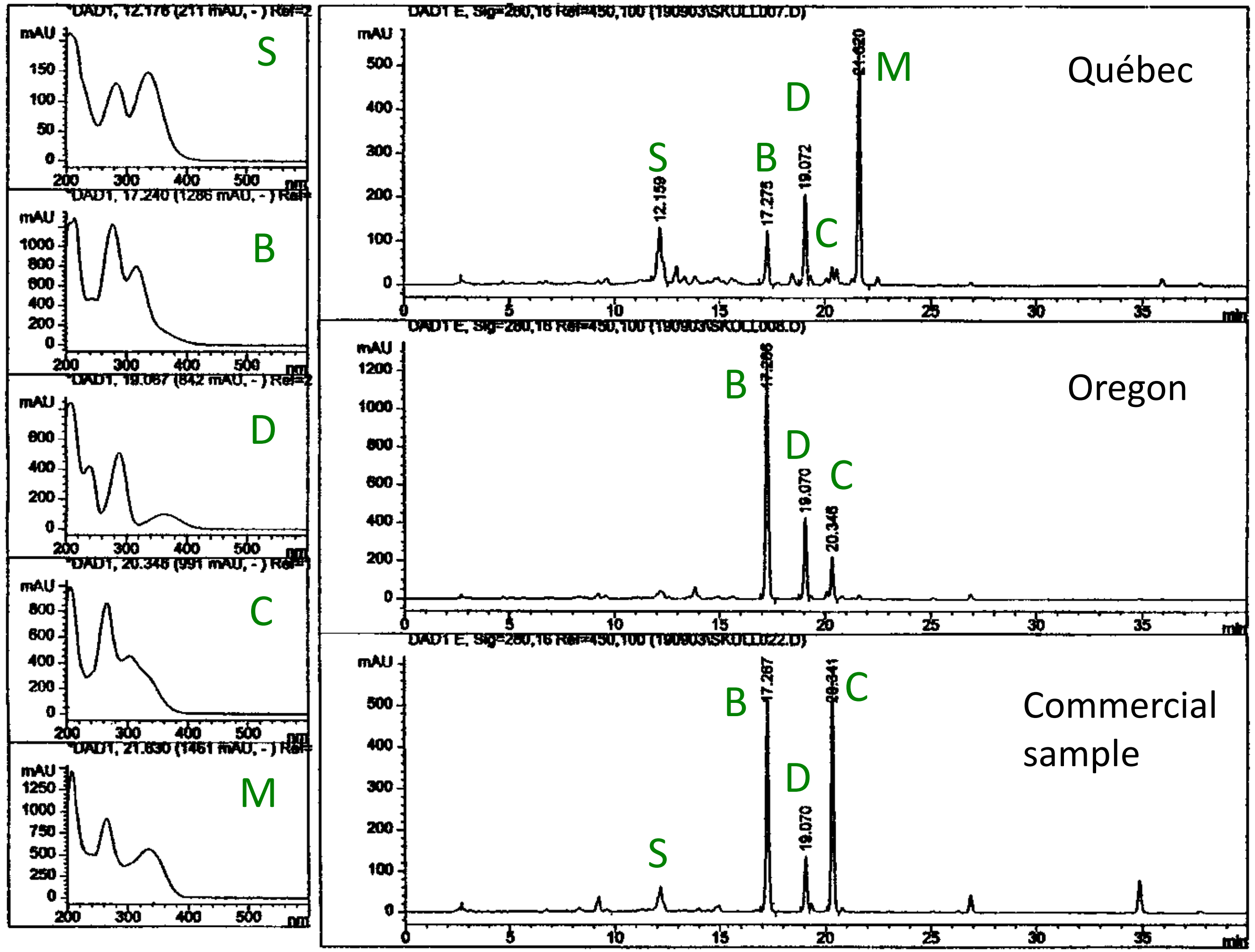
Processing Steps



Modified from http://www.berkem.com/en/expertise-en/plant-extraction

Differences in Metabolome

Scutellaria galericulata



HPLC-UV analysis with detection at 280 nm

Scutellaria galericulata

B: Baicalein-7-*O*-glucuronide (baicalin)
C: Chrysin-7-*O*-glucuronide
D: Dihydrobaicalein-7-*O*-glucuronide
M: 2’-Methoxychrysin-7-*O*-glucuronide
S: Scutallerein-7-*O*-glucuronide (scutellarin)

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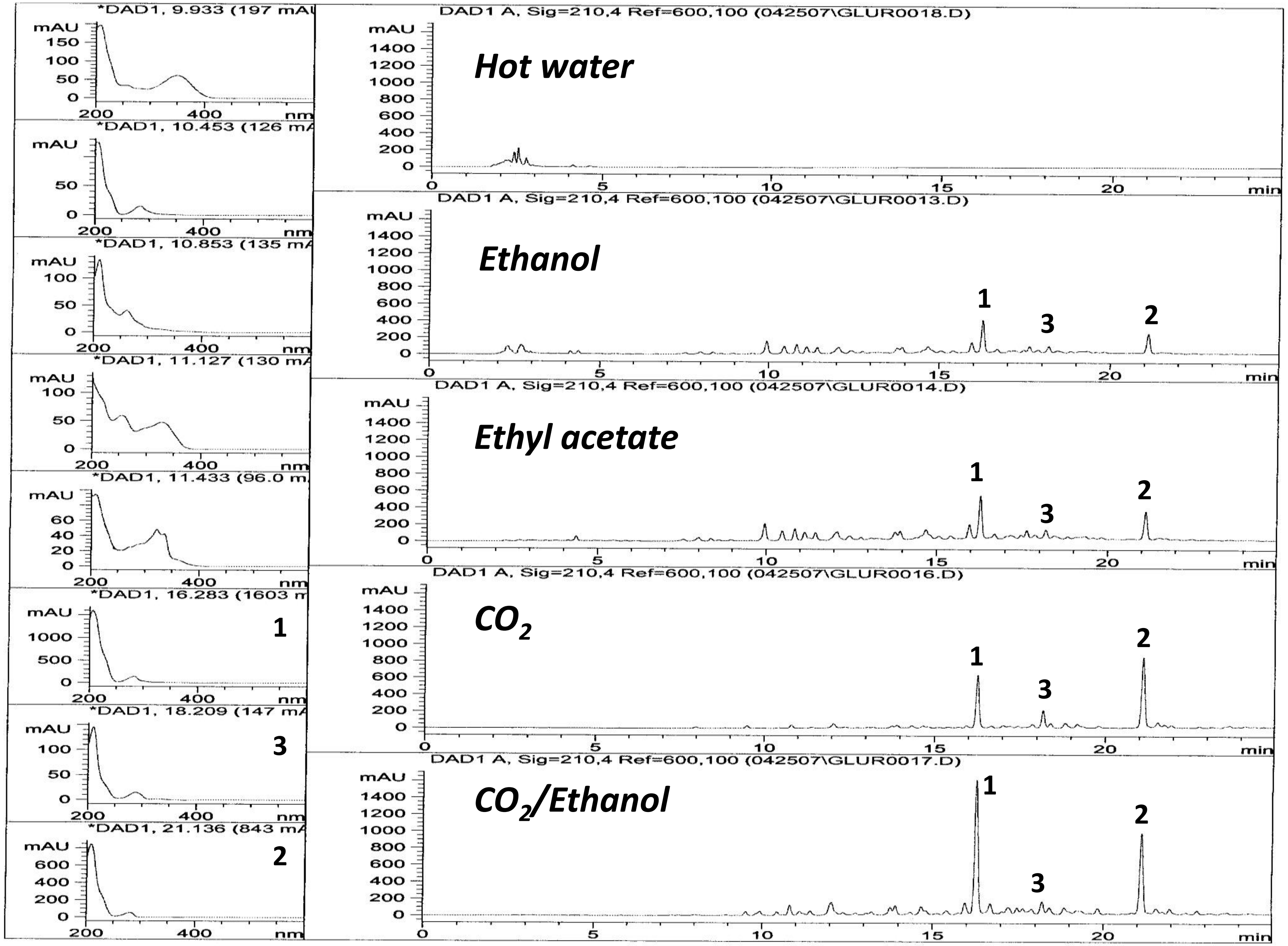
Extract Chemistry

