METHODS FOR THE ISOLATION, PURIFICATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS

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OUTLINE

- Challenges & Methods for the isolation of bioactive compounds (BAC)
- Purification & Identification methods
- CASE STUDY: Coumarins Purification & identification
BIOACTIVE COMPOUNDS

- Global market plant derived BAV - $18 B
- $26 billion predicted for 2011
- 63% anticancer drugs from natural products

**Taxol**

**Lovastatin**

**Camptothecin**

**Oyster mushroom**

**Taxus brevifolia**

**Chinese Happy tree**

GROWTH OF BIOACTIVE COMPOUNDS

WORLDWIDE BIOACTIVE COMPOUNDS PATENTS

DRUGS APPROVED IN US FROM 1981-2007

BIOACTIVE COMPOUNDS IN CITRUS

- Limonoids
- Flavonoids
- Coumarins
- Sterols
- Pectin
- Essential oils
- Lycopene & ascorbic acid
WHY PURIFICATION NECESSARY?

Limonin 5mg - $105
WHY PURIFICATION & IDENTIFICATION ESSENTIAL?

• Not available commercially
• Expensive for animal expts
• Number of positive results on biological activity
• Mechanism of these BAV by conducting various in vivo studies
• Clinical trails are needed
• Requires high pure BAC in multigram quantities
CHALLENGES FOR THE EXTRACTION OF BIOACTIVE COMPOUNDS - Raw Material

- Chemical nature - simple monomer to a highly polymerized structure

- Occur in free or conjugated forms with sugars, acids, and other organic molecules

- Stability

- Uneven distribution
Extraction is influenced by several factors

- Chemical structure, Glucosides - fruits, seeds
- Polarity of solvent, lycopene
- Sample matrix, Glucosides - dried, fresh - MeOH
- Degree of polymerization, grapes - phenolics
- Interaction with other cellular components
- Temperature, solubility, part. coeff.
- Pressure, solubility, part. coeff.
- Techniques,
- Solid-to-solvent ratio
- Particle size
CHALLENGES TO OBTAIN PURE COMPOUNDS

• Low concentrations (<0.2%) in fruits, vegetables

• Constitute to number of compounds

• Individual compound isolation in multigrams is a challenge

• Other components can have similar properties, that makes isolation / separation difficult

• Selection of raw material, Depends upon the targeted compounds, For e.g. Limonin, – Grapefruits; Lyc – Rio-red;

• Minor - start from large amount of raw materials to enrich to small quantity and go for fractionation.
POSSIBLE REASONS FOR VARIATION OF BIOACTIVE COMPOUNDS

- Cultivars - Lycopene
- Environmental conditions
- Post-harvest conditions
- Maturity
- Analysis, Methods

Grapefruits

Blood Oranges

Limonin - LG

Phenolics, HPLC
CRITICAL STEPS FOR EXTRACTION

1. Sampling/selection of raw material
2. Preservation of samples / extracts
3. Extraction of bioactive compounds
4. Separation & Detection
CRITICAL STEPS WITHIN EXTRACTION

- Sample homogenization
- Extraction (PLE, SFE, Sonication, Soxhlet, Vortex, Shaker, Stirring, Microwave, etc.)
- Filtration, centrifugation
- Hydrolysis, Derivatization
- Pre-concentration (Liquid-liquid extraction, SPE, etc.)
SELECTI ON OF FAV FOR BIOACTIVE COMPOUNDS
APPROACHES FOR NATURAL COMPOUNDS ISOLATION

• Raw material selection
• Extraction: With solvent and without solvents
• Purification: Column chromatography
  Flash chromatography
  Prep. HPLC

Analytical Techniques: TLC, HPLC, GC
Structure elucidation: NMR, LC-MS, MS-MS
EXTRACTION METHODS

Soxhlet - Solvents
Hydrotropic extraction - Non-solvent
Supercritical fluid extraction - CO2
Microwave extraction
Ultrasound extraction
Pressurized extraction
SOLVENT EXTRACTION BY SOXHLET

Citrus fruits

→ Powdered

→ Solvent

→ Extraction 6-8 h

→ Concentration

→ Bioactive fractions

Mandadi et al., Z. Naturforschung C, 62c, 179-188, 2007
• Highly water soluble organic salts with hydrophobic and hydrophilic moieties

• Increasing solubility of water insoluble compounds

• Depends not only on the nature of hydrotrope but also on the nature of solute

Dandekar et al., Z. Naturforschung, 63c, 176-180, 2008
HYDROTROPIC EXTRACTION

Cont’d.

