Introduction of Supercritical Fluids in your Workflow

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Natural Products

- Sample preparation
- Selectivity in extraction
- Easy analysis of obtained products

Extract profiling
- Screening and sample elucidation
- Research for markers
- Purification / isolation

- Finished product
- Quality control
- Consumer safety
Natural Products

SFx Technologies
using supercritical fluids
Natural Products

SFE  UPC²  MS

Prep SFC

UPC²  MS

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Why Consider Supercritical CO\(_2\) as Solvent?

- High efficiency extraction systems using CO\(_2\) as the main mobile phase have beneficial properties
- Superior solubility over traditional mobile phases for extraction
- Cost effective from extraction to compound isolation
  - Easier recovery
  - Less organic solvent consumption
  - Less disposal of solvents
  - Less steps in SOPs
  - Less post purification endeavor
  - Easier sample preparation (no derivatisation, solvents compatible as injection solvents)
- Green technique for sustainable growth
- No toxic solvents used - CO\(_2\) can be used with EtOH – organoleptic testing
Workflow
Sample Preparation

- Drying
- Grinding
- Soxhlet Extraction
- Distillation
- Evaporation
- SFE
- SPE
- Macro
- Micro
- Liquid / Liquid

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The Overview of SFE in Natural Product Research

- SFE is generally more selective than traditional solvent extraction, especially for target compounds of low to medium polarity.

- The sample complexity is minimized prior to chromatographic purification, hence simplifying and shortening the chromatographic analysis and purification.

- The target compound can be enriched prior to chromatographic purification, allowing for large sample loading and high purification productivity.
Control of Tunable Extraction Parameters Critical to Optimizing and Reproducibility

CO₂ tunable parameters and polarity for selectivity
Control of Tunable Extraction Parameters Critical to Optimizing and Reproducibility

CO$_2$ tunable parameters and polarity for selectivity
Extraction Sample and Effect of Increasing Density

- 100 Bar
- Isolated compound of interest
- 200 Bar
- 300 Bar

Extrait 1: 100 bars, 55°C, 1% MeOH, 30 min
Extrait 2: 200 bars, 55°C, 1% MeOH, 30 min
Extrait 3: 300 bars, 55°C, 1% MeOH, 30 min
Selective Extraction of Ingenol from *Euphorbia* Plant

Selectivity is key to efficient sample preparation

**Goal**

To selectively extract and enrich ingenol from a complex natural product matrix using SFE, thereby enabling further method optimization for downstream chromatographic analysis and purification.

**Background**

Natural products have been a highly productive source of leads for drug discovery and development. Unprecedented natural resources combined with technological advances in screening, separation, and synthesis, are driving the advent of new natural product drug discovery efforts. One of the key steps in natural product research is isolation from the complex matrix, which often starts at low concentrations and is hampered by complex sample matrices. This complexity presents a challenge for routine chromatography, especially preparative chromatography. A single compound and matrix can complicate the process for the analyst and reduce throughput. A repetitive process is then required until the desired amount of material is accumulated. The process is often time-consuming and labor-intensive.

Ingénol, shown in Figure 1, is a naturally occurring biactive compound being developed as a potential therapeutic agent for AIDS. Current ingenol isolation involves the extraction of *Euphorbia* plant latex using hexane, followed by chromatographic purification. The overall process suffers from extremely low yields arising from the sample complexity.
A General Workflow in Natural Products Isolation and Purification

Extraction

Purification

Simpler

Standard
SFx Technology Workflow

Slide design courtesy of Chris Hudalla, ProVerde Laboratories, USA

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Importance of analytical equipment

• Understand starting material

• Determination of extraction efficiency
  • Sonication with solvent of starting and end material and analyze

• Analysis of extracts

• Analysis of purified fractions

• Ensure product quality
Waters SFx Product Portfolio

SFE Extraction

*Instruments*
- MV-10
- SFE 100
- SFE 500
- SFE 1000
- SFE 2000
- SFE 5000
- BBES