Glycyrrhiza uralensis



Additional Challenges

- Adulterants
- Heavy metals
- Residual solvents
- Microbial contamination
- Pesticides
- Herbicides
- Radioactivity
- Polyaromatic hydrocarbons (PAHs)
- Other contaminants (plasticizers, etc.)

The ABC-AHP-NCNPR Botanical Adulterants Prevention Program

- Program to educate herbal and dietary supplement industry members about ingredient and product adulteration
- Initiated by three non-profit organizations:
    - American Botanical Council (ABC)
    - American Herbal Pharmacopoeia (AHP)
    - National Center for Natural Products Research (NCNPR) at the University of Mississippi

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All publications freely available at http://cms.herbalgram.org/BAP/

ABC AHP NCNPR
Botanical Adulterants Prevention Program

ABC AHP NCNPR
Botanical Adulterants Prevention Program

Botanical Adulterants Prevention Bulletins

Published		Written, In Edits, and/or Peer-Review	Planned/ Proposed
1. Aloe vera 2. Arnica flower 3. Ashwagandha root extract 4. Bilberry fruit extract 5. Black cohosh root & rhizome 6. Boswellia tree resin 7. Cranberry fruit extract 8. Ginkgo leaf extract 9. Goldenseal root & rhizome 10. Grape seed extract 11. Maca root 12. Olive oil 13. Oregano leaf and oil 14. Rhodiola root/rhizome & extract 15. Saw palmetto fruit 16. Skullcap herb	17. St. John's wort herb 18. Synthetic antimicrobials sold as "Grapefruit Seed Extract" 19. Tea tree leaf oil 20. Turmeric root/rhizome & extracts/curcumin	1. Cinnamon bark 2. Cordyceps fruiting body 3. Lavender flower oil 4. Milk thistle extracts 5. Pomegranate fruit & extract 6. Saffron stigma	1. Black seed (*Nigella sativa*) oil 2. Elder berry 3. Eleuthero root 4. Ginseng (Asian & American) root 5. Kava 6. Muira puama 7. Sandalwood oil 8. "Spiked" herbal extracts: caffeine, vitamin C, et al. 9. Tongkat Ali

Analytical Methods Used for Botanical Ingredient Identification

Method	Applicability	Limitations; Not applicable to
Taxonomy	- Whole living plant	- Extracts, - Powdered or cut crude plant material
Macroscopy	- Whole or cut crude plant material	- Extracts - Powdered crude plant material
Microscopy	- Whole, cut or powdered crude plant material	- Extracts
Genetics (DNA)	- Whole, cut or powdered crude plant material - Extracts possessing intact DNA from the parent plant	- Extracts without DNA - Materials processed using prolonged heat, exposure to UV light, or irradiation
UV/VIS (standalone)	- Extracts - Whole, cut or powdered crude plant material after extraction	- Analytes with no UV/VIS chromophore (e.g., sugars and sugar alcohols, many amino acids) without prior derivatization
FT-IR	- Extracts - Whole, cut or powdered crude plant material after extraction	- Extracts containing large amounts of carriers, e.g., maltodextrin
FT-NIR	- Extracts - Whole, powdered or cut crude plant material	- Materials with variable moisture content - Extracts containing large amounts of carriers, e.g., maltodextrin
MS (standalone)	- Extracts - Whole, cut or powdered crude plant material after extraction	- Very high molecular weight analytes - Non-readily ionizable molecules
NMR	- Extracts - Whole, cut or powdered crude plant material after extraction	- Certain highly polymerized molecules (e.g., high molecular weight PACs)
HPTLC	- Extracts - Whole, cut or powdered crude plant material after extraction	- Highly polar compounds
GC-FID	- Extracts - Whole, cut or powdered crude plant material after extraction	- Non-volatile compounds
GC-MS	- Extracts - Whole, cut or powdered crude plant material after extraction	- Non-volatile compounds
HPLC-UV(DAD)	- Extracts - Whole, cut or powdered crude plant material after extraction	- Analytes with no UV/VIS chromophore (e.g., sugars and sugar alcohols, many amino acids) without prior derivatization
HPLC-MS	- Extracts - Whole, cut or powdered crude plant material after extraction	- Very low and very high molecular weight analytes - Non-readily ionizable molecules (e.g. , terpenes with limited presence of functional groups)

Euphrasia officinalis Identification Challenges

- Species hybridize frequently
- Self-pollination and insect pollination → species may exhibit highly variable morphology (e.g., *E. minima*)
- Morphological distinction criteria often blurred (e.g., *E. rostkoviana* vs. *E. montana* or *E. versicolor*)
- Botanists use different scientific names for the same species

Euphrasia rostkoviana
(syn. *E. officinalis* subsp. *rostkoviana*)

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HPTLC Investigation of Commercial Eyebright Samples

- 28 botanical samples collected in the wild, including 25 *Euphrasia* spp. samples, *Odontites lutea*, *O. viscosus*, and *Bartsia alpina*
- 32 commercial samples analyzed: 25 bulk materials and 7 finished products (USA: 28; Europe: 4)
- Bulk materials originating in Bulgaria, Croatia, Macedonia, Poland, and Ukraine

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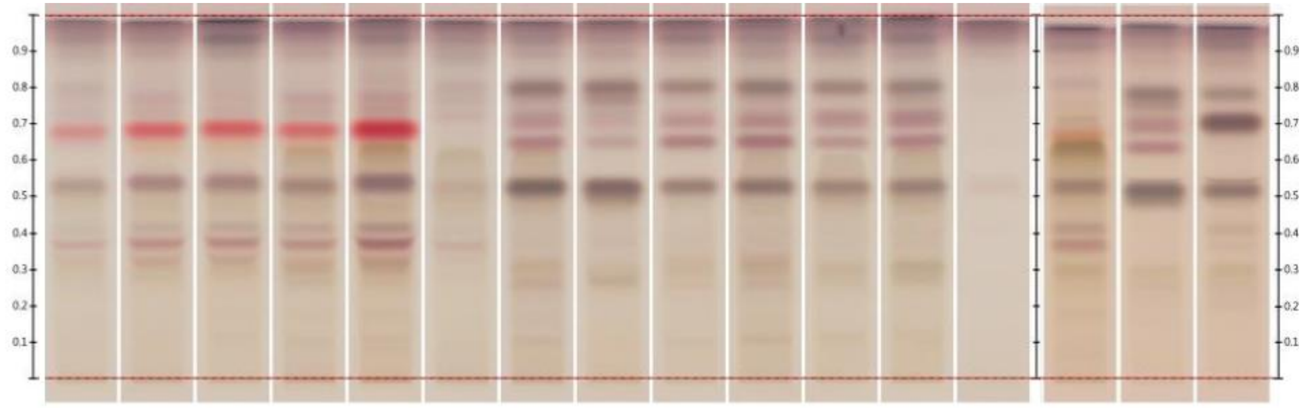
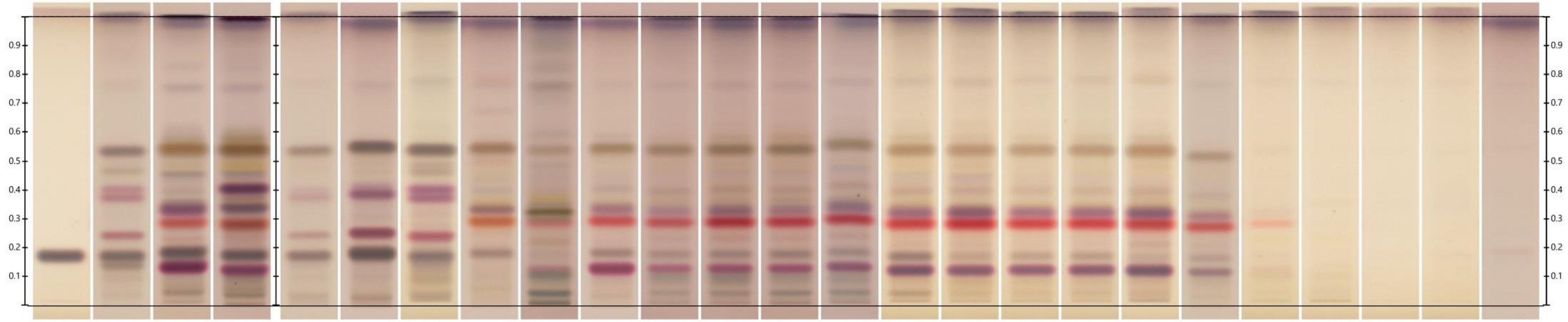
Results

Mobile phase: Dichloromethane, methanol, water (60:40:4)



Samples:
B1-B25: Bulk samples
P1-P7: Dietary supplements
A: Aucubin
Er: *Euphrasia rostkoviana*
Es: *Euphrasia stricta*
Ol: *Odontites lutea*
Ov: *Odontites viscosus*

Mobile phase: Ethyl acetate, acetic acid, formic acid, water (100:11:11:26)



Bulk materials: 8 *Odontites* spp. (all from USA), 13 *Euphrasia* spp., 4 weak or blank
Dietary supplements: 4 *Euphrasia*, 3 weak or blank

Images provided by Camag, Switzerland

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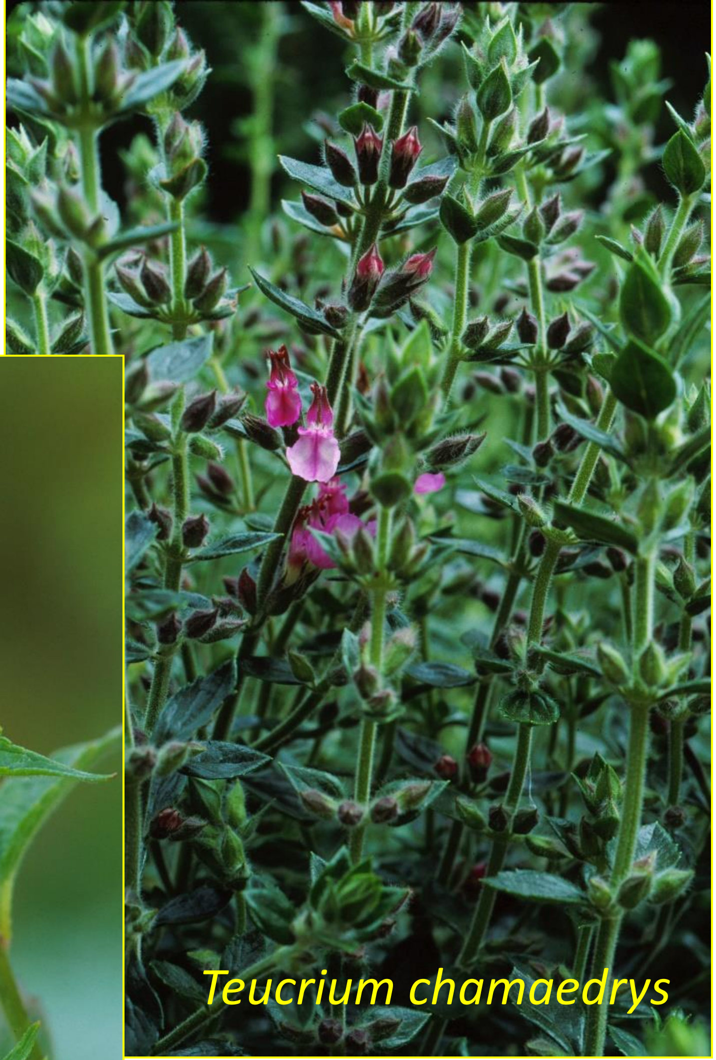
Teucrium canadense

Scutellaria lateriflora



Teucrium chamaedrys

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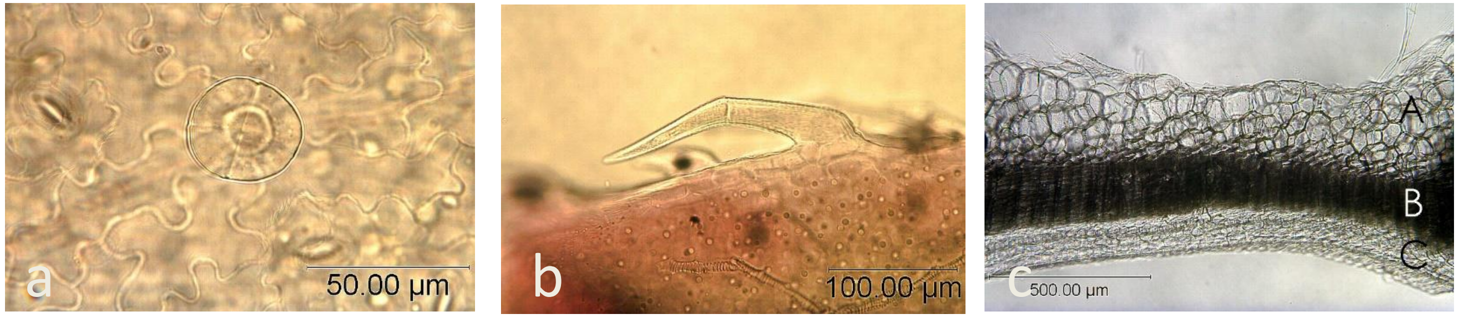
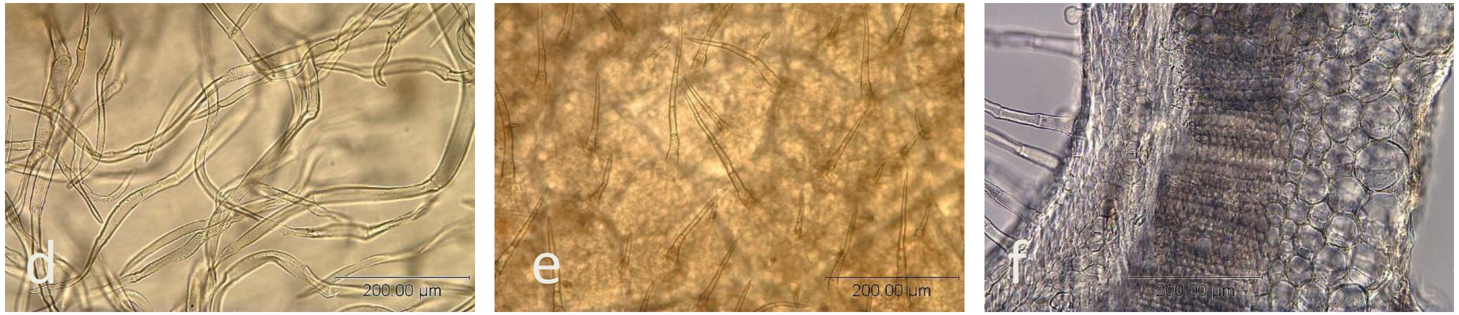
Digital Photo-Microscopy

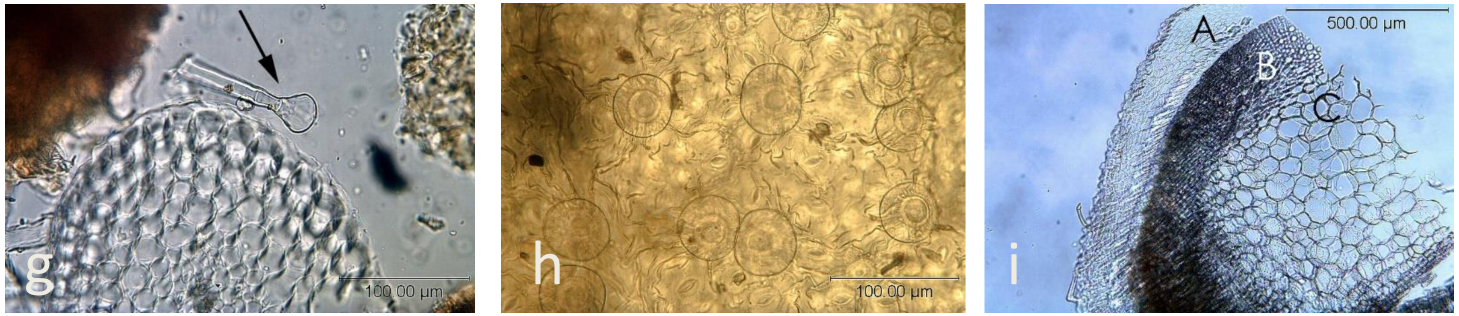
Scutellaria lateriflora



Teucrium canadense



Teucrium chamaedrys



Images provided by Alkemist Labs

Glycyrrhiza uralensis Fisch.

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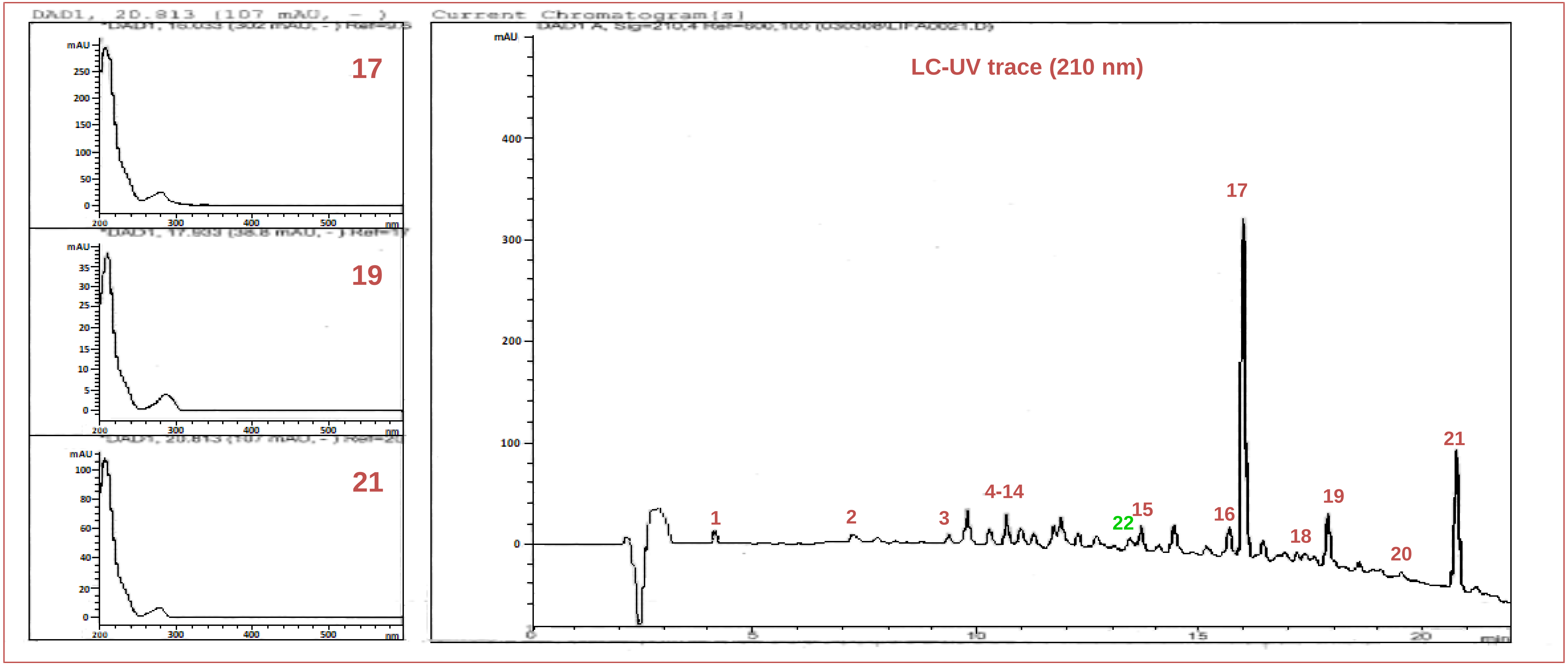
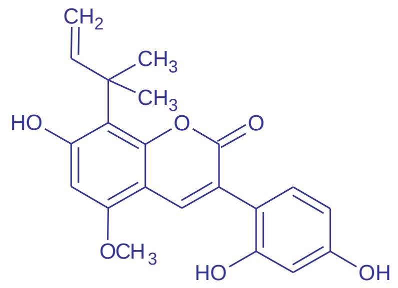
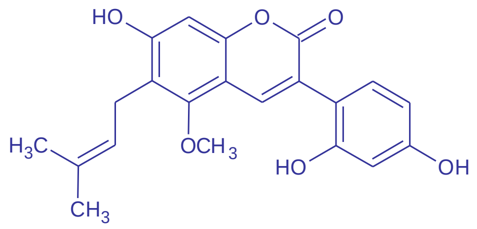
Licorice CO_2 Extract: Chemistry



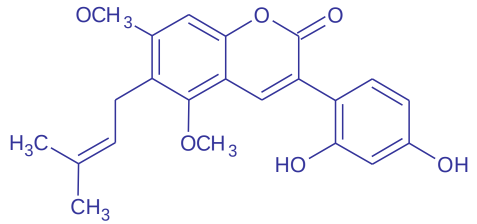
Licorice extract chemistry



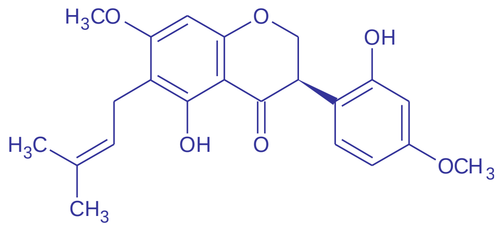
Licoarylcoumarin (3)



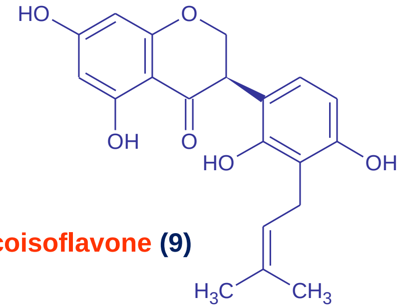
Glycycoumarin (5)



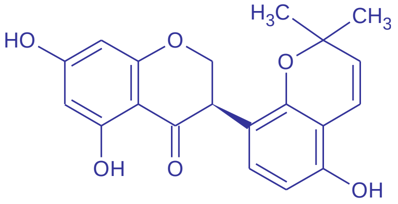
Glycyrin (15)



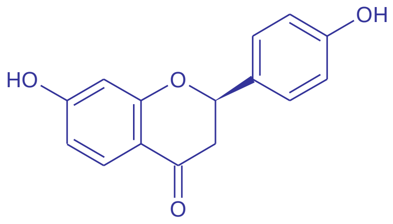
Glyasperin K (18)



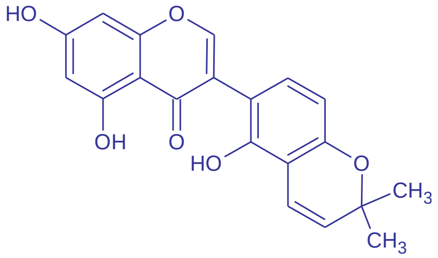
Dihydrolicoisoflavone (9)



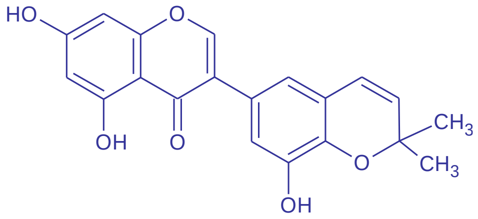
Glyasperin F (7)



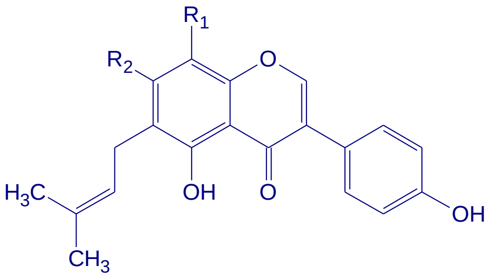
Liquiritigenin (1)



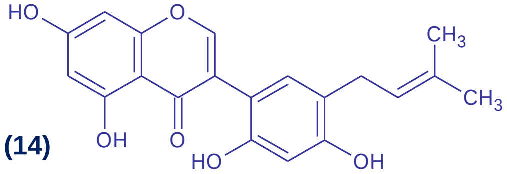
Licoisoflavone B (6)



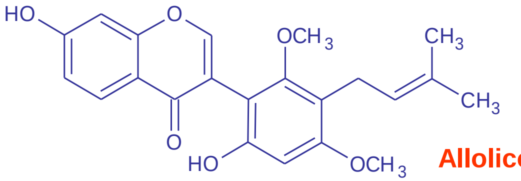
Semilicoisoflavone B (10)



R_1=OH, R_2=iPr: 6,8-Diprenylgenistein
R_1=OCH_3, R_2=H: Gancaonin G



Licoricone (14)



Allolicoisoflavone A (12)

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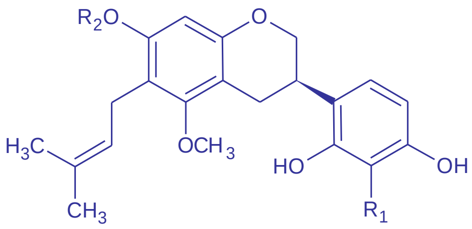
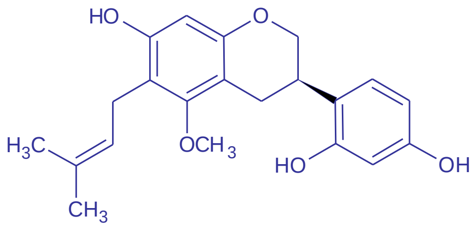
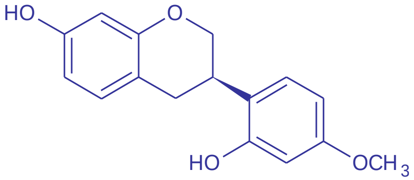
Licorice extract chemistry



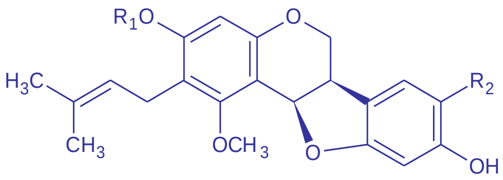
R_1=iPr, R_2=H: Licoricidin (17) 15-16%
R_1=iPr, R_2=CH_3: Licorisoflavan A (21) 5-6%
R_1=H, R_2=CH_3: Glyasperin D (16)



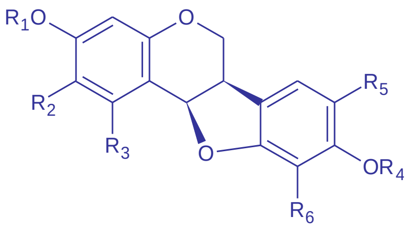
Glyasperin C (8)



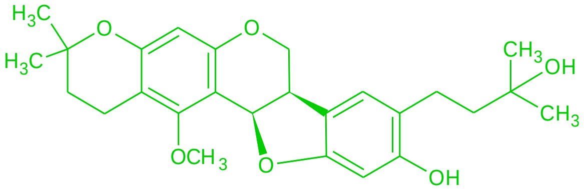
Vestitol (2)



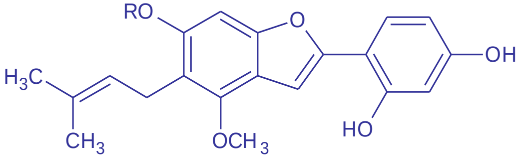
R_1=H, R_2=iPr: 1-Methoxyficifolinol (19) 2-3%
R_1=CH_3, R_2=H: Kanzonol P (20)



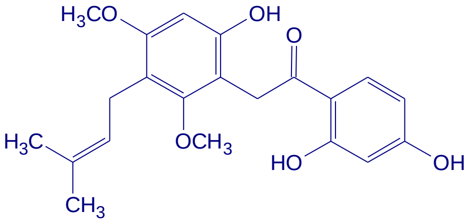
R_1=R_2=R_3=R_5=R_6=H, R_4=CH_3: Medicarpin (4)
R_1=R_2=R_4=R_5=H, R_3=OCH_3, R_6=iPr: 1-Methoxyphaseollidin (11)
R_1=R_4=R_5=R_6=H, R_3=OCH_3, R_2=iPr: Edudiol (13)



Glycycarpan (22)



R=H: Licocoumarone
R=CH_3: Gancaonin I



Licoriphenone

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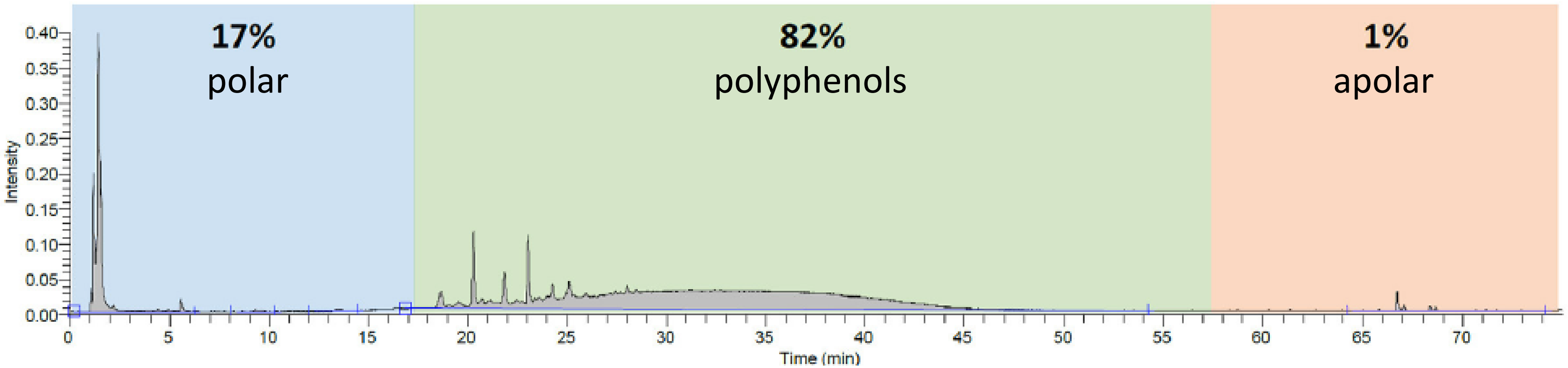
HPLC-CAD-HRMS

Vitis vinifera

CAD (100 pA)



Sica VP, et al. *Front Chem*. 2018; doi: 10.3389/fchem.2018.00334

Composition:
75% Proanthocyanins (DP>5); 7% flavan-3-ol monomers to pentamers; <1% lignans; 16% minerals & carbohydrates

Toxicity calculations:

- No concerns for mutagenicity or genotoxicity based on published data
- No DART data
- Threshold of Toxicological Concern (TTC): 90 µg/kg/day[1] per analyte

[1]Review of the Threshold of Toxicological Concern (TTC) approach and development of new TTC decision tree. *EFSA Support Publ*. 2016;13:1006.

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Multiple Analytical Methods

Aloe vera

Composition of 18 commercial decolorized aloe vera leaf extracts:

Analyte	Method	Amount [%]
Moisture	Gravimetry	2.6 - 8.0
Total ash	Ignition	6.7 - 28.8
Lipid content	Gravimetry	0.3-4.3
Protein content	Kjeldahl nitrogen	0.7 - 9.8
Crude fiber	Gravimetry	traces – 45.8
Free sugars	HPAEC-PAD	0.1 - 37.7
Organic acids	HPLC-UV	5.0 - 31.4
Polysaccharides	GPC-MALS/RI	6.9 – 45.4

All analytes combined account for 84.6 – 95.2% of the extract

Zhang Y, et al. *J AOAC Int*. 2018; 101(6):1741-1751

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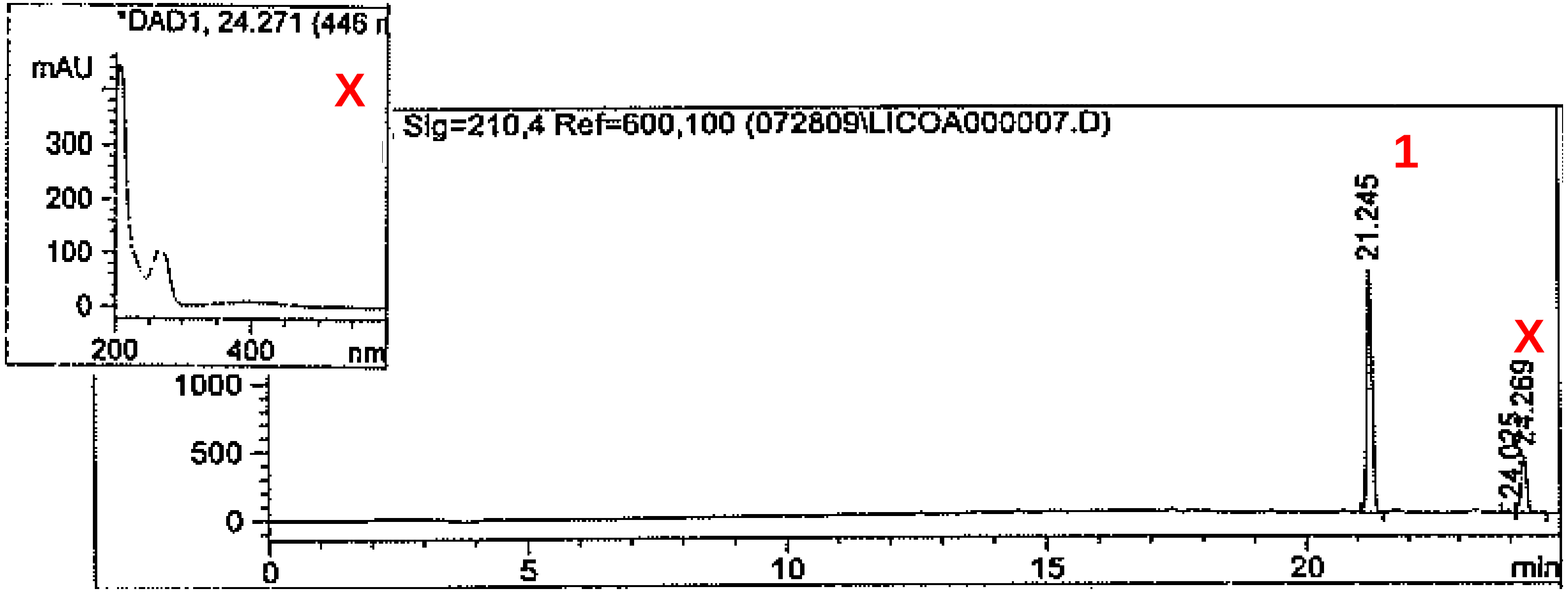
What Happens In Test Medium?

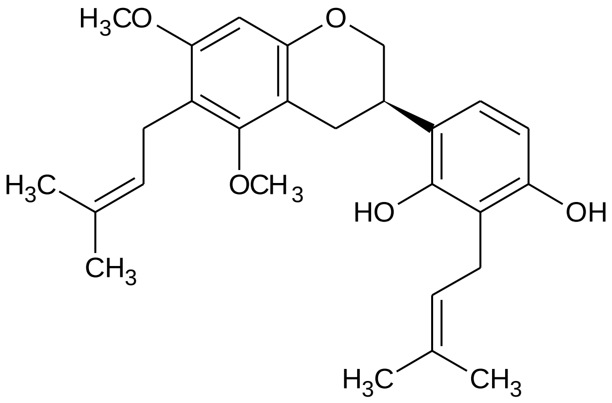
Licorisoflavan A

Stress tests as part of HPLC method validation:

- 0.1 N HCl
- 0.1 N NaOH
- 3% H_2O_2

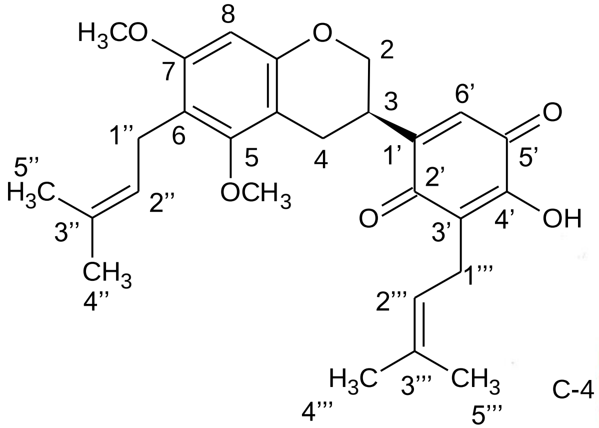


Licorisoflavan A after 22 hours in 0.1 N NaOH

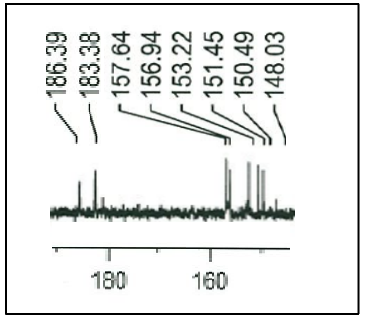


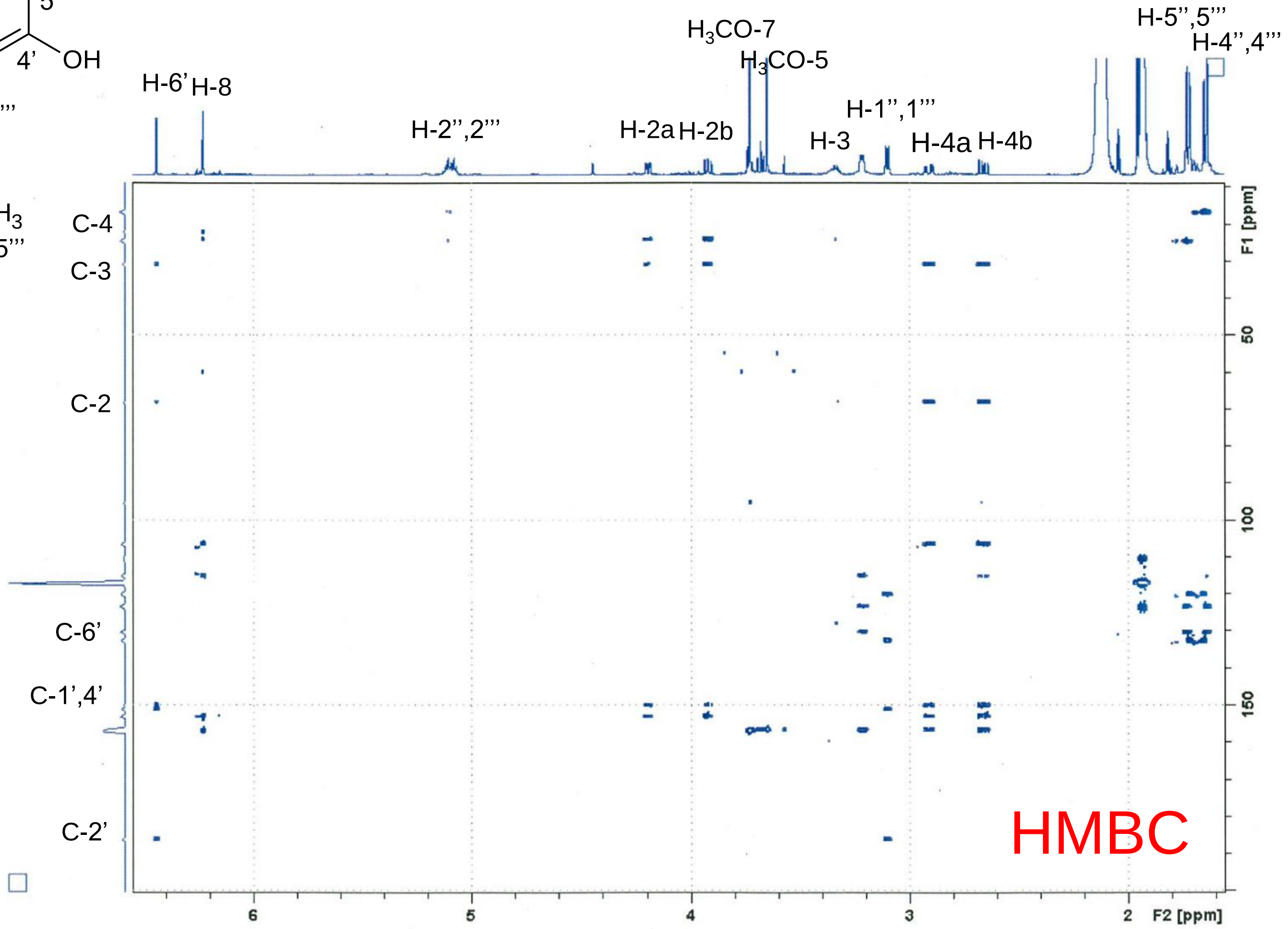
1: Licorisoflavan A

HPLC-NMR



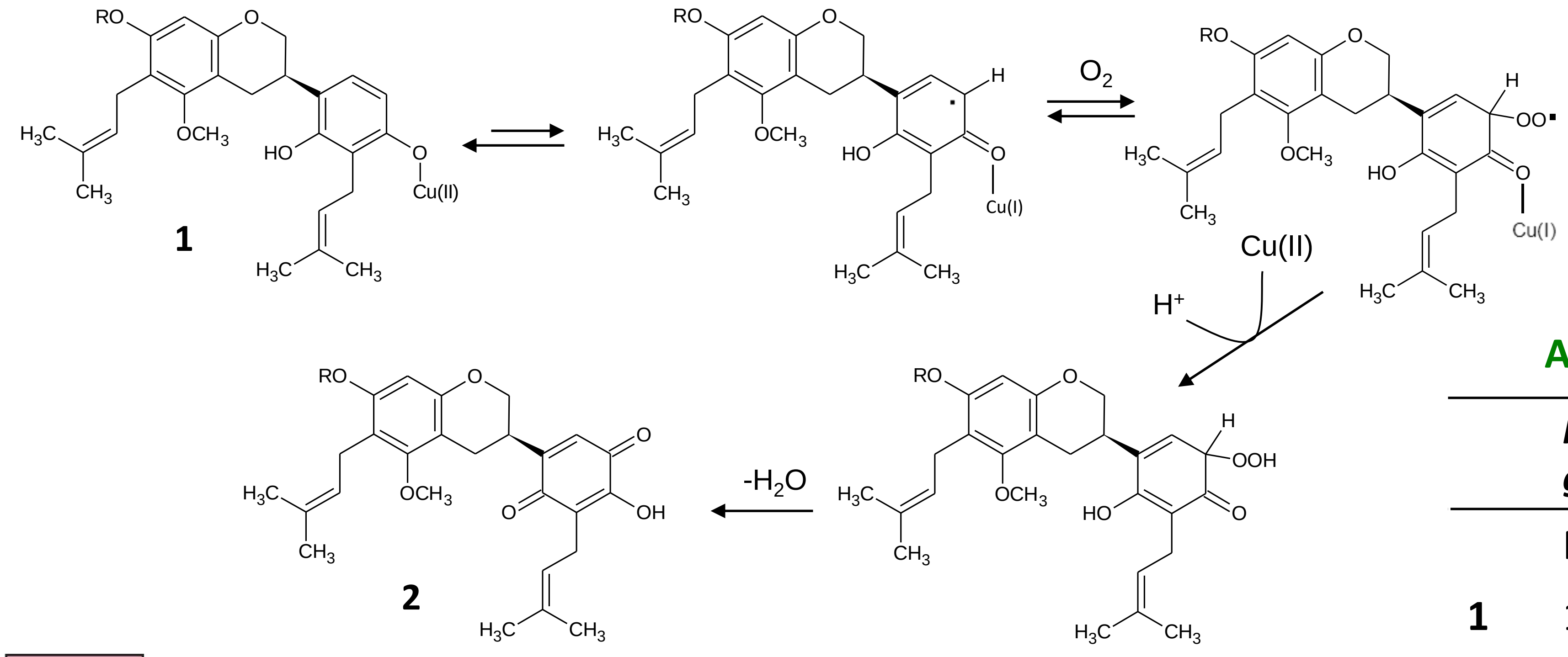
^{13}C NMR





NMR data agrees with *p*-quinone

Proposed Reaction



Antibacterial activities (µg/mL)

	Porphyromonas gingivalis		*Streptococcus mutans*	
	MIC	MBC	MIC	MBC
1	1.56	1.56	50	100
2	25	25	>200	>200

Ling KQ, Lee Y, Macikenas D *et al.*, J. Org. Chem. 2003, *68*, 1358-66
Singh US, Scanell RT, An H *et al.*, J. Am. Chem. Soc. 1995, *117*, 12691-99

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Chemical Analysis TWG

Method Considerations

- Availability of botanically authenticated reference material
- Presence of inert material (e.g., cellulose, maltodextrin, dirt)
- Adaptation of the methods for testing with mixtures, (e.g., TCM)
- Sample preparation issues (e.g., solubility of “botanicals”)
- Ability to evaluate the material in bioassay fluids
- Stability issues (e.g., ligustilide in extracts of *Ligusticum* spp.) and compatibility issues (e.g., solubility of the analyte in the mobile phase)
- Detection bias

Chemical Analysis TWG

Approach

Compilation of Literature	Literature on phytochemical compositions of botanical ingredients will be compiled
Procurement & Handling	Crude powdered raw plant materials & finished products will be procured from multiple suppliers
Authentication	Botanical taxonomy, macroscopic identification, microscopic identification, DNA barcoding, chemical analysis can be used to determine authenticity
Comprehensive Characterization	Identification & quantitation of constituents. Also moisture content, total inorganics, nutrient content, mass balance, etc.
Contaminant Analysis	Presence of pesticides, heavy metals, & mycotoxins will be determined
Bulk Stability	Stability of known marker constituents will be determined

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