Raw Material

Hydrotrope Solution

Extract

Filtered Extract

Water (pH 7 or pH 3)

Sour orange + Sodium cumen sulfonate, 45°C for 6 H

Solid Residue

Dilute Hydrotrope Solution

Dilute Extract

Product

Mixtures of limonoids & Flavonoids

Dandekar et al., Food Chemistry, 109, 515, 2008
SUPERCRITICAL FLUID EXTRACTION

- Solutes can be separated without loss of volatiles
- SC-CO2 protects the substrate from oxygen, resulting in fewer decomposition products

SFE can eliminate the concentration process

25-40 MPa - 3000- 7000 PSI

PRESSURIZED / ACCELERATED / ENHANCED SOLVENT EXTRACTION

Solid - Liquid extraction

50-200 °C / 1500- 2500 PSI

Ong, et al., J. Chromatogr., A, 2000, 904, 57;
MICROWAVE-ASSISTED EXTRACTION

Microwaves: Solid-Liquid
Non-ionising EM radiation freq. 300-300,000 MHz

Fast and rapid method for small quantity

Flammable solvents cannot be used

## SUMMARY / CONCLUSIONS

<table>
<thead>
<tr>
<th>Type</th>
<th>Sample size: solvent</th>
<th>Temp</th>
<th>Pressure/Time</th>
<th>Investment</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soxhlet</td>
<td>1-2000 g; 4-8000 ml</td>
<td>Depends upon the solvent</td>
<td>Atmospheric / 6-8 h</td>
<td>Very low</td>
<td>Efficient for polar &amp; non-polar BAC</td>
</tr>
<tr>
<td>SFE</td>
<td>25-100 g; continuous flow</td>
<td>25 – 45 Mpa; 1-2 h</td>
<td>High</td>
<td>Efficient for non-polar BAC</td>
<td></td>
</tr>
<tr>
<td>PLE</td>
<td>1-30 g 10-100 ml</td>
<td>80-200</td>
<td>1 – 10 Mpa; 10-30 min</td>
<td>High</td>
<td>Efficient for polar &amp; non-polar BAC, Safety</td>
</tr>
<tr>
<td>MAE</td>
<td>1 -20 g; 10-50 ml</td>
<td>80-150</td>
<td>Variable / 10-30 min</td>
<td>Moderate</td>
<td>Use of solvents is risky</td>
</tr>
<tr>
<td>HYDRO</td>
<td>10-100 g</td>
<td>40-60</td>
<td>Atmospheric / 6-8h</td>
<td>Low</td>
<td>Residual hydrotrop</td>
</tr>
</tbody>
</table>
OUTLINE

- Introduction
- Challenges & Methods for the isolation of bioactive compounds (BAC)
- Purification & Identification methods
- CASE STUDY: Coumarins Purification & identification
PURIFICATION TECHNIQUES

- Distillation, Fractional Dist. BP
- Fractional crystallization,
- Gel filtration, size
- Affinity chromatography
- Ion exchange chromatography
- Flash chromatography
- Preparative HPLC
SELECTION OF ADSORBENT

Proteins, AA, Alkaloids
- Sample Characteristics
  - With metal reagent
  - Charged

Stationary Phase (conditions)
- Reversed Phase Silica (RP)
- Cyano silica (RP)
- Metal Scavenger Silica (NP)
- Neutral alumina (NP)
- Florisil (NP)
- Cyano (NP)

Anthocyanins
- Acid Sensitive
- Acidic Properties
- Normal Phase Silica (NP)
- Reversed Phase Silica (RP)
- Acidic Alumina (NP)
- SAX (NP)
- Cyano (NP)