SFC Analytical

*Instruments*
- UPC²

SFC Preparative

*Instruments*
- Prep 15
- Prep 80
- Prep 100
- Prep 200
- Prep 350

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Waters SFx Product Portfolio

SFE Extraction

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SFC Analytical

*Instruments*
- ACQUITY UPC²

SFC Preparative

*Instruments*
- Prep 15
- Prep 80
- Prep 100
- Prep 200
- Prep 350
Overview of Systems Available

SFE 100  
SFE 500  
SFE 1000  
SFE 2000  
BBES

Choosing the appropriate SFE system

Extraction vessel size

Start

Suggested sample size

100  <50g
500  50-250g
1000  250-500g
2000  500-1000g
5000  1000-5000g

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Bulk / Large Scale SFC Solutions

Investigator
1 mg – 10’s mg

Prep 80q
10’s mg – 10’s g

Prep 200q
g’s – 100’s g

Prep 350
100’s g – kg’s

Scale mgs to grams to kgrams
MS Directed Purification

Prep 15

Prep 100
What is SFC? Supercritical Fluid Chromatography? Convergence Chromatography?

- **SFC is like HPLC except the primary mobile phase is supercritical CO$_2$ instead of an aqueous solution**
  - CO$_2$ is much less polar than water (similar to heptane)
  - Elution order is reversed (non-polar compounds elute first)
  - Organic solvent still used as a strong solvent (e.g. methanol, ethanol)

- **Modern SFC is like normal-phase HPLC, but better**
  - Better reproducibility
  - Fully MS compatible
  - Highly orthogonal to RPLC separations
  - Much faster—no long equilibration times
  - Minimal use of toxic organic solvents
  - Amenable to gradients (not only isocratic)
- Built upon proven UPLC Technology
  - Quantifiable increase in productivity
  - Ultra-low dispersion enable the use of small diameter particles

- Exceptional increase in available selectivity
  - Solve routine and complex separation challenges
UPC²: Compatibility with all Waters MS Technologies

For ultimate CC-MS performance, ACQUITY UPC² System coupled with:

ACQUITY QDa
- Single quadrupole detector for robust and routine performance

Xevo TQ-S
- Ultimate sensitivity

Xevo G2-S Qtof and Synapt G2-S
- Qualitative and quantitative results from a single platform
SFC - Instrumentation

Similar to UHPLC - from hardware as well as operational standpoint

Waters ACQUITY UPC² and ACQUITY UPLC

ACQUITY UPLC  Xevo TQD  ACQUITY UPC²
SFC - Instrumentation

How does it work? What is different?

Mass Spec

Make-up Pump

Back Pressure Regulator (Dynamic and Static)

PDA detector

Column Manager

Auxiliary Inject valve

Inject valve

Thermo-electric heat exchanger

mixin

CO₂ Supply

CO₂ Pump

Modifier Pump

Waste

Modifier

CO₂ Supply

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Difference between SFE and SFC
When can UPC² be used?

- Chiral = no-brainer
- Compounds with no retention in RPLC
- Compounds degrading in H2O
- Orthogonal in comparison to C18
- Normal phase
- Structural Isomers
- Future scale-up
- When MS is needed on NP methods
Chiral separations with faster time to result and increased profit

230 nm
UPC² on AD-3
Gradient: 5-40% Isopropanol

NPLC on AD-H
Heptane/IPA (9/1), isocratic
Sample pooling and QDa for increased throughput and profitability in chiral analyses

Analyse 6 components in one screening
Use the power of QDa to distinguish compounds
Screening is shortened 6 times!

Courtesy of Alex Brien, Reach Separations, UK

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Pharma sample UCB on ACQUITY UPLC BEH C18 – 210 nm

Pharma sample UCB on ACQUITY UPC² BEH – 210 nm

Courtesy of David Clicq, UCB, Belgium
UPC²
Orthogonality with UPLC C18 methods

Genotoxic impurity analysis

UPLC
ACQUITY UPLC BEH C18

UPC²
ACQUITY TORUS 2-PIC

Additional peak in UPC²!

