A Novel Technique to Eliminate the Bottle Neck and Problems Associated with Concentrating Collected Preparative Fractions

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Preparative chromatography is the predominate method employed when purifying compounds in pharmaceutical and biotechnology research. However, preparative chromatography is not without issues that plague researchers.

Namely, once the sample has been purified there is the challenge of what to do with the fractions. The purified sample may be in several collected fractions or be in such a large volume that the solvent needs to be removed before the sample can be processed or stored.

This usually involves evaporating the solvent from the sample at slightly elevated temperatures. Evaporation not only takes time 4-12 hours but the temperature and the use of modifiers in the mobile phase (TFA) that concentrate during the dry down process, leading to hydrolysis of functional groups and product degradation.
An alternative to evaporation of collected fractions is to employ the use of SPE (solid phase extraction) cartridges to absorb and concentrate the purified compound in the fraction.

The (SPE) cartridge can be dried for storage or eluted with a small amount of organic solvent for storage or quick dry down without aqueous or modifier complications. Several aspects associated to this approach were investigated, break-through monitoring, peak trapping conditions, lowering back pressure over the capture cartridge, and compound recovery.
Evaluation of concept

- Sample used polar/nonpolar
- Sample size (mg/ml)
- Fraction conditions/volume
- Dilution of collected fractions
- Prep of SPE cartridges
- Breakthrough past info
- Elution
- Verification
Prep LC-FC-SPE System

- GX-281 Liquid Handler, injection and collection capabilities
- Prep HPLC: 30 ml/min, Phenomenex Luna C-18(2) 21.2 x 150 mm, 5 micron, 100 angstrom
- Sample: Rosmarinic acid/Carvacrol, 50 mg/ml @/1 ml injection
- Collection system: Code 307, 6 ml cartridges, 15 x 45 ml collection vials (4 ml)
- 6 ml SPE: Strata, C-18-E (55um, 70A)1000mg/6ml; 20/rack
Options for Collection of Fractions:

• **Basic Approach:** Collect fractions based on collection criteria, pool all fractions for a peak, remove solvent from fraction, rotovap or evaporation with heat *required*

• **On-Line Collection:** Eluent from the column is connected to a dilution pump (water) before the detector and collection cartridge, decreasing the % organic so that the compound will absorb to the cartridge (non-eluting solvent conditions)

• **Off-Line Collection:** Determine fractions, liquid handler dilutes and mixes fraction with water, introduces each fraction to a conditioned SPE on the bed, dries the SPE with N₂, elutes pure compound with 3-4 mls of ACN, storage of sample or quick dry down no heat required
Evaluation of New Collection Concepts;

On-Line Collection:

- Flow rate through an analytical column is in the 1-4 ml/min range
- C-18 stationary phase columns use mobile phases containing a significant percentage of organic solvent (5 – 95%)
- It is important to include a diluting pump between the analytical column and the collection cartridge in order to establish non-eluting solvent conditions
- A 1:15 ratio of column flow rate to dilution pump is required to obtain non-eluting solvent conditions over the entire gradient range
• Total flow rate through the collection cartridge will be in excess of 16 ml/min
• At these flow rates, the force of the mobile phase through the collection cartridge alone can possibly cause break-through (1)
• Pressure generated over the cartridge will often exceed the maximum limits of the pumps and detector flow cell (2)
• Operating at lower flow rates is a possibility, but the run time may become 5-10 times longer
• In Preparative Mode: On-Line Dilution becomes a researchers worst nightmare, at a 10 ml/min flow rate the dilution pump would have to be pumping at 150 ml/min, which in turn means that the collection cartridge is exposed to 160 ml/min flow rate, break-through is inevitable not to mention channeling and cartridge damage

Graph 1: Represents break-through of caffeine from a C-18 collection cartridge, 6 ml, after 3 injections, 4 ml/min flow rate, 60 ml/min dilution flow rate, 64 mls/min flowing through SPE cartridge

Black: Analytical injection of caffeine 10 mg/ml, 20 ul injection
Blue: Break-through of caffeine after 1 collection
Red: Break-through of caffeine after 3 collections (> 50% loss)
Selective approach to Collection:

- On-Line collection and concentration of fractions via a collection cartridge is an alternative approach to basic fraction collection
- Characteristics of this approach relative to flow rate, sample load and pressure limit its viability for preparative purifications