Proteins, AA, Alkaloids
- Basic Properties
- High Polarity
- Reversed Phase Silica (RP)
- Cyano Silica (RP)
- Neutral alumina (NP)
- Amine Silica (NP/RP)
- Basic Alumina (NP)
- SCX (NP)
- Neutral alumina (NP)
- Cyano silica (NP)

Most of the BAC
- Low or Medium Polarity
- Reversed Phase Silica (RP)
- Cyano Silica (RP)
- Neutral alumina (NP)
- Dii Silica (NP)
- Cyano Silica (NP)
- Neutral Alumina (NP)
- Reversed Phase Silica (RP)
PURIFICATION BY ION EXCHANGE & ADSORBANT RESINS

Molasses

Seed extract

Prep. HPLC

Pure compounds

Flash chromatography

Jayaprakasha et al., US patent, 2007/0237885 A1
FLASH CHROMATOGRAPHY

- Clark Steel (1978) – purification, Silica gel
- Glass columns high flow rates - 5 ml/min, faster
- Low molecular weight natural, synthetic
- Irreproducible
- **Modern flash techniques**
- Use of convenient disposable flash cartridges.
- Speed up the purification process.
SAMPLE LOADING

- **Dry Loading**
  - Pre-adsorption of sample onto silica for low solubility samples

- **Wet “dry loading”**
  - Pipetting sample onto pre-packed solid load cartridge.

- **Liquid Injection**
  - Through valve allows for column equilibration.

- **Liquid injection directly onto column**
  - Equilibration is skipped and sample is run through dry column (for speed).
STRUCTURE ELUCIDATION

Quantification, HPLC, GC
Purity, HPLC, GC, TLC

Spectroscopic methods

Structure elucidation

Stereochemistry

Optical activity, i.e., polarimetry
Thin Layer Chromatography (TLC)

- Very sensitive, rapid, very accurate for the natural products
- Sample is spotted onto TLC plate using a glass capillary
- Plate is “developed” using a solvent system
ANALYTICAL TECHNIQUES Cont.,

- High Performance Liquid Chromatography (HPLC)
- Gas Chromatography (GC)

IDENTIFICATION BY NUCLEAR MAGNETIC RESONANCE (NMR) SPECTRA

NMR is the most powerful tool available for organic structure determination.

Variety of nuclei:
$^1$H, $^{13}$C, $^{15}$N, $^{19}$F, $^{31}$P

NUCLEAR SPIN

- Nucleus with an odd atomic number or an odd mass number has a nuclear spin.
- The spinning charged nucleus generates a magnetic field.
- When placed in an external field, spinning protons act like bar magnets.
CHEMICAL SHIFT

- Dependence of nuclear magnetic energy levels on the electronic environment in a molecule
- Measured in parts per million
- Called the delta scale
NMR SIGNALS

- **Number of signals** - different kinds of protons
- **Location** how shielded or deshielded the protons
- **Intensity** - number of protons of that type
PROTONS IN A MOLECULE

Depending on their chemical environment, protons in a molecule are shielded by different amounts.

Methanol - CH3OH

- 3H - 1 signal -3.4 ppm
- 1 - signal - 3.6 ppm

more shielded, absorb at a higher field

less shielded, absorbs at a lower field
More electronegative atoms deshield more and give larger shift values.

Effect decreases with distance.

Additional electronegative atoms cause increase in chemical shift.
# Typical Values

<table>
<thead>
<tr>
<th>Type of Proton</th>
<th>Approximate $\delta$</th>
<th>Type of Proton</th>
<th>Approximate $\delta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>alkane ($\text{--CH}_3\text{)}$</td>
<td>0.9</td>
<td>$\text{\textgreater C=C\textless }$</td>
<td>1.7</td>
</tr>
<tr>
<td>alkane ($\text{--CH}_2\text{--}$)</td>
<td>1.3</td>
<td>Ph $\text{--H}$</td>
<td>7.2</td>
</tr>
<tr>
<td>alkane ($\text{--CH}\text{--})$)</td>
<td>1.4</td>
<td>Ph $\text{--CH}_3$</td>
<td>2.3</td>
</tr>
<tr>
<td>$\text{O}$</td>
<td></td>
<td>R $\text{--CHO}$</td>
<td>9–10</td>
</tr>
<tr>
<td>$\text{\textbar C\text{-CH}_3\text{)-}$}</td>
<td>2.1</td>
<td>R $\text{--COOH}$</td>
<td>10–12</td>
</tr>
<tr>
<td>$\text{\textbar C\equiv C\text{-H})}$</td>
<td>2.5</td>
<td>R $\text{--OH}$</td>
<td>variable, about 2–5</td>
</tr>
<tr>
<td>R $\text{--CH}_2\text{--X}$</td>
<td>3–4</td>
<td>Ar $\text{--OH}$</td>
<td>variable, about 4–7</td>
</tr>
<tr>
<td>($X = \text{halogen, O}$)</td>
<td></td>
<td>R $\text{--NH}_2$</td>
<td>variable, about 1.5–4</td>
</tr>
</tbody>
</table>

*Note:* These values are approximate, as all chemical shifts are affected by neighboring substituents. The numbers given here assume that alkyl groups are the only other substituents present. A more complete table of chemical shifts appears in Appendix 1.
1D & 2D NMR SPECTROSCOPY

- **INEPT (Insensitive Nuclei Enhancement by Polarization Transfer)**
- **DEPT (Distortionless Enhancement by Polarization Transfer)**
- **SEFT (Spin-echo Fourier Transform)**

Carbon status – primary, secondary, tertiary, quaternary

- **COSY** - Homonuclear correlation spectroscopy
  - Proton - proton correlations

- **HSQC** - Heteronuclear single quantum coherence
- **HMQC** - Heteronuclear Multiple Quantum Coherence
  - Carbon and Proton direct attachments

- **HMBC** - Heteronuclear Multiple Bond Correlation
  - Carbon and Proton on adjacent carbon (2 or 3) attachments

- **TOCSY (or) HOHAHA** - Total Correlation Spectroscopy
  - Carbon and Proton on neighboring carbon through hetero atom.
SUMMARY / CONCLUSIONS

Adsorption / affinity chromatography - Good separation technique for BAC

Flash chromatography is rapid, and reproducible

TLC is more actuate for the confirmation of the purity, fast rapid, reliable

HPLC - good tool for the quantification

2D NMR will help for the accurate assignments of NMR signals
OUTLINE

- Introduction
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OBJECTIVE

- Extraction of bioactive compounds from limes
- Purification of coumarins by flash chromatography
- Identification of isolated compounds by NMR and mass spectral analysis
EXTRACTION OF COUMARINS

Whole limes, juiced & freeze dried

Extraction with chloroform

Extract

Spent

Concentrated under vacuum
Sample Name: limepeel-nf
Date: 06 Mar 2008
RediSep Column: 12 g
Run length: 23 min
Detection wavelength (red): 210 nm
Flow Rate: 30 ml/min
Equilibration Volume: 101 ml
Initial Waste: 0 ml
Rack: 18 mm tubes
Peaks Tube Volume: 18.0 ml

Tube Collection Pattern: Standard Peaks collected only

Peak Detection
Slope-based
Sensitivity: High
Peak Width: 1 min
Threshold: 0.2 AU
Solvent A: (ACN, Reservoir A2)
Solvent B: (acetone, Reservoir B2)

Run Notes:
## FLASH FRACTIONS & COMPOUNDS

<table>
<thead>
<tr>
<th>Peak</th>
<th>Tube</th>
<th>Peak</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42 to 42</td>
<td>4</td>
<td>48 to 60</td>
</tr>
<tr>
<td>2</td>
<td>43 to 43</td>
<td>5</td>
<td>51 to 53</td>
</tr>
<tr>
<td>3</td>
<td>44 to 47</td>
<td>6</td>
<td>54 to 63</td>
</tr>
</tbody>
</table>

- **Compound 1**: 42 to 47
- **Compound 2**: 48 to 60
- **Compound 3**: 54 to 63
A). 3mM Phosphoric acid

B). ACN

$\lambda_{\text{max}}$ - 254 nm, 0.5ml/min

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>3mM Phosphoric acid (%)</th>
<th>Acetonitrile (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>45</td>
<td>55</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>30</td>
<td>25</td>
<td>75</td>
</tr>
</tbody>
</table>
HETERONUCLEAR MULTIPLE-QUANTUM COHERENCE (HMQC) SPECTRA OF COMPOUND 2 (DI METHOXYCOUMARIN)

Aromatic region

Aliphatic region
MULTIPLE-BOND CH CORRELATION (HMBC) SPECTRA OF DIMETHOXYCUMARIN
HETERONUCLEAR MULTIPLE-QUANTUM COHERENCE (HMQC) SPECTRA OF COMPOUND 3 (ISOIMPINELLIN)

Aromatic region

Aliphatic region
HETERONUCLEAR MULTIPLE-QUANTUM COHERENCE (HMBC) SPECTRA OF ISOIMPINELLIN

1H NMR

13C NMR
IDENTIFICATION EI/MS

MS- TOF analysis of 5,7-dimethoxycoumarin
MS-TOF analysis of Isopimpinellin

Isopimpinellin
exact mass

C13H10O5 – 246.2

m/z

Relative Abundance

100
90
80
70
60
50
40
30
20
10
0

100
200
300
400
500
600

188.03
160.03
175.06
69.05
81.08

Exact Mass 246.05

Exact Mass 231.04

Exact Mass 205.02

Exact Mass 189.01

Exact Mass 185.00

Exact Mass 183.00

Exact Mass 181.00

Exact Mass 169.00

Exact Mass 161.00

Exact Mass 159.00

Exact Mass 157.00

Exact Mass 155.00

Exact Mass 153.00

Exact Mass 151.00

Exact Mass 149.00

Exact Mass 147.00

Exact Mass 145.00

Exact Mass 143.00

Exact Mass 141.00

Exact Mass 139.00

Exact Mass 137.00

Exact Mass 135.00

Exact Mass 133.00

Exact Mass 131.00

Exact Mass 129.00

Exact Mass 127.00

Exact Mass 125.00

Exact Mass 123.00

Exact Mass 121.00

Exact Mass 119.00

Exact Mass 117.00

Exact Mass 115.00

Exact Mass 113.00

Exact Mass 111.00

Exact Mass 109.00

Exact Mass 107.00

Exact Mass 105.00

Exact Mass 103.00

Exact Mass 101.00

Exact Mass 99.00

Exact Mass 97.00

Exact Mass 95.00

Exact Mass 93.00

Exact Mass 91.00

Exact Mass 89.00

Exact Mass 87.00

Exact Mass 85.00

Exact Mass 83.00

Exact Mass 81.00

Exact Mass 79.00

Exact Mass 77.00

Exact Mass 75.00

Exact Mass 73.00

Exact Mass 71.00

Exact Mass 69.00

Exact Mass 67.00

Exact Mass 65.00

Exact Mass 63.00

Exact Mass 61.00

Exact Mass 59.00

Exact Mass 57.00

Exact Mass 55.00

Exact Mass 53.00

Exact Mass 51.00

Exact Mass 49.00

Exact Mass 47.00

Exact Mass 45.00

Exact Mass 43.00

Exact Mass 41.00

Exact Mass 39.00

Exact Mass 37.00

Exact Mass 35.00

Exact Mass 33.00

Exact Mass 31.00

Exact Mass 29.00

Exact Mass 27.00

Exact Mass 25.00

 Exact Mass 23.00

 Exact Mass 21.00

 Exact Mass 19.00

 Exact Mass 17.00

 Exact Mass 15.00

 Exact Mass 13.00

 Exact Mass 11.00

 RT: 0.56 AV: 1 NL: 2.20E8

 MS-TOF analysis of Isopimpinellin

 Foods for Health

 JRP-SAMPLE,S4 #129 RT: 0.56 AV: 1 NL: 2.20E8

 T: + c Full ms [50.00-600.00]
CRITICAL APPROACHES FOR THE ISOLATION & IDENTIFICATION

- Identifying the bioactive compounds (BAC) of our interest
- Understanding the interaction of BAC with sample matrix
- Stability of the BAC
- Optimization of extraction solvents
- Selection of purification methods, adsorbent, elution solvents
- Nature of compounds, MW, volatality, polarity of BAC
ACKNOWLEDGEMENTS

USDA-CSREES

Vegetable & Fruit Improvement Center | Texas A&M University System
Thank You