Courtesy of Alex Brien, Reach Separations, UK
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Problematic compounds in C18 methods
Can be analysed via UPC²

- UPLC – low retention + tailing
- UPC² – higher retention + sharp peaks

Separation of isomers

Courtesy of Arjen Gerssen, Rikilt, Netherlands
Broad Applicability of UPC²

Polarity limits of chromatographic techniques

- **Lipophilic**
  - Log P: 10
  - Lipids (triglycerides)
  - Liposoluble vitamins
  - Few plant components

- **Log P**
  - 5
  - Most of usual drugs

- **Hydrophilic**
  - Log P: -5
  - Metabolites
  - Amino acids/peptides
  - Nucleotides/Nucleosides
  - Antibiotics
  - Polysaccharides

**NPLC**
- Requires a dedicated LC system.
- Poorly MS compatible.
- Hazardous solvent.

**IEX**
- Requires a dedicated LC system.
- Poorly MS compatible.

**HILIC**
- Suitable till a certain polarity limit.
- High ACN consumption.
- Limited retention of polar and ionisable compounds.

**RPLC**
- Same instrument without drastic changes in analytical conditions.

**SFC**
- Polarity limit of SFC must be defined.

 Courtesy of A. Grand-Guillaume Perrenoud, D. Guillarme, Pr J-L. Veuthey, University of Geneva
UPC² - Synapt for Lipidomics

Our Workflow for Lipidomic Quantitation

Year 2014:

Chromatography – MS

- HILIC
- NP
- UHPSFC

- Special LC modes: RP, Ag, chiral, 2D;
  MS: ion mobility

Shotgun MS

- ESI-QqQ
- MALDI-Orbitrap

- PI, NL
- HR

MS/MS

Data processing

Multivariate data analysis

Biomarker discovery

Courtesy of Michal Holcapek, University of Pardubice, Czech Republic
Our Workflow for Lipidomic Quantitation

Year 2015:

Chromatography – MS

Shotgun MS

UHPSFC

ESI-QqQ

MALDI-Orbitrap

MS/MS

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Biomarker discovery

Courtesy of Michal Holcapek, University of Pardubice, Czech Republic
UHPSFC/ESI-MS of All Lipid Classes

Nonpolar lipids

• Separation of 30 polar and nonpolar lipid class standards within 6 min!

Polar lipids

~ Conditions: column UPC\(^2\) ACQUITY BEH (100x3 mm, 1.7 \(\mu\)m, Waters), positive-ion ESI-MS, 60° C, modifier MeOH/water (99:1) + 30 mM ammonium acetate, flow 1.9 mL/min, ABPR 1800 psi, standards of lipid classes

Natural products - cosmetics
Structure elucidation via UPC²-Synapt

SFC based extractome analysis

Courtesy of Alexandre Grand-Guillaume Perrenoud, University of Geneva & Nestlé, Switzerland
**UPC² - Xevo TQ-S for Polar doping agents**

### Tested Doping Agents

<table>
<thead>
<tr>
<th>S3 – Beta-2 Agonists</th>
<th>S6 – Stimulants</th>
<th>S6 – Stimulants</th>
</tr>
</thead>
<tbody>
<tr>
<td>bambuterol</td>
<td>amfepramone</td>
<td>benzphetamine</td>
</tr>
<tr>
<td>fenoterol</td>
<td>amphetamine</td>
<td>cathine</td>
</tr>
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<td>formoterol</td>
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<td>salmeterol</td>
<td>benzylpiperazine</td>
<td>methedrone</td>
</tr>
<tr>
<td>terbutaline</td>
<td>carphedon</td>
<td>epedrine</td>
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<tr>
<td>S5 – Diuretics</td>
<td>clophetorex</td>
<td>ethospermine</td>
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<tr>
<td>acitazolamide</td>
<td>cocaine</td>
<td>ethamivan</td>
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<td>fencamino</td>
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<td>fenetyline</td>
<td>fenbufibrate</td>
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<td>bumetanide</td>
<td>fenfluramine</td>
<td>fencamfamine</td>
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<td>canrenone</td>
<td>fenproporex</td>
<td>heptaminol</td>
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<tr>
<td>chlortalidone</td>
<td>furfenorex</td>
<td>p-hydroxyamphetamine</td>
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<tr>
<td>chlorothiazide</td>
<td>mfenorex</td>
<td>isomethestene</td>
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<tr>
<td>clopamide</td>
<td>mephenentermine</td>
<td>methsathinone</td>
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<tr>
<td>eplerone</td>
<td>mesocarb</td>
<td>MDMA</td>
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<tr>
<td>ethacrynic acid</td>
<td>methamphetamine</td>
<td>methylephedrine</td>
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<td>norfenfluramine</td>
<td>methylenidate</td>
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<td>xipamide</td>
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<table>
<thead>
<tr>
<th>S7 – Narcotics</th>
<th>S6 – Stimulants</th>
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</thead>
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<tr>
<td>buprenorphine</td>
<td>pseudoeohedrine</td>
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<td>dextromoramide</td>
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<td>sibutramine</td>
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<td>sufentanil</td>
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<td>methoxyamphetamine</td>
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<tr>
<td>morphine</td>
<td>6-hydroxybromantan</td>
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<td>oxycodone</td>
<td>chlorphentermine</td>
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<td>oxymorphone</td>
<td>methoxyphenamine</td>
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<td>pethidine</td>
<td>N-ethylnicotinamide</td>
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<td>codeine</td>
<td></td>
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<td>hydrocodone</td>
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<tr>
<td>6-acetylmorphine</td>
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<table>
<thead>
<tr>
<th>S1 – Anabolic Agents</th>
<th>S4 – Hormone and Metabolic Modulators</th>
</tr>
</thead>
<tbody>
<tr>
<td>androstatriendione (ATD)</td>
<td>aminoslotethimide</td>
</tr>
</tbody>
</table>


**Courtesy of Lucie Novakova, Charles University, Czech Republic**
UPC² - Xevo TQ-S for Polar doping agents

(2) ANALYSIS OF RELATIVELY POLAR DOPING AGENTS

UHPLC-MS/MS

Micrónica content [%]

ESI POSITIVE ▲ ESI NEGATIVE

UHPSFC-MS/MS

CO₂

MeOH content [%]

ESI POSITIVE ▲ ESI NEGATIVE

Courtesy of Lucie Novakova, Charles University, Czech Republic
Cosmetic industry

Sunscreens

The most frequently used sunscreens

Courtesy of Maria Khalikova, University of Pardubice, Czech Republic
Cosmetic industry
Sunscreens

BZ3, IMC, MBC, DHHB, OCR, EDP, BDM, EMC, HS1, ES, HS2, DBT, ET, DRT, MBP, EMT

HPLC-UV
C18 125 x 4 mm, 5 μm, T=60 °C
gradient EtOH:water (cont. 1% FA and 20 mM of 2-hydroxypropyl-cyclodextrin)

HPLC

A.Chisvert et al. / Analytica Chimica Acta 790 (2013) 61–67

Courtesy of Maria Khalikova, University of Pardubice, Czech Republic
Cosmetic industry
Sunscreens

High reproducibility
Method is validated

Courtesy of Maria Khalikova, University of Pardubice, Czech Republic
Supercritical fluid chromatography for GMP analysis in support of pharmaceutical development and manufacturing activities

Michael B. Hicks, Erik L. Regalado, Feng Tan, Xiaoyi Gong, Christopher J. Welch

REPRODUCIBILITY

Fig. 4. Evaluation of the retention and peak area reproducibility performance of the UPC² SFC system for analyzing 96 injections of MR-2 using experimental conditions described in the experimental section.

INCREASED RESOLUTION
CONCLUSIONS

- Reduce Solvent Costs
- Increase Speed & Throughput
- Enantiomer isomer Separations
- Simplify Workflow
- Orthogonality
- Issues with Range of Polarity
- Structural isomer separations
- Selectivity
- Green Chemistry

SFC/UPC²/SFE
Thank You!

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- Arjen Gerssen, Rikilt, Netherlands
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You for your attention!