- ALTERNATIVE APPROACH:
  - Off-Line Collection:
    - Allow basic preparative fraction collection
    - Control of fractions volumes collected
    - Possibility of more than one fraction per peak
    - Cherry pick the fractions of interest
    - Activate SPE cartridge
    - Elute purified compound in small pure organic
Off-Line Collection Approach:

- Preparative purification chromatography
- Collect fractions based on collection conditions, limiting volume per tube
- “Cherry Pick” fractions to be loaded onto the SPE cartridges, for concentration and aqueous removal
- Dry the SPE cartridges with N₂
- Elute the concentrated compound off the SPE cartridge with 100% ACN (3-4 mls)
- Quick dry removal of ACN without heat
Preparative LC-FC-SPE:

- The preparative chromatogram represents the separation of Rosmarinic acid and Carvacrol, 50 mgs each on column, 100 mgs total, 30 ml/min flow rate, 21.2 x 150 mm Luna C-18(2)

- Fractions were collected based on level with a 4 ml volume limit per tube*

- Based on predetermined criteria or visual observation the sample list is populated for SPE cartridge evaluation, compound concentration and aqueous removal
Off-Line Collection:

Activation and Loading of SPE cartridges:

- SPE cartridges are conditioned prior to sample loading based on manufactures requirements
  - 15 mls of Methanol
  - 15 mls Water

- Selected fractions are diluted with 20 mls of water/mixing

- Diluted fractions are dispensed into SPE cartridges, dried and eluted with ACN
Evaluating SPE Break-Through;

As the diluted preparative collected fraction was introduced to the SPE cartridge each 5 ml volume was captured to determine compound break-through. The data is consistent with several evaluations within/between unique runs. The chromatographic overlays represent the 4 separate collections of the fraction introduced to the SPE cartridge.
Recovery via SPE:

A 1 ml injection of the eluent from the SPE cartridge demonstrates that the SPE cartridge is very capable of retaining, concentrating and removing the aqueous portion from the collected fraction.

Recoveries: Rosmarinic Acid: 96-99%

Carvacrol : 95-101%
Recovery via SPE:

- 1 week later

The SPE cartridges were stored after Nitrogen dry down for one week. After the week the SPE cartridges were eluted as previously stated with ACN. There was no loss of compound recoveries being the same as stated in a prior slide Rosmarinic acid: 96-99% and Carvacrol 95-101%
Cost Assessment of the Off-Line Collection Approach:

- No additional instrument investment
- No manual intervention
- Cost per SPE cartridge; $2-3.5
- SPE cartridges can be reused
- Approximately 2.5 hours per 20 fractions, which includes all conditioning steps for the SPE cartridges, a slow diluted fraction addition and the N₂ dry down (1 minute per SPE)
Alternative SPE:

- Concerns have been raised regarding extremely polar compounds and the ability of SPE cartridges to efficiently absorb and concentrate the diluted fraction.
- Porous carbon material packed in the SPE drastically increases the efficiency of the SPE for very polar compounds, HyperCarb.
- A wide range of SPE cartridges in various dimensions and packing material are available offering numerous options for trapping diluted fractions.
Summary and Conclusions;

• Alternative approaches to basic preparative collection of fractions and evaporation are appealing, because......
• Evaporation/heat and time associated with fractions containing purified samples may effect total recovery (decomposition/hydrolysis)
• Allowing fraction collection within a preparative run is basic
• Implementing automated post collection options eliminates possible degradation of purified compound
• The entire off-line dilution collection via SPE is automated and can be completed on the bed of the GX-281, hence injection, fraction collection and off-line dilution collection does not require any additional instrument investment
• Recovery for the two compounds Rosmarinic acid and Carvacrol were excellent > 95% and no degradation was observed
• There was no observed break-through of the compounds when they were exposed to the SPE cartridge
• The rate of diluted fraction addition to the SPE is controlled by the liquid handler drastically minimizing or eliminating channeling and pressure issues within the SPE cartridge
• The SPE cartridges containing the concentrated compound can be used as a storage device for the compound, eluting can occur at a later time, minimizes the hazard associated with transporting liquids between lab or to different sites
• No apparent compound degradation or loss was observed when the SPE cartridge was stored and eluted days later

References: