APPLICATION SOLUTIONS

FOR PREPARATIVE-SCALE CHROMATOGRAPHY

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APPLICATION SOLUTIONS FOR PREPARATIVE-SCALE CHROMATOGRAPHY

VERSATILE PURIFICATION AND ISOLATION SOLUTIONS

Preparative-scale chromatography plays a critical role in applications where compounds must be synthesized, identified, isolated, purified, characterized, screened, and tested. Purification is used to isolate compounds for drug discovery experiments, to isolate unknown impurities or metabolites, and to purify standards, natural products, and biomolecules. Criteria for performing purification, such as experimental conditions and column and instrument requirements, can vary greatly due to both the number of samples and the amount required.

Waters takes complexity out of purification with instrumentation, columns, and software that enable you to more easily purify milligrams to multiple grams, in an integrated system that can be configured to automatically process hundreds of samples. You benefit from high purity and recovery of desired compounds – with minimal system intervention.

In this application compilation, see how our versatile purification systems can improve your laboratory's efficiency, throughput, and productivity.

With Waters purification systems, your lab is ready for tomorrow's analyses

Waters offers scalable systems for purification, from the AutoPurification™ System for high-throughput, mass-directed fraction collection from hundreds of samples, to simple low-pressure mixing systems for purifying a few samples a day.

Waters' robust and reliable systems improve your ability to manage increasing workload demands, including unattended instrument operation.

- Flexible configurations enable scale-up from analytical to preparative chromatography.
- Optimum Bed Density (OBD™) Columns offer the highest sample loading and unmatched column stability for preparative chromatography.
- User-friendly console and software features help you manage solvents and samples, whether you do manual or automated injections.
- Remote system status monitor tracks sample queue, instrument, and solvent and waste container status online, giving you confidence for unattended operation.
- FractionLynx™ Software simplifies fraction collection, automating the collection of detected fractions, tracking samples and fractions, and then presenting the data in an easy-to-view format. The software can trigger collection using a variety of detector signals including UV/Visible, evaporative light scattering (ELS), mass detection (MS), and analog.
- With Open Access Software, chemists can walk up to a system, enter their sample, and have results delivered to their desktop, while an administrator monitors the system and manages instrument access.
- Easy-to-upgrade systems enable your lab to grow while using the most appropriate purification functionality and capacity to meet your application's requirements.

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UV-Directed Purification of a Small-Scale Organic Synthesis

Andrew Aubin
Waters Corporation, Milford, MA, USA

INTRODUCTION

During the drug discovery process, organic compounds are often synthesized and then isolated from reaction mixtures. These isolated compounds are seldom pure – they are usually contaminated with reaction precursors, small amounts of similar compounds, and reaction by-products formed during the reaction.

In order to characterize these compounds or use them for other purposes, it is necessary to purify them. The purification process may use any number of techniques (liquid/liquid extraction or recrystallization, for example). These techniques are often slow and not easily automated.

Liquid chromatography can be used to purify compounds from these reaction mixtures if classical techniques are not entirely successful, not desirable, or if a high level of purity is desired.

This application note describes the small-scale purification of a synthesized drug product, acetylsalicylic acid, Figure 1, using preparative-scale liquid chromatography. A simple technique for determining suitable separation conditions will also be described.

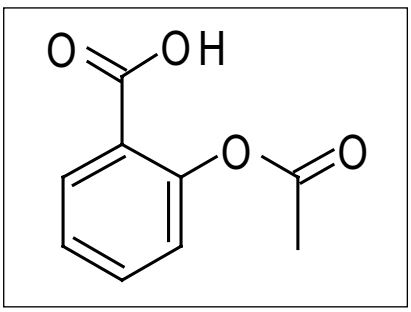

Figure 1. Acetylsalicylic acid.

EXPERIMENTAL

Analytical LC conditions

LC system:	Alliance® HPLC System
Detector:	PDA Detector
Column:	XTerra® RP18, 4.6 x 100 mm, 5 µm
Column temp.:	Ambient
Flow rate:	1.5 mL/min
Mobile phase A:	Water
Mobile phase B:	Acetonitrile
Mobile phase C:	2% Formic acid in water
Data collection:	Empower™ 2 Software

Preparative LC conditions

LC system:	Waters Purification System
Pump:	2545 Quaternary Gradient Module
Injector:	FlexInject, 10 mL loop
Collector:	Fraction Collector III
Detector:	2489 UV/Vis @ 280 nm (semi-prep flow cell)
Column:	XTerra Prep, 30 x 150 mm, 5µm
Column temp.:	Ambient
Flow rate:	64 mL/min
Mobile phase A:	Water
Mobile phase B:	Acetonitrile
Mobile phase C:	2% Formic acid in water
Data collection:	MassLynx™ Software with FractionLynx™ Application Manager

Figure 2. The Waters UV-directed purification system.

Preparative chromatography system

The preparative chromatography system (Figure 2) consisted of the 2545 Quaternary Gradient Module, a low-pressure mixing solvent delivery module capable of flow rates up to 150 mL/min; the 2489 UV/Visible Detector; the Fraction Collector III; and the FlexInject Manual Dual Injector Module.

The system was controlled using MassLynx Software with the FractionLynx Application Manager. FractionLynx controls fraction collection triggering, tracks samples, fractions, and associated data through its easy-to-use browser.

This preparative LC system configuration is designed to purify a few fractions a day, and, since Waters' versatile purification systems are upgradeable, the system can easily be expanded as laboratory workloads increase.

Synthesis

Acetic anhydride (1.5 mL) was added to 1.0 grams of salicylic acid along with one drop of concentrated sulfuric acid. The entire mixture was placed in a water bath at 55 °C for 30 min with occasional stirring. The mixture was cooled to room temperature and the resulting crystals washed with water (~ 200 mL). The washed crystals were dissolved in 2.0 mL of dimethyl sulfoxide, of which 5.0 µL of were removed and diluted to 1.0 mL in methanol; this solution was used for HPLC method development. The remaining DMSO solution was set aside for preparatory HPLC.

RESULTS AND DISCUSSION

To determine the optimal separation conditions for the preparative purification, a series of four analytical separation scouting runs were performed (Figure 3). Each gradient separation used the same starting conditions (85% A, 10% B, and 5% C) and the same gradient time (10 min). Organic solvent content was varied in each of the four runs over the gradient time:

Run 1, 10% B to 90% B

Run 2, 10% B to 75% B

Run 3, 10% B to 50% B

Run 4, 10% B to 25% B

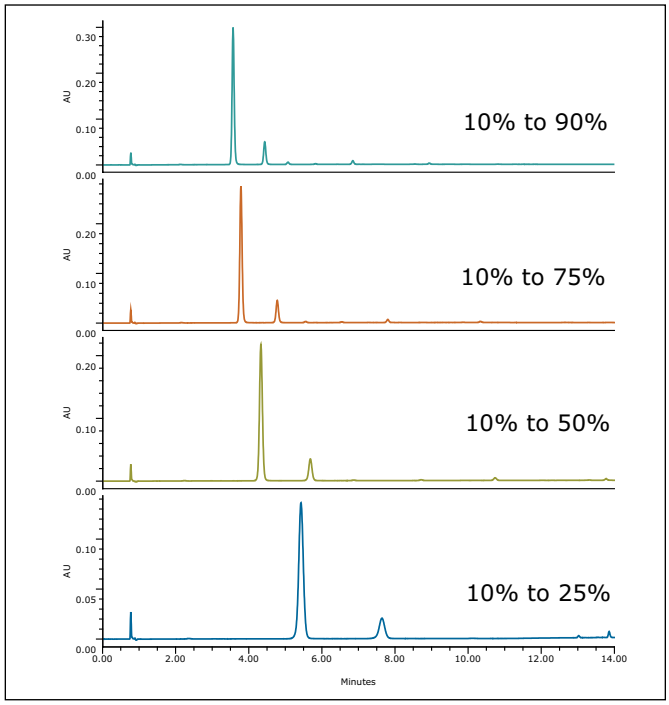

Figure 3. Results from the four analytical scouting runs.

Comparison of the four chromatograms (Figure 3) shows that the fourth run provided maximum resolution for the two main peaks in the mixture, allowing for higher sample loads and ultimately higher yields. Based on these runs, calculations showed that the crude mixture had an approximate purity of 74%, based on UV area %. In cases where the four scouting runs do not provide suitable resolution, data from those runs could be modeled using chromatography modeling software such as Molnar-Institute's DryLab. This data was also used to determine the experiment's optimal detection wavelength, which was determined to be 280 nm.

The analytical method was scaled to preparatory using the Basic Gradient Scaler function of the Waters Prep Calculator (Figure 4), generating a gradient table appropriate for the 30 x 150 mm preparatory column (Table 1). The fraction collector was set up to receive the collected fractions into flasks. Utilizing the FractionLynx Application Manager, fraction collection was triggered based on UV (280 nm) signal, and subsequently stopped when the UV absorbance reached 0.13 AU. These values can be adjusted as required.

Time (min)	Flow (mL/min)	% A	% B	% C
0.0	64	85	10	5
15.0	64	70	25	5
22.5	64	5	90	5
24.0	64	85	10	5

Table 1. The prep-scale gradient table.

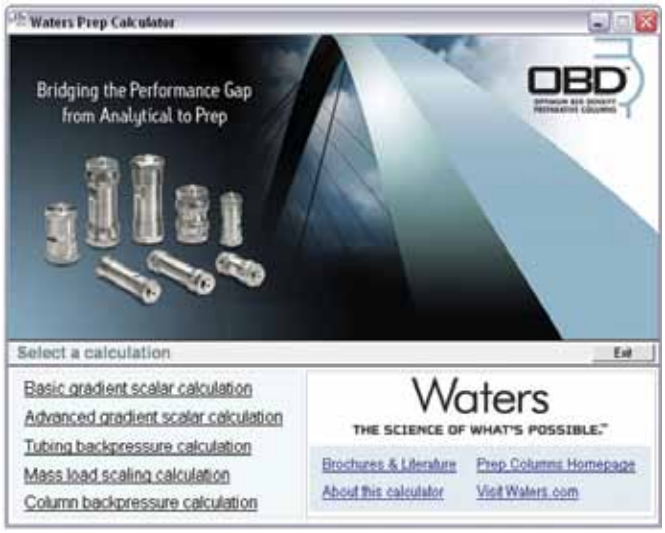

Figure 4. Waters Prep Calculator Software, ***www.waters.com/prepcalculator****.*

The entire solution generated from the synthesis was injected onto the preparatory column (Figure 5). The target peak (Peak 1) was collected from 4.5 to 7.6 minutes. Peak 2 was known to be salicylic acid and was discarded post-collection. Immediately following collection, a small portion of the peak 1 fraction was removed and analyzed for purity (Figure 6). The purity of that collected fraction, based on UV area %, was calculated to be >99.9%. The fraction was dried down and yielded 526 mg of crystalline material. As a final confirmatory check, a small portion of the purified crystals were analyzed and found to have a purity of >99.5%.

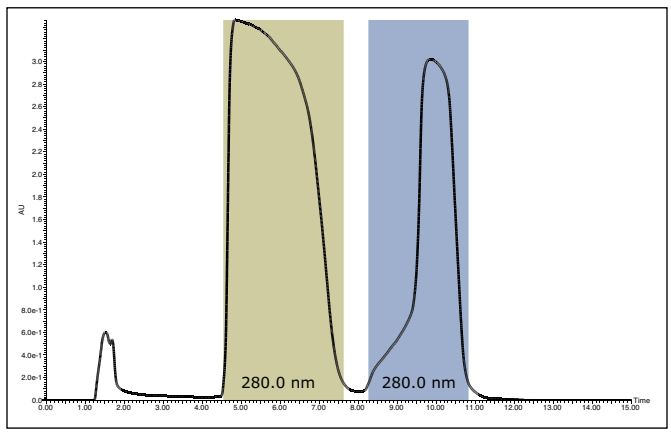

Figure 5. UV-directed purification of organic synthesis mixture. The shaded area represents the collected peak fractions.

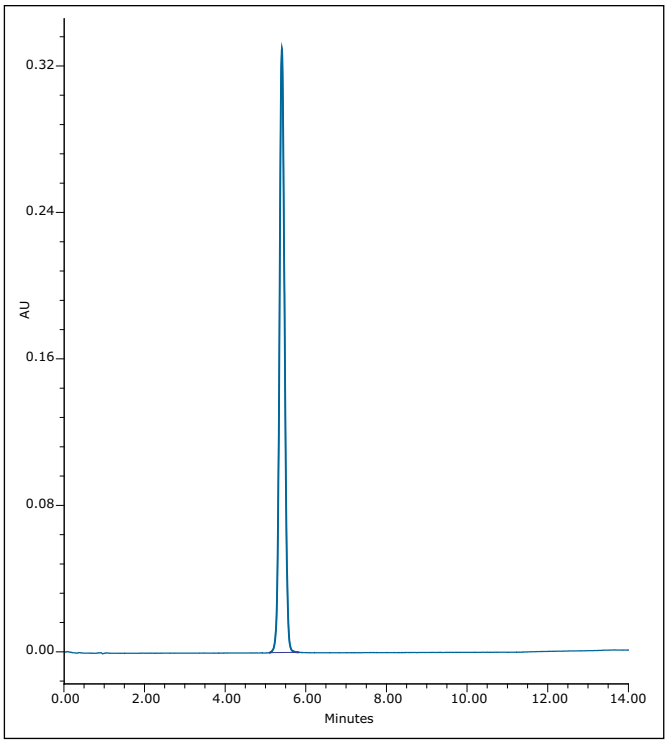

Figure 6. Purified fraction of acetylsalicylic acid.

CONCLUSION

- A simple, easy-to-use system for the purification of a small-scale organic synthesis mixture was described. The system consisted of the 2545 Quaternary Gradient Module, a manual injector, the 2489 Dual Wavelength Detector, a Waters Fraction Collector III, all controlled by MassLynx Software running the FractionLynx Application Manager.
- An increase in purity from 74% to greater than 99% was accomplished using UV-directed purification for the isolation of acetylsalicylic acid from a synthesis mixture.
- A total of 526 mg of acetylsalicylic acid was isolated.
- The straightforward purification and isolation possible by this preparative LC system enables discovery laboratories to subsequently perform further characterization of such a compound, or use it for another purpose. Additionally, the system configuration is easily expandable as the number of fractions that need to be purified increases.

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Waters Corporation
34 Maple Street
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T: 1 508 478 2000
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Preparatory Chromatography of Natural Product Extracts Utilizing a UV-Based Open Access Walk-Up Purification Strategy

Andrew Aubin, Ronan Cleary, and Darcy Shave
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

This application note describes techniques to simplify the choosing of an appropriate first-try purification method for a chemist's raw extract using an Open Access purification system. Simple method selection criteria (based on TLC analysis) allow separation methods to be tailored to an extract, as opposed to a single generic method for all extracts. This allows chemists to reduce the number of re-analysis and re-purification steps; ultimately, speeding up the entire purification process.

WATERS SOLUTIONS

Component-based preparative HPLC modules

MassLynx™ Software

FractionLynx™ and Open Access application managers

SunFire™ Prep C_{18} Column with OBD™ Technology

KEY WORDS

Schisandra, Cinnamon, Kudzu, Open Access, Preparatory chromatography, FractionLynx Application Manager

INTRODUCTION

Extraction of active medicinal compounds from plant materials is accomplished in many diverse ways. Ultimately, the chemist is left with a raw extract that contains potentially active compounds along with many other non-active constituents. Following extraction, the next logical step is purification or isolation of those compounds of interest. Preparatory liquid chromatography is often utilized to accomplish this task.

The diversity of compounds that may be extracted is large and an adequate purification requires access to different chromatographic methods for effective isolation. An Open Access liquid chromatography system that may have many methods available for use by walk-up chemists is, therefore, desirable. Methods can vary in column size, run time, gradient conditions, solvents, modifiers, and other variables. These options can make it difficult for a chemist to choose the best method for their extract and delay the isolation and development of the compounds of interest.

To simplify the choosing of an appropriate first-try method for each chemist's extract, an Open Access system using simple method selection criteria (based on TLC analysis) is described.

By using separation methods tailored to their extracts, chemists are better able to obtain higher purity compounds. By selecting a suitable method, as opposed to a single generic method for all extracts, chemists essentially reduce the number of re-analysis and re-purification steps; ultimately, speeding up the entire process.

EXPERIMENTAL

Samples and Extraction

Three different plant materials were extracted for the trial; ground schisandra berries *(Schisandra Chinenis)*, cinnamon pieces *(Cinnamomum Zeylanicum)*, and kudzu root powder *(Pueraria lobata)*. 10 grams of dry sample were shaken for one hour with a 50-mL mixture of 60:40 methanol/water. Extracts were centrifuged and the clear extract used without further manipulation. No attempt was made to optimize the extractions.

Thin Layer Chromatography

Extracts were separated on 5 x 20 cm silica gel TLC plates (Partisil LK6F Silica Gel 60A with fluorescence indicator, Whatman, Maidstone, England) using a mixture of hexane and ethyl acetate. Following development, samples were visualized by exposing the plate to UV light and observing the spots. Rf values were generated from these observations.

Preparative Liquid Chromatography

Preparatory HPLC analyses were performed using a system consisting of a Waters® 2545 Quaternary Gradient Module, a 2489 UV/Visible Detector, a Preparative Chromatography Rack, and a Fraction Collector III using a SunFire Prep C_{18} OBD, 5 µm, 19 x 100 mm Column. The entire system was controlled using MassLynx Software with both FractionLynx and Open Access application managers. Method parameters, mobile phase components, and gradient conditions are outlined in Table 1, shown on page 3.

RESULTS AND DISCUSSION

To simplify the process of deciding "Which separation method should I use?", users must first gather some information about the sample. Ideally, a series of analytical scale HPLC separations would be run and a preparatory method derived, developed, and scaled up from these results. In many cases, this level of detail is too time-consuming and unnecessary. This is particularly true when dealing with one-time extracts in the earliest stages of investigation.

A simpler approach is to conduct an initial evaluation using thin layer chromatography, evaluate the results, and create an appropriate preparatory LC method based on the calculated Rf value of the compound of interest.

In this example, extracts were first analyzed by TLC as shown in Figure 1. With the TLC results, users can either make a method with specific separation characteristics or use a method already created on the HPLC system, as shown in Table 2.

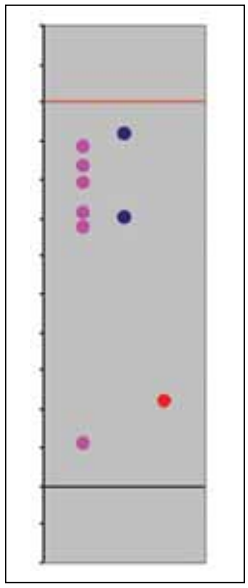

Figure 1. TLC results for (left to right), schisandra berry, cinnamon, and kudzu extracts. Spots are indicative of UV active compounds, polar compounds toward the bottom, non-polar compounds toward the top.

Gradient 1	Separation gradient 1 is for use when the Rf is less than 0.50
Gradient 2	Separation gradient 2 is for use when the Rf is greater than 0.50 and less than 0.75
Gradient 3	Separation gradient 3 is for use when the Rf is greater than 0.75 and less than 1.00

Table 2. Preparative gradients based upon Rf values.

Gradient One			
Time	%A	%B	%C
0	85	10	5
2	85	10	5
10	45	50	5
12	0	95	5
14	0	95	5
16	85	10	5

Gradient Two			
Time	%A	%B	%C
0	85	10	5
2	65	30	5
10	25	70	5
12	0	95	5
14	0	95	5
16	85	10	5

Gradient Three			
Time	%A	%B	%C
0	85	10	5
2	35	60	5
10	0	95	5
12	0	95	5
14	0	95	5
16	85	10	5

Flow:	25 mL/min
Temp.:	Ambient
A:	Water
B:	Methanol
C:	2% Formic acid in water
Detection:	UV @ 254 nm
Injection vol.:	1 mL

Table 1. Preparative chromatography method parameters.

To further simplify the HPLC purification process, method selection can be simplified using a single Open Access login page as displayed in Figure 2. After clicking the login button, users simply inject the sample via the manual injector. Results can be printed or emailed to users following completion of the run.

Gradients were designed to ensure that even if users choose a less-than-optimal method, all potential compounds of interest were eluted from the column and collected. An example of this can be seen in Figure 3 where a chromatogram shows the cinnamon extract separated using gradient 2. Two very late eluting compounds were collected long after the compound targeted by TLC.

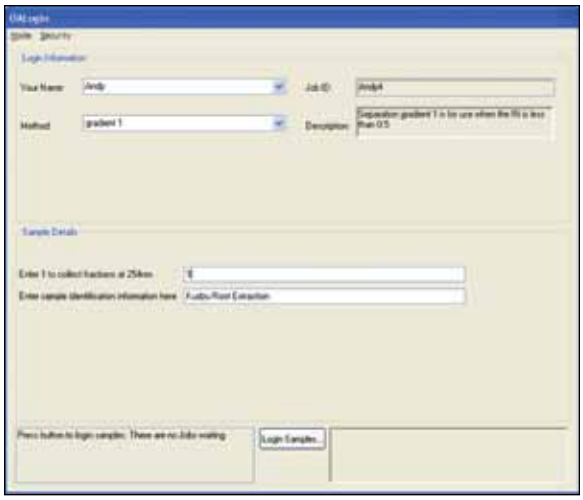

Figure 2. Open Access login page.

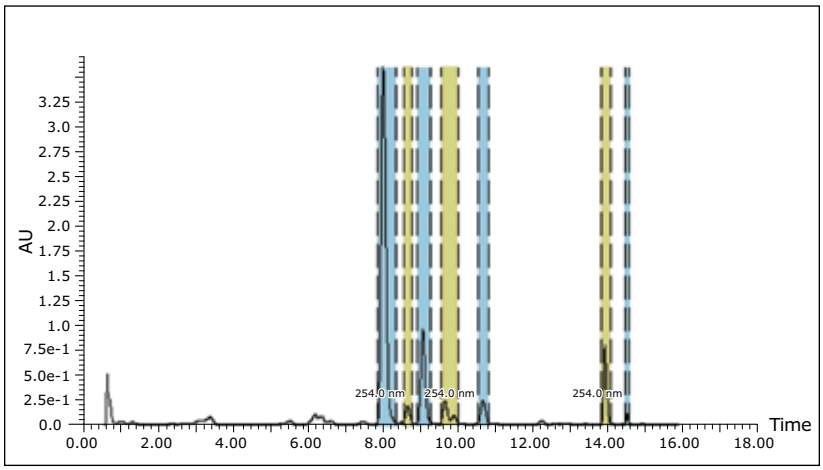

Figure 3. Cinnamon extract separated using gradient 2.

Figures 4 and 5 show both the kudzu root extract and schisandra berry extract separated using the described techniques. Chromatograms from the three natural product extracts are similar to TLC plate results, demonstrating that this progression of techniques provides a suitable workflow. As soon as the analysis is completed, sample results are emailed to users or printed as desired. System configuration and setup is enabled through a System Administrator who determines login access, method selection, and report generation. The System Administrator can also increase or decrease the number of methods available for analysis selection. The 2545 Quaternary Gradient Module allows four-solvent selection, providing the availability for selection of multiple buffers or alternative organic modifiers. The Preparative Chromatography Rack conveniently holds multiple columns and a pair of injectors to increase flexibility for the method selection choice.

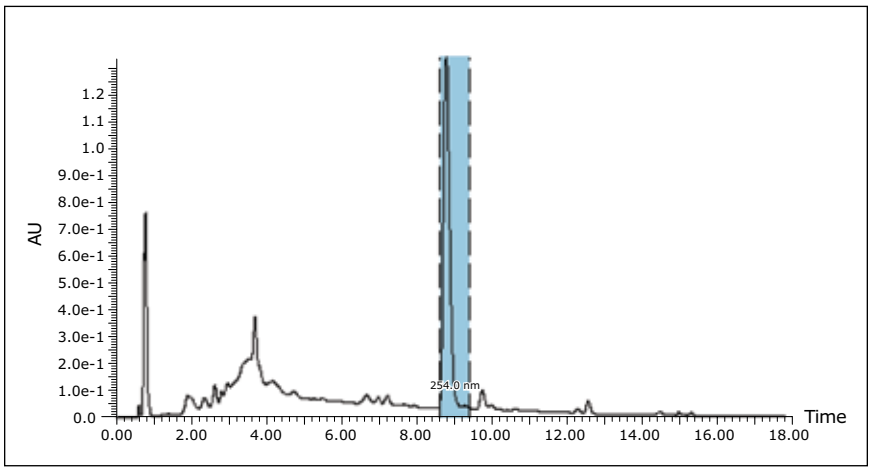

Figure 4. Kudzu root extract separated using gradient 1.

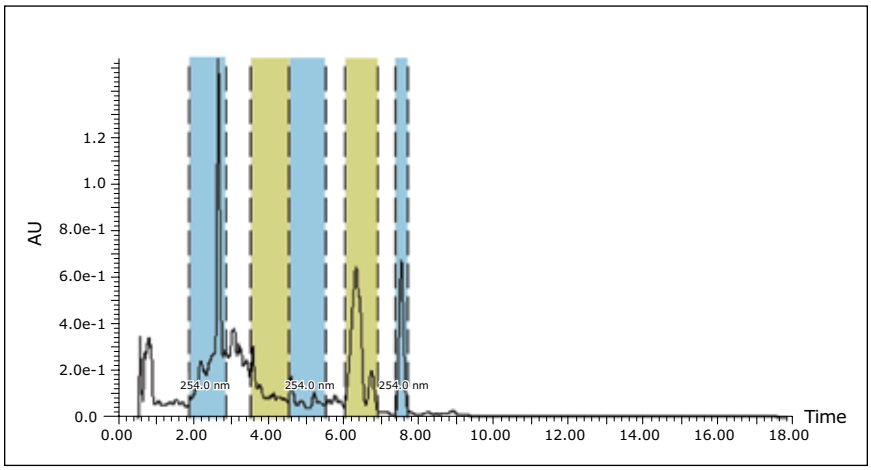

Figure 5. Schisandra berry extract separated using gradient 3.

As a final confirmation of the system functionality, the fraction collected from the kudzu root extract was re-analyzed using an analytical HPLC system, as shown in Figure 6. The results show a clean, single peak that was isolated and collected from the kudzu root, which can move to the next steps in the investigative process.

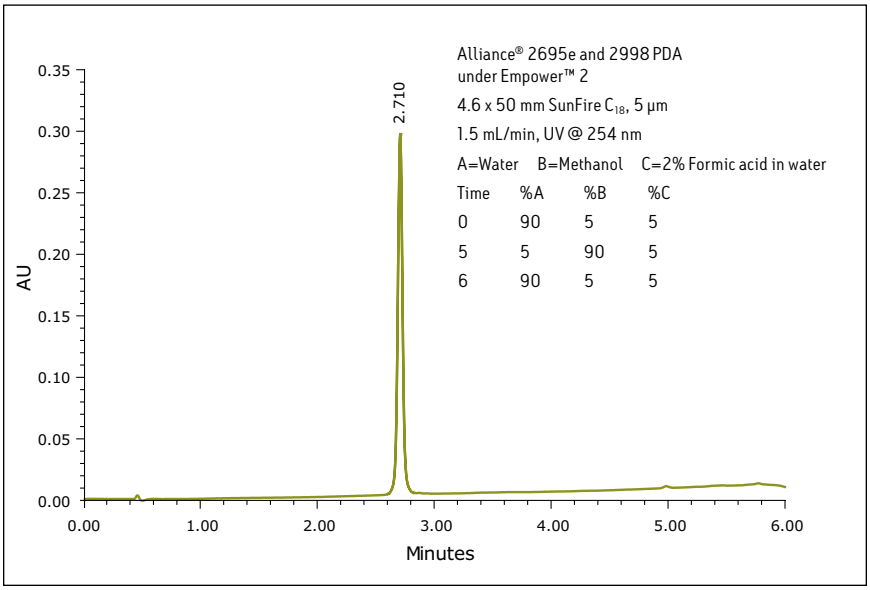

Figure 6. Single fraction collected from kudzu root extract separated using gradient 1.

CONCLUSIONS

- Waters Open Access systems give chemists the ability to analyze their own samples close to the point of production by simply walking up to the LC system, logging their sample information, injecting their samples, and walking away.
- Method parameters can be customized to suit the needs of the individual laboratory workflow.
- As many methods as required can be presented to an individual chemist, optimizing the collection of potential active compounds.
- By using methods tailored to their compounds, chemists are able to obtain higher quality fractions from their mixtures in the shortest possible time; thereby, speeding up the investigation process.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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A Modular Preparative HPLC System for the Isolation of Puerarin from Kudzu Root Extracts

Andrew Aubin and Ronan Cleary
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

A modular preparative HPLC system provides flexibility without compromising performance for isoflavone isolation. Empower™ 2 Software offers total system control including fraction collection.

WATERS SOLUTIONS

Component-based preparative HPLC modules

Empower 2 Software

SunFire™ Columns

KEY WORDS

Preparative HPLC, Kudzu, Puerarin

INTRODUCTION

Kudzu (*Pueraria lobata*) is a climbing, woody, or semi-woody perennial vine with a tuberous root. The roots of kudzu contain a number of potentially useful isoflavones, including daidzein, daidzin, genistein, genistin, and quercetin. Kudzu is also a unique source of the isoflavone puerarin as shown in Figure 1. Consumption of kudzu root extracts is thought to reduce alcohol intake and reduce alcohol withdrawal symptoms. Antibacterial, anti-cancer, anti-inflammatory, and antioxidant effects have also been noted.[1]

Generally, the goal in natural product purification is to isolate individual component compounds that may have biological activity. Sufficient amounts of these potentially active compounds need to be isolated in order to facilitate characterization of unknown compounds or for use in other studies, such as clinical trials and bioassays. The isolated compounds must be of high purity so that any results obtained can be attributed to the compound under study rather than small amounts of companion compounds. Many techniques for extraction, isolation, and purification of natural products have been previously described.[2]

Preparative reversed-phase high performance liquid chromatography (RP-HPLC) is a separation technique that is widely used in this endeavor. It is considered a rapid, reliable, and robust technique that has wide applicability over many classes of compounds. This application note describes the isolation of the isoflavone puerarin from the roots of the kudzu plant using a modular preparative HPLC system.

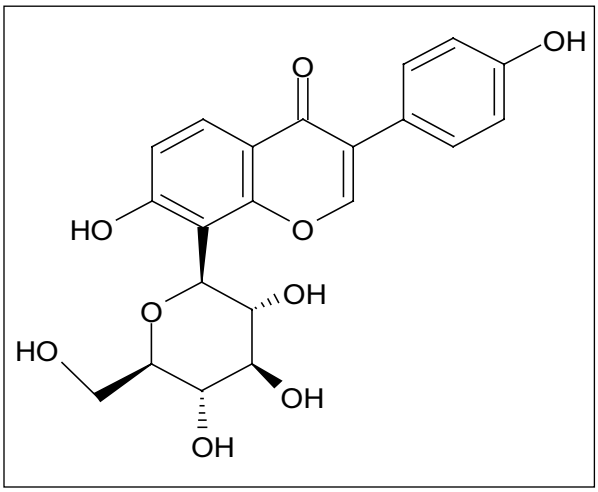

Figure 1. Chemical structure of puerarin.

EXPERIMENTAL

Extraction

Kudzu root powder (50 g) was added to 250 mL of 9:1 water/methanol and shaken for one hour, allowed to stand overnight, and shaken for an additional hour. This extract was centrifuged at 3000 RPM for 20 minutes, passed through Whatman #1 filter paper, and used without further treatment.

Separation

Preparative chromatographic separations were carried out using a Waters® Modular HPLC System as shown in Figure 2, which consisted of the following components:

Pump:	2535 Quaternary Gradient Module
Detector:	2489 UV/Visible Detector
Injector:	2707 Autosampler configured with a 1-mL loop
Collector:	Fraction Collector III
Columns:	Initial Prep Trial – SunFire C_{18}, 5 µm, 10 x 100 mm
	Final Prep Method – SunFire C_{18}, 5 µm, 19 x 100 mm Column

Figure 2. Waters Modular Preparative HPLC System.

Analytical chromatographic separations, implemented for method development and final purity checks, were carried out using the Alliance® HPLC 2695 and a 2998 PDA Detector with a SunFire C_{18}, 5 µm, 4.6 x 100 mm Column. Both the analytical and prep systems, including fraction collection, were controlled using Empower 2 Software to collect, manage, process, and report chromatography data. Two initial analytical scale separations were developed (one gradient for purity checks and one isocratic for purification) with the conditions described on the next page.

RESULTS AND DISCUSSION

Gradient HPLC analysis of the prepared extract indicated a puerarin concentration of ~0.5 mg/mL and an overall purity of 59.6% by UV area percent as shown in Figure 3. Using a previously developed analytical isocratic HPLC method (data not shown), the separation was scaled to the 10-mm I.D. prep column using the Preparative OBD™ Column Calculator shown in Figure 4. The Preparative OBD Column Calculator, a free download, provides an easy-to-use tool that aids in analytical-to-preparative scaling calculations (www.waters.com/prepcalculator). The Preparative OBD Column Calculator was used to convert the analytical separation method to the preparatory separation methods described in this application note. The use of the Preparative OBD Column Calculator has been described in a previous application note.[3]

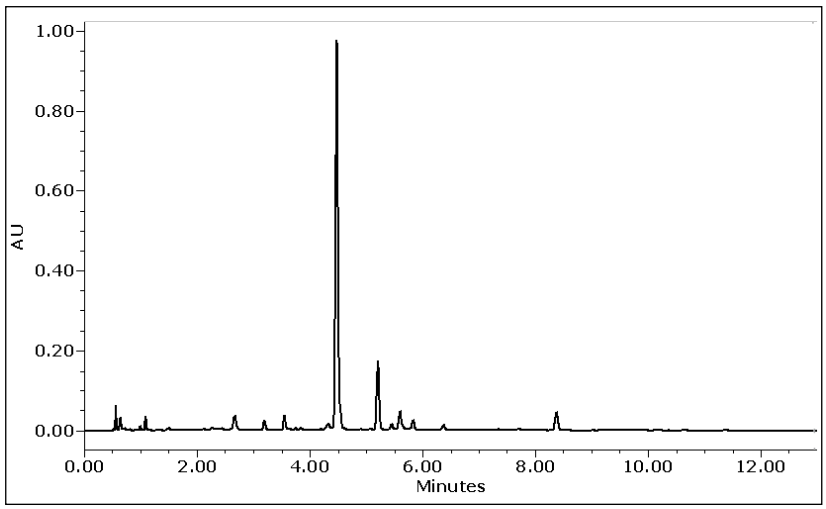

Figure 3. Analytical gradient separation of kudzu root extract.

Figure 4. Waters Preparative OBD Column Calculator.

Analytical Gradient Conditions

Column temp.:	Ambient
Flow rate:	1.5 mL/min
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile
Gradient:	95% A:5% B to 20:80 over 20 minutes
Detection:	UV at 251 nm

Analytical Isocratic Conditions

Column temp.:	Ambient
Flow rate:	1.5 mL/min
Mobile phase A:	88.5% Water + 0.1% formic acid
Mobile phase B:	11.5% Acetonitrile
Detection:	UV at 251 nm

Preparative separations were geometrically scaled from the analytical methods and are described below.

Preparative Isocratic Conditions

Column temp.:	Ambient
Flow rate:	7 or 25 mL/min (for 10 and 19 mm I.D. respectively)
Mobile phase A:	88.5% Water + 0.1% formic acid
Mobile phase B:	11.5% Acetonitrile
Injection vol.:	500 µL using Autosampler with 10-mm column or 2000 µL using Line D of the 2535 with 19-mm column
Detection:	UV at 251 nm

Using a 500-µL injection, acceptable reproducible preparative separations were achieved at 2.1% RSD retention time, as indicated in Figure 5. In order to increase the throughput, the method was further scaled to a 19-mm I.D. column. Injection volume scaling calculations indicated that a 2-mL injection was needed to keep the chromatography constant. Because the 2707 Autosampler was configured with a 1-mL loop, sample loading via the D line of the 2535 Quaternary Pump was used. In this loading technique, sample was introduced using a gradient table outlined in Figure 6. The gradient method was initiated via Empower 2 Software using the "Inject Immediate Sample Function." Although the Autosampler is not used, it does not have to be removed from the system, which allows users to quickly choose sample introduction via the pump or autosampler.

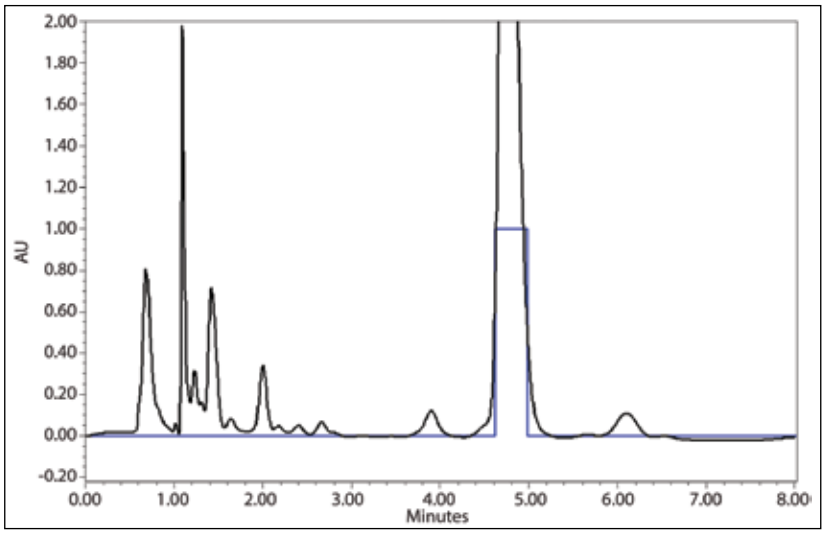

Figure 5. Prep (10 mm I.D.) separation of kudzu root extract using autosampler injection (black line is the UV output, blue line is the fraction collector state, 0.0=off, 1.0=on).

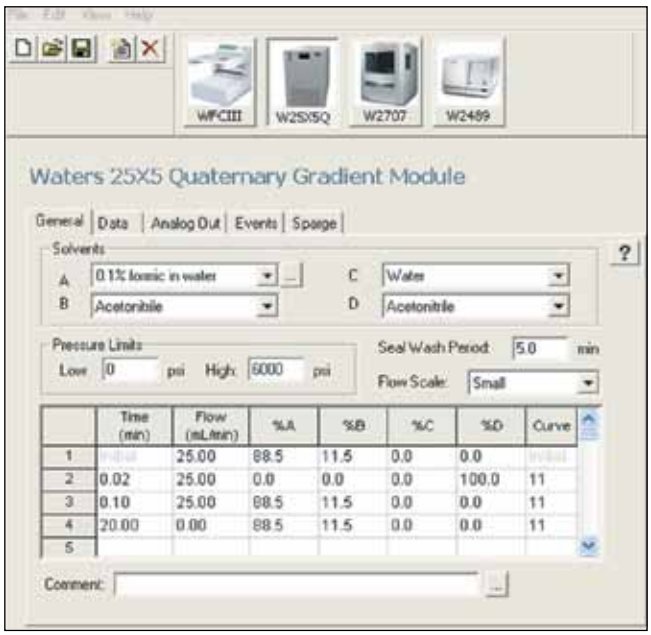

Figure 6. Empower Gradient Table with "Line D" loading.

Acceptable, reproducible prep separations were again achieved with the 19-mm column shown in Figure 7. Fraction collection was set up using Empower 2 Software for threshold collection within a window. In this mode, a collection window is defined (in this case 3.0 to 6.0 minutes), and any peaks eluted outside of that window are not collected. Peaks eluting within the window must meet peak threshold criteria (in this case ~0.60 AU) to be collected. In addition, the fraction collector was set to collect multiple injections per position. Each peak is collected into the same bed position, as opposed to each peak being collected into a separate container. This allows for bulk-style purification and eliminates the need to pool collected fractions from identical sample injections. Ten separate injections totaling ~11.5 mg of puerarin were made on the 19-mm column with a total volume collected of 92 mL. Subsequent analysis of the collected fractions shown in Figure 8 had a puerarin concentration of 0.099 mg/mL with a UV area purity of >97.5%. The overall yield of puerarin from the kudzu extract was 79.6%.

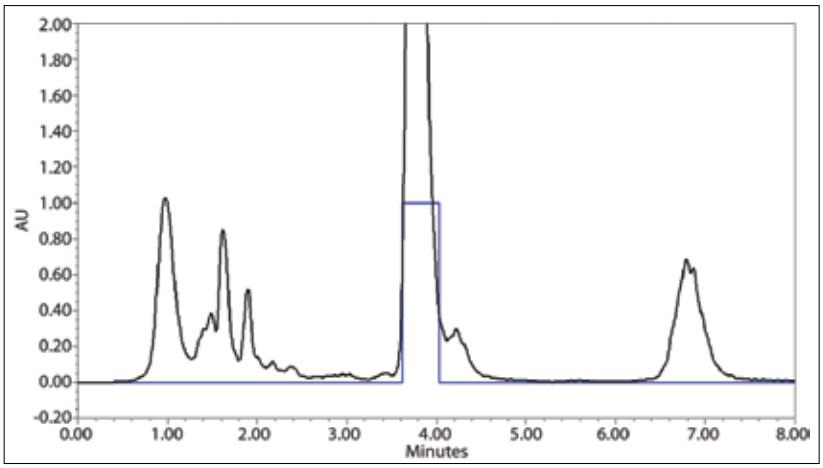

Figure 7. Prep (19 mm I.D.) separation of kudzu root extract using "Line D" loading.

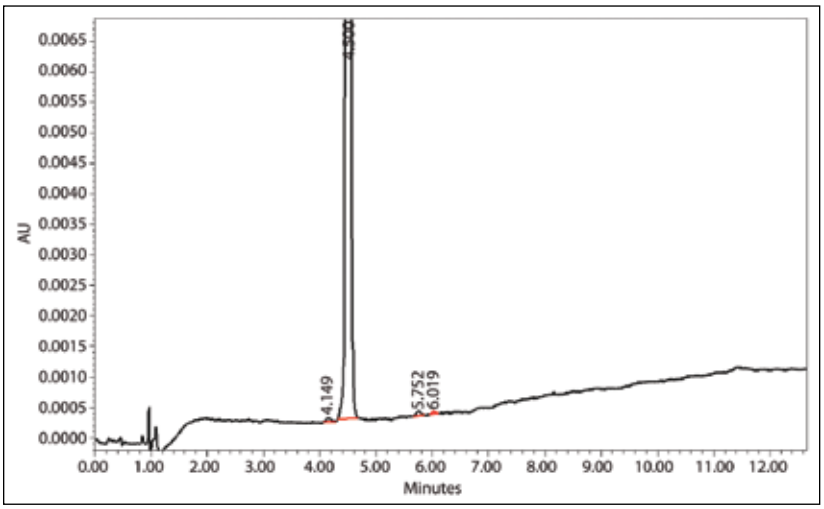

Figure 8. Analytical gradient separation of combined purified fraction.

To speed dry-down time, the system was used to concentrate the collected 92-mL fraction. The 92 mL collected was first diluted to 1 L with water. The column was then equilibrated with 99:1 water/acetonitrile. The entire diluted fraction was placed onto the column via the load valve embedded on the front of the pump. Once the entire sample was on the column, a simple step gradient to 100% acetonitrile was run and the eluted peak was captured in <10 mL, a 10X concentration factor. This small, mostly organic fraction was then dried to liberate crystalline puerarin.

CONCLUSIONS

Puerarin, an extract of kudzu root, was isolated and purified using Waters modular preparative HPLC system. Separations were analytically developed and geometrically scaled to two sizes of prep columns. The bulk purifications performed in an automated manner, eliminating the need for constant user intervention. Finally, the collected fractions were concentrated using the same system and column that was used to isolate the compound of interest.

- The system was shown to be flexible, running multiple columns (10 and 19 mm I.D.), using a variety of injection techniques (Autosampler, Line D, load valve), and easily adjustable fraction collection parameters using Empower 2 Software.
- Total system software control via Empower 2 Software provided system flexibility by allowing easy modification of all method parameters.
- The system was used for both purification and concentration steps without requiring modification to the system.
- The initial extract contained 50.0% puerarin and was purified to greater than 97.5%, and concentrated in a small volume ready for dry-down.

References

1. PDR for Herbal Medicines, 4th Ed., 2007; Herbal Monographs: 501-505.
2. Sarker SD, and Latif Z. Natural Products Isolation 2nd edition, 2006.
3. Aubin A, and Cleary R. Analytical HPLC to Preparative HPLC: Scale Up Techniques using a Natural Product Extract, Waters Application Note, 2009.

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Developing Focused Gradients for Isolation and Purification

Jo-Ann M. Jablonski, Thomas E. Wheat, and Diane M. Diehl
Waters Corporation, Milford, MA, USA

INTRODUCTION

Chromatographic separations for isolation and purification are governed by the same physical and chemical principles as analytical separations. In prep experiments, however, scientists isolate compounds at high mass loads, often on large columns, and require better resolution to enhance purity and recovery of the collected materials. Although creating a shallower gradient is a good first approach to enhancing resolution, changing the gradient slope for the whole separation leads to broader peaks and an increase in total run time. Focused gradients, an alternative to universally shallower gradients, decrease the gradient slope for only that portion of the chromatogram that needs increased resolution, providing more resolution between closely eluting peaks without increasing the total run time. A focused gradient can be defined based on a scouting run or directly from a first prep run.

EXPERIMENTAL APPROACH

Steps for gradient development:

- Determine system volume for prep scale
- Run scouting gradient
- Design focused gradient
- Run the focused gradient on the large scale column

EXPERIMENTAL CONDITIONS

Instrumentation

LC system:	Waters 2525 Binary Gradient Module, 2767 Sample Manager, Column Fluidics Organizer, 2996 Photodiode Array Detector, AutoPurification™ Flow Cell
Column:	XBridge™ Prep OBD™ C_{18} Column 19 x 50 mm, 5 µm (Part Number 186002977)
Flow rate:	25 mL/min
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile
Wavelength:	260 nm

Sample mixture

- Sulfanilamide, 10 mg/mL
- Sulfathiazole, 10 mg/mL
- Sulfamethazine, 20 mg/mL*
- Sulfamethizole, 10 mg/mL
- Sulfamethoxazole, 10 mg/mL
- Sulfisoxazole, 4 mg/mL
- Total concentration, 64 mg/mL in DMSO

**Peak selected for focused gradient.*

RESULTS AND DISCUSSION

Determine system volume for prep scale

- Remove column and replace with a union.
- Use Acetonitrile as A, and Acetonitrile with 0.05 mg/mL uracil as B (eliminates non-additive mixing and viscosity problems).
- Monitor 254 nm.
- Collect 100% A baseline for 5 min.
- At 5.01 min, program a step to 100% B, and collect data for an additional 5 min.
- Measure absorbance difference between 100% A and 100% B.
- Measure time at 50% of that absorbance difference.
- Calculate time difference between start of step (5.01 min) and 50% point.
- Multiply time difference by flow rate.

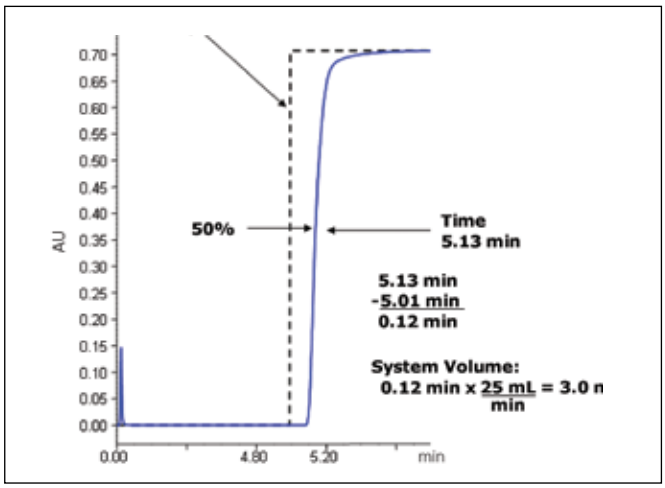

Figure 1. System volume determination.

The system volume is defined as the volume from the point of gradient formation to the head of the column. The system volume is used in designing the focused gradient. As shown in Figure 1, the system volume for the instrument configuration used in this experiment is 3.0 mL.

Run scouting gradient on sample

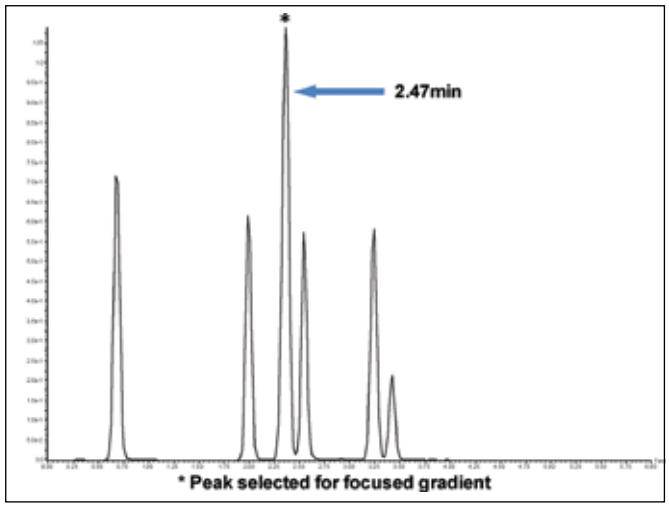

Figure 2. Large scale chromatogram of six compound sample mixture. Chromatographic method has a 0.39 min hold at the initial condition. The gradient is from 5-50% B in 5 minutes.

Designing the focused gradient

Step 1

The solvent concentration that elutes peak 3 at 2.47 minutes was formed at an earlier time. As shown in Figure 3, the offset between the detector and the point of gradient formation is equal to the system volume plus the column volume. The offset, then, for this particular system is equal to the 3 mL system volume determined earlier plus the volume of the 19 x 50 mm prep column (11.9 mL), or 14.9 mL. At a flow rate of 25 mL/min, it takes 0.59 minutes for the solvent concentration to reach the detector. The elution time of 2.47 minutes minus the offset time of 0.59 minutes is 1.88 minutes. Since the initial large scale gradient has a hold of 0.39 minutes, the time when the percentage of acetonitrile that elutes the peak was formed at 1.88 minutes minus 0.39 minutes, or 1.49 minutes into the 5 minute gradient.

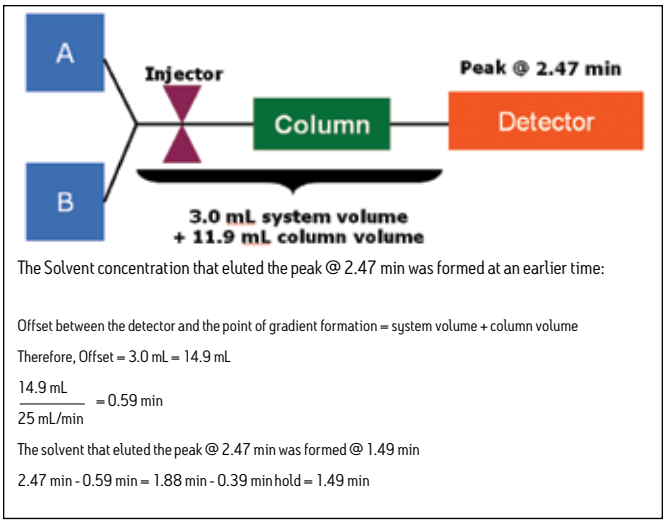

Figure 3. System diagram with offset calculations.

Designing the focused gradient

Step 2

Calculate the percentage of acetonitrile that elutes the peak at 2.47 minutes. The original large scale gradient goes from 5-50% B in 5 minutes with an initial hold of 0.39 minutes.

$$\frac{45\%}{5 \text{ min}} = 9\% \text{ per min}$$

AND

$$\frac{9\%}{\text{min}} \times 1.49 \text{ min} = 13.4\% \text{ acetonitrile}$$

Another way to calculate the percentage of acetonitrile:

$$\frac{1.49 \text{ min}}{5.00 \text{ min}} \times 45\% = 13.4\% \text{ acetonitrile}$$

The percentage of acetonitrile calculated from the gradient that elutes the peak at 2.47 minutes is 13.4%; because the gradient starts at 5% acetonitrile, the actual concentration of acetonitrile that elutes the peak is 13.4% + 5%, or 18.4% acetonitrile.

Designing the focused gradient

Step 3

A focused gradient intended to separate closely eluting peaks in the middle of the gradient should start at the original small pilot scale conditions, usually 0-5% B. Once the sample injection occurs, quickly ramp the gradient to the percentage of acetonitrile that is 5% below the expected percent acetonitrile concentration that will elute the peak of interest. Make the shallow, focused portion of the gradient proceed at one-fifth of the slope that was used in the scouting gradient. A five-fold shallower gradient can be expected to give better resolution between closely eluting peaks. End the focused portion of the gradient 5% above the expected percent acetonitrile concentration that will elute the peak of interest. The original gradient goes from 5-50% B in 5 minutes, or 45% change in 5 minutes. This is a 9% change per minute in acetonitrile concentration (round the 9% to 10% to simplify). The new gradient slope should then be one-fifth of 10%, or 2% change per minute. A 2% change per minute for a 10% change in acetonitrile concentration means that the focused gradient time segment for separating peaks 3 and 4 should have a duration of 5 minutes. Once the focused portion of the gradient is completed, quickly ramp the percentage of acetonitrile to 95% B to wash the column. End the gradient at initial conditions after equilibrating the column.

5-45% B = 9% per min (rounded to 10% per min)
2% per minute gradient slope

$$10\% \text{ acetonitrile} \times \frac{1 \text{ min}}{2\%} = 5 \text{ min}$$

New focused gradient for isolating peak at 2.47 min:

Time	Flow	%A	%B
0	25	95	5
1	25	86.6	13.4
6	25	76.6	23.4
7	25	5	95
7.4	25	5	95
7.5	25	95	5
10.5	25	95	5

Focused Gradient Compared with Scouting Gradient

Scouting Gradient

Time	Flow	%A	%B
0	25	95	5
0.39	25	95	5
5.39	25	50	50
5.89	25	5	95
6.89	25	5	95
7.39	25	95	5
10.50	25	95	5

Focused Gradient

Time	Flow	%A	%B
0	25	95	5
1	25	86.6	13.4
6	25	76.6	23.4
7	25	5	95
7.4	25	5	95
7.4	25	95	5
10.50	25	95	5

Running the focused gradient

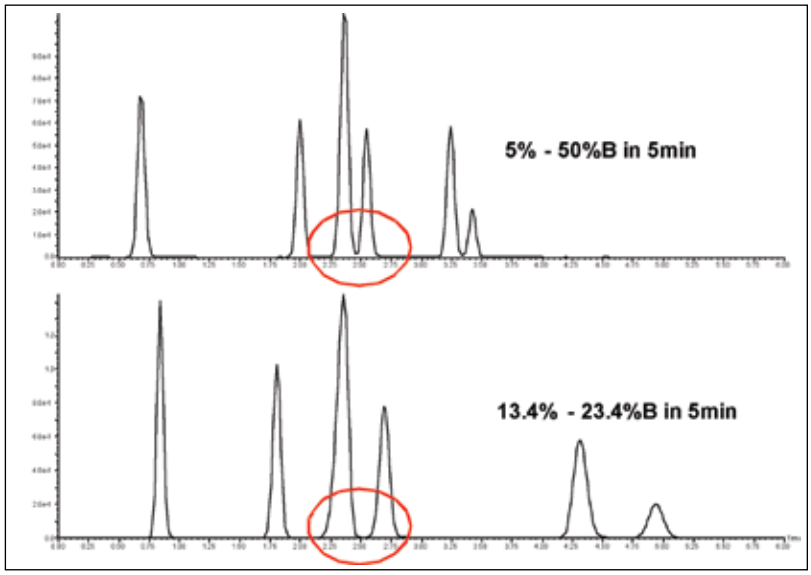

Figure 4. Top trace – Six compound mixture separation with scouting prep gradient (5%-50% B in 5 min). Bottom trace – Six compound mixture separation with focused large scale gradient to separate peaks 3 and 4 (13.4%-23.4% B in 5 min).

The focused gradient clearly improves the separation between peaks 3 and 4 in the chromatogram in Figure 4. Peaks 5 and 6 shift because they are influenced by the focused portion of the gradient, which continues to elute compounds at the shallower slope until the higher percentage of acetonitrile programmed for column washing permeates the column. Shallow, focused gradients allow the chromatographer to obtain pure products and better recoveries due to better resolution of crude mixture components without an increase in run time.

CONCLUSION

Scientists isolate compounds at high mass load when purifying products for future experiments. Focused gradients can improve an isolation by refining the resolution between closely eluting peaks without increasing the run time. Knowledge of the system volume permits the direct optimization of the prep gradient. Using focused gradients can increase the product yield and purity without increasing solvent consumption and waste generation. The focused gradient approach to developing isolations, therefore, helps to control purification costs.

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Analytical HPLC to Preparative HPLC: Scale-Up Techniques using a Natural Product Extract

Andrew Aubin and Ronan Cleary
Waters Corporation, Milford, MA, USA

INTRODUCTION

Chromatographic separation methods can be developed on any scale. To minimize the consumption of sample and solvents, there is a benefit in developing separation methods on a small scale and transferring them to a larger scale. Taking into account the important parameters and applying appropriate scaling factors, in a logical manner, enables users to scale up from analytical chromatography to larger-scale preparative separations easily and successfully. In this application note, the analytical-scale separation of Kudzu (*Pueraria lobata*) root extract is used to demonstrate the calculations and techniques used to move from a 4.6 mm I.D. analytical column separation through 10, 19, and 30 mm I.D. preparatory column separations.

Kudzu is a climbing, woody or semi-woody, perennial vine with a tuberous root. The roots of Kudzu contain a number of potentially useful isoflavones, including daidzein, daidzin, genistein, genistin, and quercetin. Kudzu is also a unique source of the isoflavone puerarin. Kudzu root extracts are thought to reduce alcohol intake and reduce alcohol withdrawal symptoms. Antibacterial, anti-cancer, anti-inflammatory, and antioxidant effects have also been noted.[1]

Figure 1. AutoPurification System.

EXPERIMENTAL

Extraction

Kudzu root pieces (20 g) were added to 100 mL of 9:1 water/methanol and shaken for one hour, allowed to stand overnight, and shaken for one additional hour. This extract was centrifuged at 3000 RPM for 20 minutes and used without further treatment.

Separations

Chromatographic separations, at all scales, were carried out using the Waters® AutoPurification™ System (Figure 1), which consisted of the following components:

Pump	Waters 2545 Binary Gradient Module
Detectors	Waters 2998 Photodiode Array
	Waters 3100 Mass Detector
Injector/collector	Waters 2767 Sample Manager
Column management	Waters System Fluidics Organizer

An initial analytical-scale separation was developed on a 4.6 x 50 mm Waters SunFire™ C_{18}, 5-µm column, using the conditions described below.

Column temp.:	Ambient
Flow rate:	1.5 mL/min
Mobile phase A:	Water + 0.1% Formic acid
Mobile phase B:	Methanol
Gradient:	5% to 70% B over seven minutes
Injection vol.:	20 µL
Detection:	UV (200 to 400 nm) and
	MS Full Scan 150 to 700 *m/z*

The resulting chromatogram (Figure 2) showed a number of resolved compounds and was considered an acceptable candidate for scale-up.

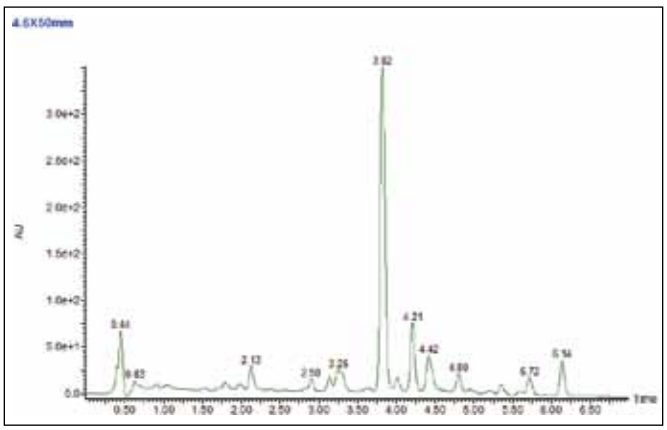

Figure 2. Analytical separation (4.6 mm I.D.) of Kudzu root extract.

RESULTS AND DISCUSSION

Scale-up method

A systematic approach to scale up will provide the best possible result. The ultimate goal is to maintain chromatographic resolution between key components and enable users to better predict chromatographic performance between analytical and preparative chromatography.

There are a number of key factors to consider when approaching this scale-up process.

Column chemistry

The heart of the separation is the column. Ideally, you should choose column chemistries that are identical. If the analytical and preparative columns are of different chemistries, it becomes very difficult to predict the preparative separation based on the analytical results. Waters offers a wide range of column chemistry choices available in analytical- and preparative-scale dimensions. As well as the chemistry itself, particle size should also be considered. Columns of the same particle size will provide similar resolution of critical pairs at both separation scales. Column length also influences the separation efficiency; columns of identical length, when scaled, give similar separation power. It is possible to scale to shorter or longer columns, but keep in mind that the separation will change.

Injection volume

To maintain peak shape and loading capacity, the injection volume needs to be suitably scaled using the following equation:

$$Vol_{PREP} = Vol_{ANALYTICAL} \bullet \frac{D^2_{PREP}}{D^2_{ANALYTICAL}} \bullet \frac{L_{PREP}}{L_{ANALYTICAL}}$$

where Vol is the injection volume (µL), D is the inner diameter of the column (mm), and L is the column length (mm). For example, a 20 µL injection on a 4.6 x 50 mm column corresponds to a 341 µL injection on a 19 x 50 mm preparative column.

Flow rate

To maintain separation quality, the flow rate must be scaled based on column dimensions. With columns of identical particle size, the following equation is used to geometrically scale flow rate:

$$F_{PREP} = F_{ANALYTICAL} \bullet \frac{D^2_{PREP}}{D^2_{ANALYTICAL}}$$

where F is flow rate (mL/min) and D is the inner diameter of the column (mm). For example, a 1.5 mL/min flow rate on a 4.6 mm I.D. column equates to a 25.6 mL/min flow rate on a 19 mm I.D. column.

Gradient scaling

When columns are of identical length, no changes to the gradient profile are required. If scaling to longer or shorter columns, the gradient segment volume must be maintained to preserve the separation profile.

The Waters Optimum Bed Density (OBD™) Prep Calculator, a free download, (Figure 3) is an easy-to-use tool that aids in these analytical-to-preparative scaling calculations (**www.waters.com/prepcalculator**). The Waters OBD Prep Calculator was used to convert the analytical separation method to the preparatory separation methods described in this application note.

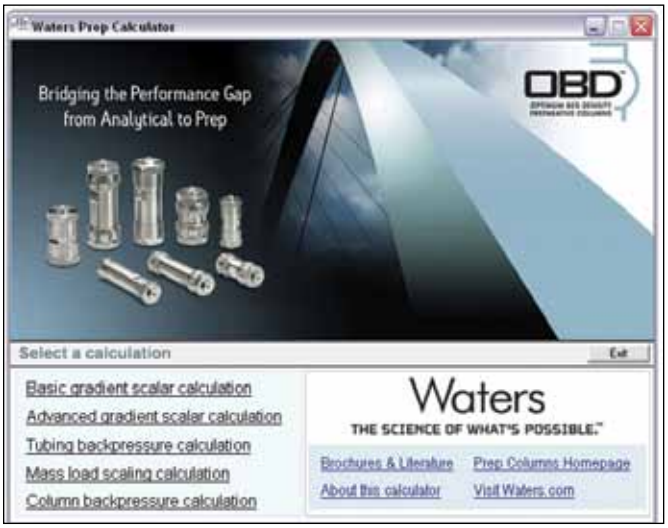

Figure 3. Waters OBD Prep Calculator.

Using the Waters OBD Prep Calculator

To calculate injection volume and flow rates, select the mass load scaling calculation (Figure 4) from the opening screen. Input your analytical and preparative column dimensions, analytical flow rate, and injection volume and the calculator returns the correct preparative values.

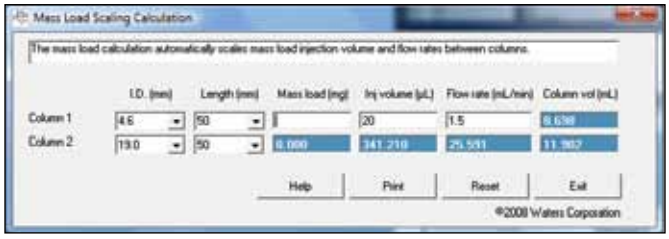

Figure 4. Waters OBD Prep Calculator mass load scaling calculation.

If your column lengths are identical, you can simply input the preparative flow rates into your gradient table using the same gradient segment times as your analytical method. Alternatively, for gradient methods, choose the basic gradient scalar calculation (Figure 5) from the opening screen, select your analytical and preparative column dimensions, input your analytical gradient table, and click the Calculate button. The preparative gradient table is automatically calculated and shown on the bottom half of the page. The Waters OBD Prep Calculator User Guide gives detailed instructions on use of all calculator functions.

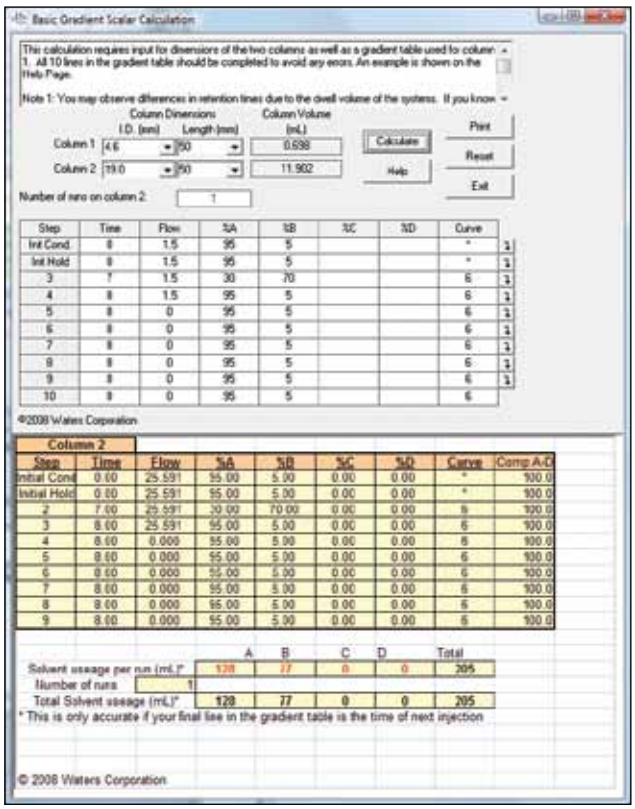

Figure 5. Waters OBD Prep Calculator basic gradient scalar calculation.

RESULTS

To demonstrate the previously described techniques, the analytical separation method described in the experimental section was scaled to three different preparative dimension columns (10.0, 19.0, and 30.0 mm I.D.). The scaled flow rates and injection volumes (all calculated using the Waters OBD Prep Calculator) are shown in Table 1.

Inside diameter (mm)	Flow rate (mL/min)	Injection volume (L)
4.6	1.5	20
10.0	7.1	95
19.0	25.6	341
30.0	63.8	851

Table 1. Waters OBD Prep Calculator scaled flow rates and injection volumes.

All of the preparative columns are SunFire Prep C_{18} OBD, 5 µm, 50 mm in length, and all of the separations were performed on the same system as the analytical-scale chromatography. As can been seen in Figure 6, regardless of the scale, the chromatography (UV TIC) is very similar. When compared to the original 4.6-mm I.D. scale (Figure 2), it can be seen that in terms of resolution and retention time the chromatography is again very similar.

This simple experiment demonstrates that a systematic approach to scale up meets the goal of maintaining chromatographic resolution between key components, and enables users to better predict chromatographic performance between analytical and preparative chromatography. This exercise also demonstrates the unique capability of the Waters AutoPurification System, which allows users to perform both analytical and preparatory chromatography on the same system with no performance compromise.

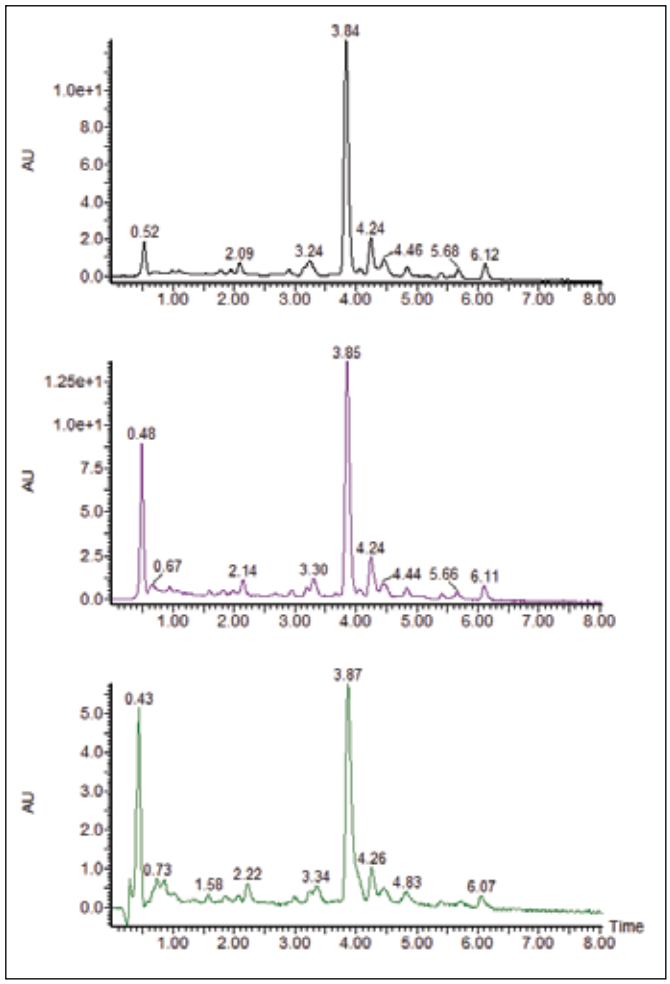

Figure 6. Scaled preparatory separations, 10 mm I.D. (top), 19 mm I.D. (middle), 30 I.D. mm (bottom).

SUMMARY

Analytical chromatography can be successfully scaled to preparatory chromatography easily by using a systematic approach.

- The use of identical column chemistry and identical column lengths maintains separation quality.
- Waters' proprietary Optimum Bed Density (OBD) column design offers excellent sample loading and column stability in an extensive array of chemistries and configurations.
- The Waters Prep OBD Calculator aids in the scaling calculations.
- Using the Waters AutoPurification System, separation methods can be developed on an analytical scale and transferred to preparatory scale on the same system, reducing a laboratory's overall capital investment.
- Developing methods on the analytical scale and transferring them to preparatory scale reduces solvent and sample consumption, while reducing waste disposal cost, compared to developing separation methods only at the preparatory scale.

References

1. PDR for Herbal Medicines. Thompson Healthcare Inc, Montvale NJ, U.S.A. 2007; 4th Ed.

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F: 1 508 872 1990
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Purification Workflow Management

Ronan Cleary and Paul Lefebvre
Waters Corporation, Milford, MA, USA

INTRODUCTION

A standard requirement for drug discovery screening of synthetic libraries is that the test compounds must have a minimum purity. Purity is based on the area percent of an LC chromatogram from a detector such as UV, evaporative light scattering (ELS), MS with a total ion chromatogram (TIC), or a combination of multiple detectors. If the screening compounds do not meet this standard, purification is required. Managing the flow of samples, subsequent fractions, and all the associated data through this process can often be difficult and time consuming.

This application note illustrates how a sample is efficiently taken through a three-step purification process utilizing the AutoPurify™ capabilities within the Waters® FractionLynx™ Application Manager for MassLynx™ Software, and the AutoPurification™ System for MS-directed analysis. This comprehensive informatics solution enables automation from the initial evaluation, through the purification, to analysis of the collected fraction.

DISCUSSION

The AutoPurify functionality uses the results of the analytical analysis to determine the purification process. By performing an analytical evaluation of the sample, the presence of the target compound is confirmed and its purity measured (Figure 1).

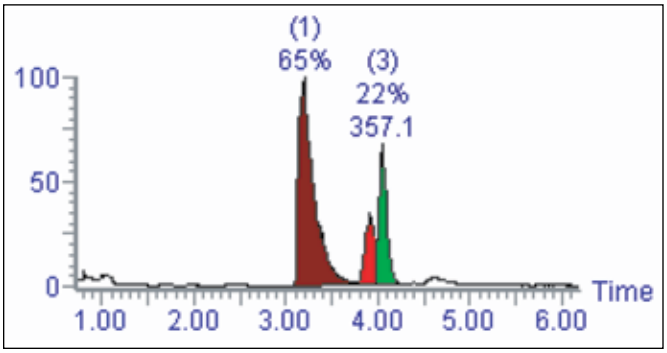


Figure 1. TIC chromatogram of the analytical-scale analysis of the crude sample.

The software will decide which shallow gradient should be used to perform the purification (Figure 2).

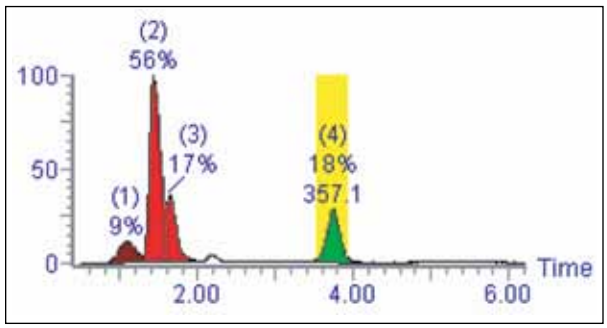


Figure 2. TIC chromatogram after purification, with fraction collection indicated by the shaded area.

Then, it automatically performs analysis of the collected fractions (Figure 3).

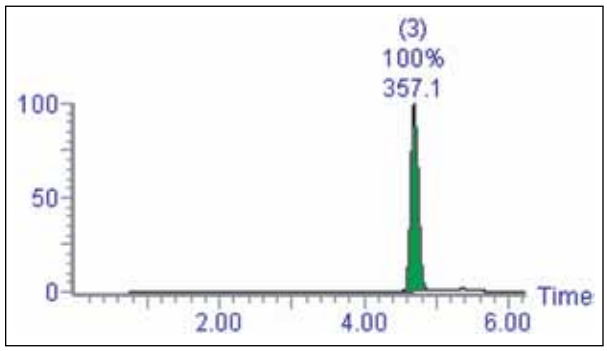


Figure 3. TIC chromatogram of the analysis of the collected fraction.

Information determined from analysis of the fractions can be used to help with post-purification handling such as fraction pooling and transfer to an evaporator. A report can be exported in different file formats such as .xml, .csv, and .tab, to easily interface with other sample handling software packages.

Step 1: Analytical interpretation

In the first of the three-step process, the purity of the target mass is identified by integrating the chromatogram. In the example shown in Figure 4, the area percent of the target determined from the TIC (22%) is then used to calculate the sample purity.

The area percent can also be determined by total absorbance current, wavelength, or analog signal. The purity of the target is then classified as "pass," "tentative," or "fail," based on user-defined limits. In this example, less than 10% pure means purification will not occur, 10% to 80% purity requires purification, greater than 80% is pure enough, and does not require further purification.

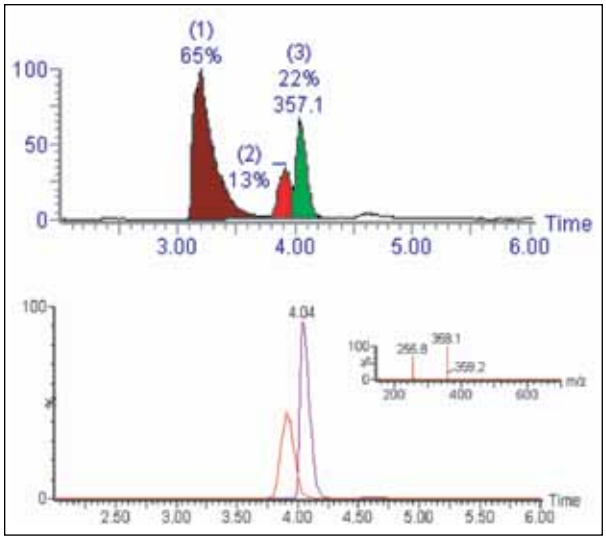


Figure 4. Analytical evaluation of mass 357.1 is 22% of the TIC, and the target sample is co-eluting with peak 2. An overlay chromatogram of the two co-eluting peaks, with the spectrum, indicates the potential fraction contamination that could occur.

In a manual process, the analyst would evaluate the separation, and adjust the gradient to achieve the best results. However, in an open access environment or where large numbers of samples are being handled, automation is necessary.

Step 2: The purification process

In the second step of the process, purification occurs. The software will determine the purification method best suited to improving the separation by choosing one of six different shallow gradients. Using the analytical retention time of the target, the appropriate shallow gradient-based method will be chosen.

Shallow gradients, also referred to as narrow gradients, allow for optimal target separation from closely eluting impurities, thus improving the purity of the resulting fraction. Each narrow gradient, whose time window is indicated by the colored lines (Figure 5), is created to cover a different timed section of the analytical gradient.

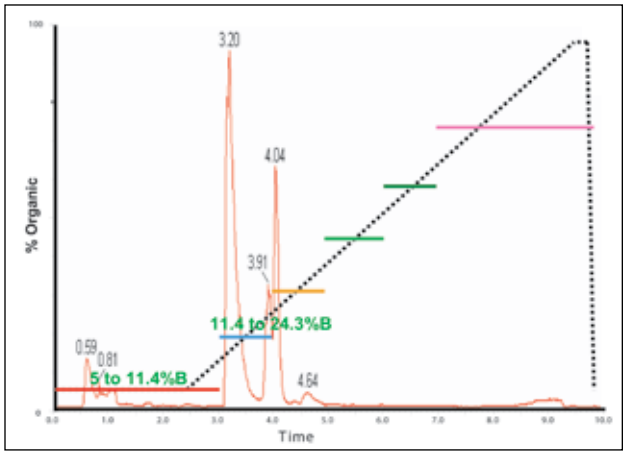


Figure 5. Graphical representation of analytical and prep gradients.

The analytical gradient is indicated by the dotted black line, and shows the solvent change over the course of the gradient to be from 5% to 95% B. With the relationship between the analytical retention time and the elution organic composition known, the software can choose which of the narrow gradients will be used to automatically purify the samples during the purification stage of the process.

When the software evaluates the analytical sample, it creates a browser report defining the recommended strategy. The user has the opportunity to change the strategy if necessary. The part of the report that refers to the strategy is the results pane (Figure 6). In this example, there are several other samples analyzed, but the one that is of interest is that last one on the list, A123008.

Sample ID	Ti...	Sample Purity	Purity Test	Masses	Strategy
A123004	4.61	46.13	TENTATIVE	375.00	NarrowC
A123005	-	-	N/A	-	None
A123006	3.42	68.15	TENTATIVE	226.90	NarrowB
A123007	-	-	N/A	-	None
A123008	4.04	21.96	TENTATIVE	357.10	NarrowC

Figure 6. Browser results pane with sample purity and prep strategy displayed.

The sample in this case eluted at 4.04 min (Figure 7), so the narrow gradient chosen for the purification was "Narrow Gradient C," the one that targeted the solvent change that occurred between 4 and 5 minutes. This gradient is denoted by the green line, which changes from 24% to 37% organic over 6.5 min, and is defined graphically as below.

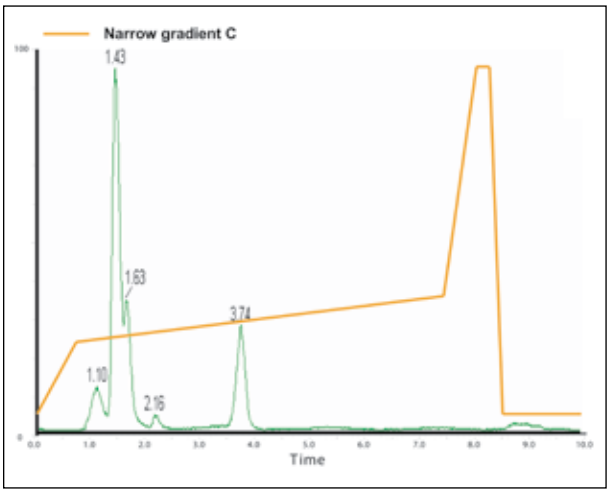


Figure 7. Representation of the narrow prep gradient chosen for the purification of the compound eluting at 4.06 min, with improved separation showing the isolated peak at 3.74 min collected.

The improved separation is more clearly displayed when the chromatograms of the two co-eluting compounds, as seen in Figure 4, are extracted and their chromatograms reviewed. Figure 8 shows the two chromatograms of masses 255 and 358, overlaid, and the improved separation achieved.

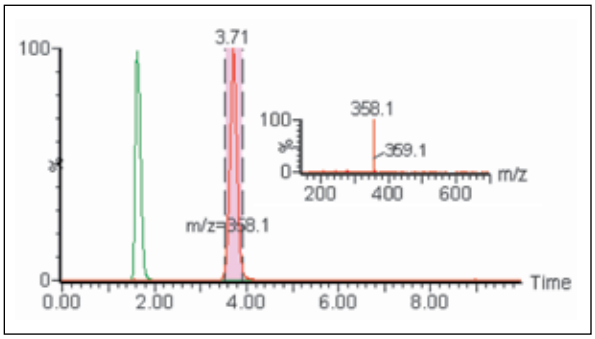


Figure 8. Overlay of the chromatograms of the two masses that were co-eluting earlier, showing the improved separation that was achieved. Spectra highlight the success also.

Step 3: Fraction analysis

With the first two steps of the process complete, the user can also decide to analyze the fractions (Figure 9). AutoPurify creates a sample list containing the fractions required for analysis and automatically runs them.

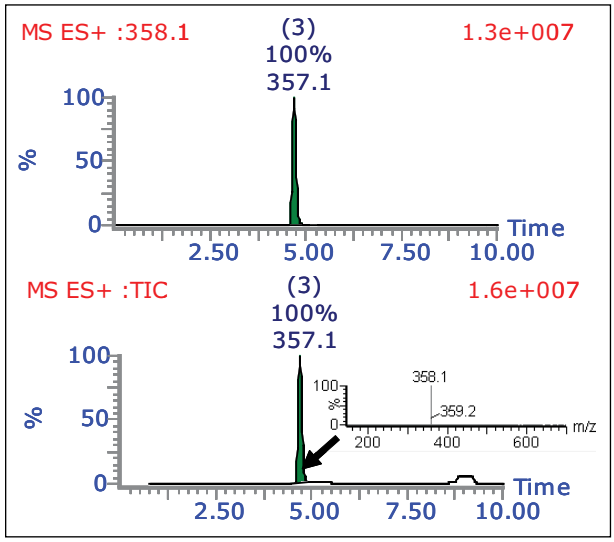


Figure 9. Fraction analysis post-collection, and post-fraction mixing by the injector/collector. TIC shows no other compounds present in the collection vessel.

To ensure that the portion of the sample taken for analytical analysis is representative of the entire collected fraction, it may be necessary to pre-mix fractions prior to injection (done with the injector/collector). Once homogenized, analysis can be performed on an analytical scale.

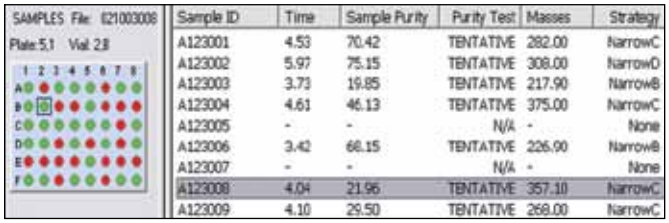
Automating the process

Automation of the three-step purification process is accomplished through AutoPurify.

A FractionLynx browser is created after each of the three stages to display results of the analysis and to report the recommended strategy for the next stage in the process. The software can automatically create and run the list of samples that are to continue to the next step. The user has a choice whether to allow the three stages to run unattended, or to manually review the results of each stage and edit the software's decision.

The determined strategy can be adjusted as necessary by the user through the interactive browsers that are produced. By automating the process, decisions can be made after regular work hours, allowing the work to continue unattended, saving time and resources.

The root name of the data, the sample ID, sample list, and the FractionLynx browser, A123, as shown in Figure 10, are edited by the software and carried through the purification process to make sample and results tracking easier.



Sample ID	Time	Sample Purity	Purity Test	Masses	Strategy
A123001	4.53	70.42	TENTATIVE	282.00	NarrowC
A123002	5.97	75.15	TENTATIVE	308.00	NarrowD
A123003	3.73	19.85	TENTATIVE	217.90	NarrowB
A123004	4.61	46.13	TENTATIVE	375.00	NarrowC
A123005	-	-	N/A	-	None
A123006	3.42	68.15	TENTATIVE	226.90	NarrowB
A123007	-	-	N/A	-	None
A123008	4.04	21.96	TENTATIVE	357.10	NarrowC
A123009	4.10	29.50	TENTATIVE	268.00	NarrowC

Figure 10. Browser report created after the analytical evaluation. The resulting strategy is displayed using different colors for the injection plate. Green = mass is found, purity level between 20% and 80%, and sample requires purification; and red = mass is either not found or sample is already pure enough, and purification will not be performed.

Analytical interpretation

FractionLynx browsers also include chromatograms and spectral information that are not shown in this application note. The portion of the browser file in Figure 10 shows sample purity and the prep strategy decision that was determined after the samples were analyzed on an analytical scale.

The preparative sample list is automatically created and run after the analytical analysis. Once the purifications are complete, the results are processed and a new FractionLynx browser report is generated (Figure 11).

Figure 11. Purification results, indicating where the fractions were collected, including fraction volume and spectral purity. Blue = collected fraction of the sample highlighted in the injector plate, green = passed spectral purity assessment, burgundy = review required, and red = failed purity assessment.

Purification process

Upon completion of the processing of the purification results, a sample list is generated and automatic analysis of the fractions generated is performed (Figure 12).

Figure 12. Fraction analysis results, indicating the sample purity of the collected fractions.

Fraction analysis

The final report shows the locations of the fractions, chromatograms, and spectra. The information in the reports can then be easily exported in different file formats such as .xml, .csv, and .tab, to easily interface with sample handling software packages such as liquid handlers or weighing devices.

CONCLUSION

This application note shows how a library of compounds can easily and efficiently be purified using the AutoPurify capabilities within the FractionLynx Application Manager. The software is capable of automating the entire purification process, from the original analytical purity assessment, to purification, and finally to the analysis of the fractions.

AutoPurify allows the process to be performed intelligently. Analytical results are used to determine if the target is present and its purity. Based on these criteria, only samples that truly require purification continue on through the process. Samples that do not contain the target compound, not enough of the target, or are already pure enough can simply be excluded from purification.

The benefits of using AutoPurify can be measured in time savings, reduced solvent consumption, and overall productivity gains. This is noticeable in several main areas:

- Automated evaluation of samples before purification prevents unnecessary purification from being performed by removing samples that do not require purification.
- Computerized evaluation of samples throughout the entire process saves analysts from having to manually review batches between stages of the process, and enables the subsequent analysis to be performed immediately – without waiting for the analyst to be present.
- Computerized determination of methods required during the process saves analysts from having to make or decide which gradients should be used to improve separations.
- The use of narrow gradients allows for the use of shorter, more focused gradients, saving time and solvents.
- Automation from stage to stage allows for unattended operation, combining all the savings of the process.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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Scaling a Separation from UPLC to Purification using Focused Gradients

Ronan Cleary, Paul Lefebvre, and Marian Twohig
Waters Corporation, Milford, MA, USA

INTRODUCTION

Purification laboratories face many of the same challenges that their counterparts in analytical laboratories face: the need to increase throughput and efficiency without sacrificing quality and quantity. Successful performance of a purification lab is measured in the ability to produce pure fractions in sufficient quantities in a timely manner.

UltraPerformance LC® (UPLC®) has been widely accepted by chromatographers because of the improvements over HPLC in sensitivity, resolution, and speed of separations. Now scientists are beginning to explore the use of this technology in the sample screening process as a screening tool to evaluate samples prior to purification.

A typical run time for analytical screening in a preparative lab is 10 minutes. By capitalizing on the efficiency of UPLC, a 10-minute run time can be shortened to as little as one minute. This offers substantial time savings enabling for greater capacity, but also fits into the "fail fast and fail cheap" motto adopted by many pharmaceutical companies.

This application note will discuss the use of focused gradients to maintain selectivity and resolution and to allow UPLC screening to be applied to preparative samples. This will offer the substantial time savings associated with UPLC to customers in the preparative environment.

Figure 1. The mass-directed AutoPurification System.

EXPERIMENTAL

A standard solution of pharmaceutical-like compounds was prepared to simulate the conditions under which many purification systems operate.

UPLC conditions

LC system:	ACQUITY UPLC® System with ACQUITY UPLC Photodiode Array (PDA) Detector
Column:	ACQUITY UPLC BEH C_{18}, 1.7 µm, 2.1 x 50 mm
Injection vol.:	2.0 µL
Flow rate:	0.8 mL/min, 2.1 x 50 mm
Mobile phase A:	0.05% Formic acid in acetonitrile
Mobile phase B:	0.05% Formic acid in water
Gradient:	Generic 5% to 95% over two minutes Focused Gradient

HPLC conditions

LC system:	AutoPurification™ System
Column:	XBridge™ Prep OBD™ C_{18}, 5 µm, 19 x 50 mm XBridge C_{18}, 5 µm, 4.6 x 50 mm
Injection vol.:	200 µL
Mobile phase A:	0.05% Formic acid in acetonitrile
Mobile phase B:	0.05% Formic acid in water
Flow rate:	22 mL/min
Gradient:	0 to 0.25 min, 2% B to initial % B 0.25 to 1.61 min, initial % B to end % B 1.61 to 1.86 min, end % B to 95% B 1.86 to 2.71 min, 95% B 2.71 to 2.72 min, 95% B to 2% B

MS conditions

MS system:	3100 Mass Detector
Ionization mode:	Positive
Switching time:	0.05 sec
Capillary voltage:	3 Kv
Cone voltage:	60 V
Desolvation temp.:	350 °C
Desolvation gas:	500 L/Hr
Source temp.:	300 °C
Acquisition range:	150 to 700 amu
Acquisition rate:	5000 amu/sec

RESULTS AND DISCUSSION

In order to maintain the selectivity and resolution achieved by analytical analysis, the overall cycle time of a preparative analysis must be increased almost nine-fold.[1] This long cycle time is not practical for most separation scientists. Therefore, we look to focused gradients to maintain selectivity and resolution in UPLC screening.

The UPLC separation of the sample shows the compound of interest eluting at 0.48 min, and is partially resolved from the peak at 0.51 min.

The separation is first directly scaled to a 19 x 50 mm XBridge Prep OBD C_{18} Column. The XBridge chemistry is built on the same second-generation bridged ethyl hybrid (BEH) particle as the ACQUITY UPLC BEH chemistry, in order to maintain the selectivity and resolution of the analytical analysis. To maintain the resolution and selectivity, the overall cycle time must be increased over nine-fold.

In a preparative environment, where the compound of interest is being isolated from the other components in the sample, retaining analytical resolution is not as important as isolating and collecting the compound of interest.[2]

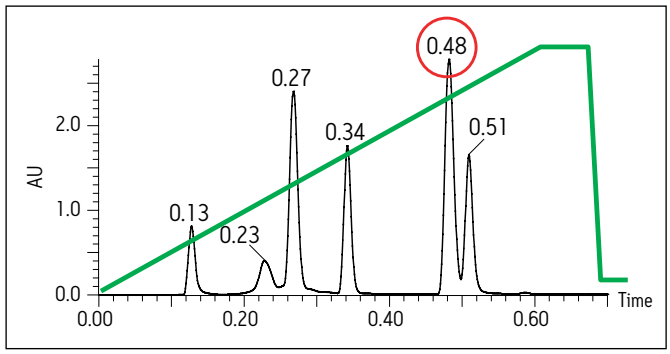

Figure 2. ACQUITY UPLC analytical separation.

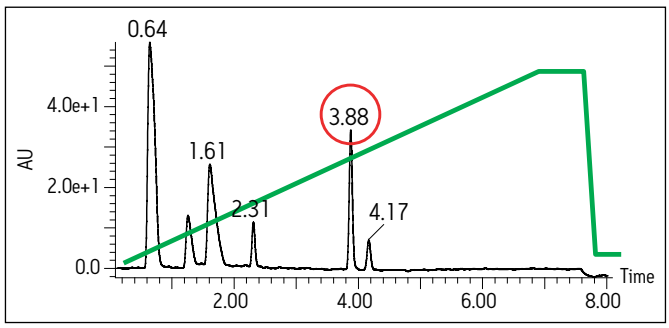

Figure 3. Direct scale-up maintains resolution and selectivity, with a run time of eight minutes.

A set of focused gradients can be created based on the relationship between percent composition and retention time. The system dwell time is used to determine that relationship.[3]

Here, in the analytical screen the mobile phase is 2% organic solvent at 0.17 minutes and 17.5% at 0.295 minutes, and so a series of gradients can be created.

The theory behind the focused gradients is the same for HPLC and for UPLC, but the time window for the UPLC gradient is much smaller.

Based on Table 1, method C is selected to isolate the compound that eluted at 0.48 min in the UPLC analysis. Using the focused gradient, the separation and isolation of the compound was carried out in three minutes.

UPLC library purity screening

This same methodology can be applied to the purity screening and purification of a large sample library. The ACQUITY UPLC System's large capacity (22 384-well plates) and the rapid analysis cycle time provide the ideal tool for high throughput library screening. Data is processed and handled using AutoPurify,™ part of the FractionLynx™ Application Manager.[4]

Method	Time (min)	Time (min)	% B start	% B end
A	0.17	0.295	2	17.5
B	0.295	0.42	17.5	33
C	0.42	0.545	33	48.5
D	0.545	0.67	48.5	64
E	0.67	0.795	64	79.5
F	0.795	0.92	79.5	95

Table 1. UPLC retention time windows and corresponding focused preparative gradient composition.

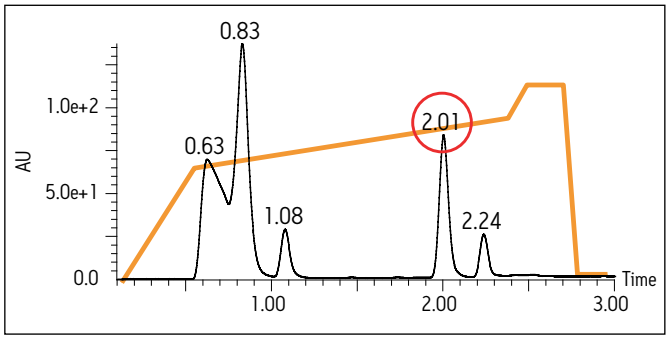

Figure 4. Separation of the compound of interest using a three-minute focused gradient.

Focused library purification

AutoPurify automatically selects the samples requiring purification and the corresponding focused preparative method.

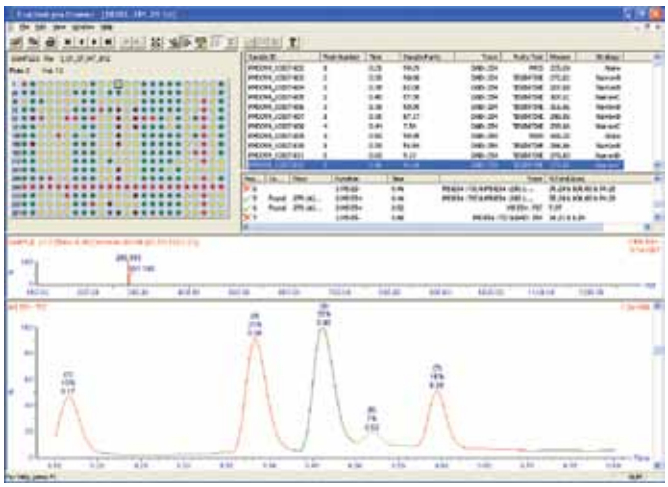

Figure 5. AutoPurify processing report showing the color coded purity and found/not found of a 348-well plate.

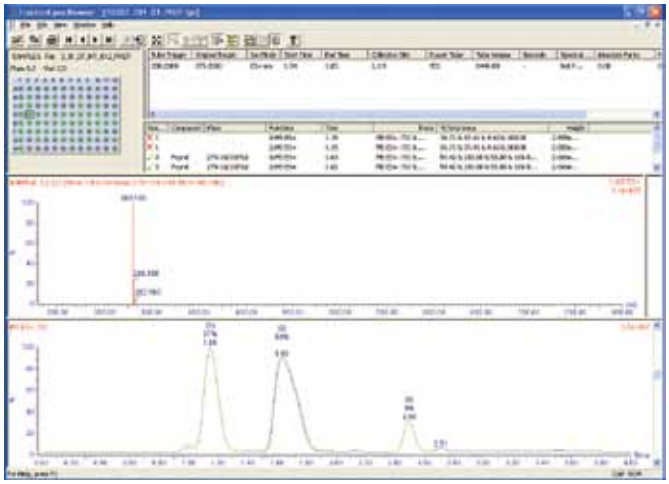

Figure 6. AutoPurify processing of the UPLC screening library.

UPLC fraction analysis

The substantial time savings associated with analytical screening can be magnified by incorporating UPLC into the analysis of the collected fractions. The collected fractions are analyzed to determine the new sample purity, and sample lists are automatically generated for each step of the process. By incorporating fraction analysis by UPLC into the workflow, the efficiency of the lab is further increased.

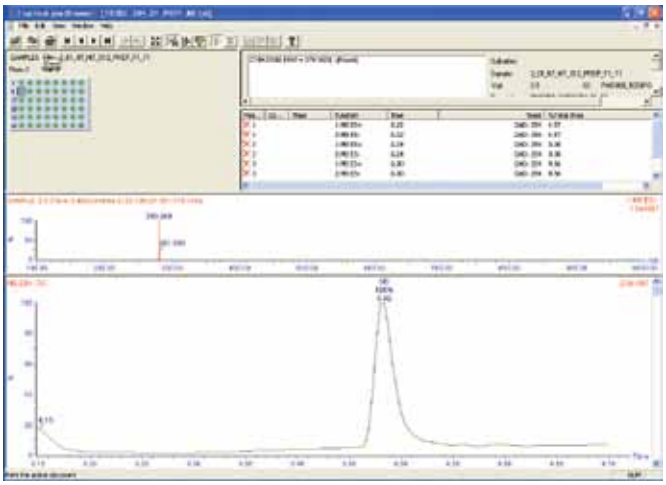

Figure 7. AutoPurify processing of the UPLC analysis of the collected fractions.

CONCLUSION

- Scale-up from UPLC to preparative HPLC in an efficient manner is possible with the use of focused gradients.
- The efficiency of UPLC can be carried through to purification, offering a substantial increase in throughput and productivity.
- The AutoPurify capabilities of FractionLynx allows for automation from the initial UPLC QC, through purification, to UPLC fraction analysis.
- AutoPurify is also capable of automatically selecting a focused preparative gradient based on the analytical results, giving better quality purification and eliminating the need for expert manual invention.

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1. Xia F, Cavanaugh J, Diehl D, Wheat T. Seamless Method Transfer from UPLC Technology to Preparative LC, Waters Application Note. 2007; 720002028EN.
2. Cleary R, Lefebvre P. The Impact of Focused Gradients on the Purification Process, Waters Application Note. 2007; 720002284EN.
3. Jablonski J, Wheat T. Optimized Chromatography for Mass Directed Purification of Peptides, Waters Application Note. 2004; 720000920EN.
4. Cleary R, Lefebvre P. Purification Workflow Management, Waters Application Note. 2006; 720001466EN.

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Waters Corporation
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Evaluating the Tools for Improving Purification Throughput

Paul Lefebvre, Warren Potts, Ronan Cleary, and Robert Plumb
Waters Corporation, Milford, MA, USA

INTRODUCTION

Chemists are constantly looking for ways to improve the overall throughput of their purification system. Time is the limiting factor for throughput, and there are two areas where time savings can be achieved: the amount of time required to perform a separation, and the amount of time between injections. Making the purification system as efficient as possible requires optimizing and minimizing both of these times. The challenge, however, is minimizing these times without impacting the purity and recovery of the fractions.

In this application note, we examine tools available for increasing the overall throughput of a purification system. We will use information from the analytical separation to optimize the purification, and will examine the steps required between injections to then determine the most efficient way to minimize run time.

Figure 1. The Waters mass-directed AutoPurification System.

OVERVIEW

In order to correctly compare time-saving techniques, we first established a baseline separation to define a standard analysis and collection time. We purified 10 drug-like compounds with a generic 10-minute preparative gradient. This baseline analysis time was then used as the comparison time for the analysis performed when the different time-saving chromatographic functionalities were applied.

The major areas for improving throughput are:

- Decreasing the time required for the analysis
- Decreasing the time between injections

One approach for decreasing the analysis time uses shallow or narrow gradients. Approaches for decreasing the time between injections include column regeneration techniques and automatically ending the purification run after the desired target has been collected.

METHODS AND DISCUSSION

Components

The Waters® AutoPurification™ System is comprised of:

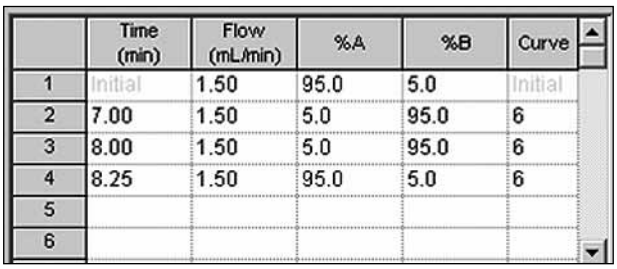
- 2545 Binary Gradient Module (BGM)
- 2767 Sample Manager
- System Fluidics Organizer (SFO)
- 2996 Photodiode Array Detector
- 3100 Mass Detector
- 515 makeup pump
- Passive flow splitter, 1:1000
- All components are controlled by MassLynx™ and FractionLynx™ software

The 10-sample library consisted of various drug-like compounds at a sample concentration of about 20 mg/mL dissolved in DMSO.

The chromatographic methods used water with 0.1% formic acid as mobile phase A, and acetonitrile with 0.1% formic acid as mobile phase B. Methanol was used as the makeup solvent for the preparative analysis.

Analytical gradient

SunFire™ C_{18} 4.6 x 50 mm, 5 µm, 1.5 mL/min total flow gradient and a 10-minute total run time.



	Time (min)	Flow (mL/min)	%A	%B	Curve
1	Initial	1.50	95.0	5.0	Initial
2	7.00	1.50	5.0	95.0	6
3	8.00	1.50	5.0	95.0	6
4	8.25	1.50	95.0	5.0	6
5					
6					

Figure 2. Analytical gradient table.

Generic preparative

SunFire C_{18} 19 x 50 mm, 5 µm, 25 mL/min total flow gradient. The same gradient table, as shown in Figure 2, was used. The only difference was the flow rate.

Narrow or shallow preparative gradient

SunFire C_{18} 19 x 50 mm, 5 µm, 25 mL/min total gradient. The start and end percent B composition is variable and dependant on the sample retention time during its analytical analysis.

Time (Minutes)	Composition (%B)
0.00 to 0.5	5 to %B start
0.50 to 1.67	%B start to %B end
1.67 to 2	%B end to 95
2 to 3	95
3 to 5	End

Table 1. Narrow gradient table. See Table 2 for percent B start and end.

Gradient Name	Analytical Retention Time	%B Start	%B End
A	0.00 to 1.67	5	20
B	1.67 to 2.84	20	35
C	2.84 to 4.0	35	50
D	4.00 to 5.17	50	65
E	5.17 to 6.34	65	80
F	6.34 to 7.5	80	95

Table 2. The narrow gradients used relative to the analytical retention time.

The time window in which the analytical sample eluted defines the conditions for the prep run. For example, if the compound eluted at 4.04 min, then the purification method would ramp up the organic percentage so that is was 50% at 0.5 min.

Baseline throughput

The generic gradient was used to perform the purification of 10 samples and the overall run time was measured. This time is used to compare the improvements.

Sample	Retention Time (min)	Run Time (min)	Time Between Injections (min)
1	1.18	10	2
2	5.20	10	2
3	1.35	10	2
4	4.67	10	2
5	3.18	10	2
6	2.55	10	2
7	2.41	10	2
8	5.06	10	2
9	2.02	10	2
10	2.63	10	2
Total Run Time		**120 minutes**	

Table 3. The overall throughput with the generic gradient. The total run time was 120 minutes.

Narrow gradients

Narrow gradients can be used to improve preparative chromatographic resolution.[1] However, if the resolution is adequate in the analytical separation, a shorter narrow or focused gradient can be used to increase throughput. The short method will focus its gradient on the same organic concentration, but in a shorter time frame.

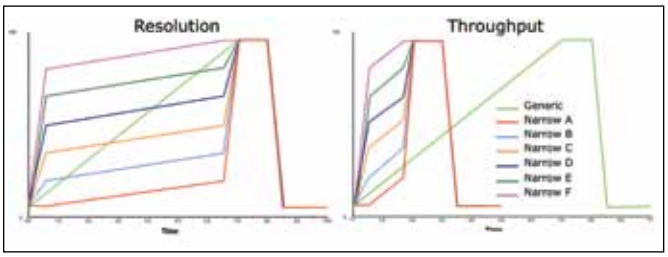


Figure 3. The different narrow gradients possible to focus on either improved resolution or throughput.

Figure 4 shows an example of one of the 10 samples being purified by both a generic and a narrow gradient. The target was successfully isolated using narrow gradient D. The results show that the resolution is maintained over the focused section of the gradient (the blue bracket). Note that there is a loss in resolution, as expected, in the non-focused areas of the gradient. This would have to be considered when the compound elutes at the very beginning or end of the focused gradient.

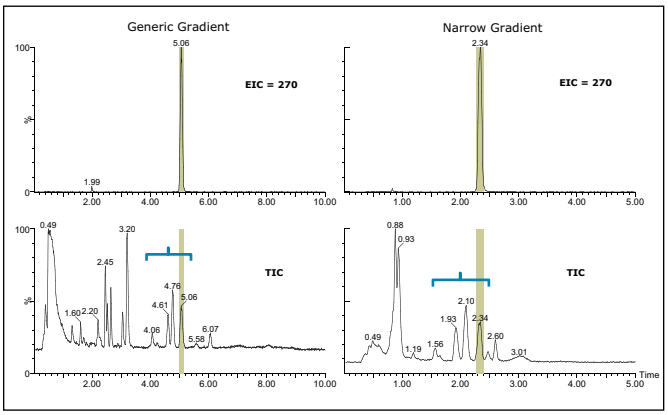


Figure 4. Comparison of the 10-minute generic and the 5-minute narrow purification. The blue bracket corresponds to the focused area of the gradient, where the resolution is maintained.

Sample	Generic Retention Time (min)	Narrow Gradient	Narrow Retention Time (min)	Run Time (min)	Time Between Injections (min)
1	1.18	A	1.38	5	2
2	5.20	E	1.65	5	2
3	1.35	A	1.74	5	2
4	4.67	D	1.94	5	2
5	3.18	C	1.75	5	2
6	2.55	B	1.90	5	2
7	2.41	B	1.95	5	2
8	5.06	D	2.34	5	2
9	2.02	B	1.30	5	2
10	2.63	B	2.08	5	2
Total Run Time		**70 minutes = 1.7 Fold Increased Throughput**			

Table 4. The overall throughput increases by 1.7 fold when incorporating narrow gradients, compared to using a generic gradient.

Rinsing and equilibration

It is important for high-quality chromatography that the column is rinsed and re-equilibrated with the appropriate volume of solvent, typically defined in column volumes. Insufficient rinsing can cause carryover, and equilibration time also has a significant impact on the overall throughput, with inadequate equilibration leading to retention time variability, poor chromatographic peak shape, or even sample breakthrough. The quantity of rinsing solvent is dependant upon the sample matrix, the retentiveness of the column, and the elutropic strength of the rinsing solvent. Typically, two to three column volumes is required to rinse. For equilibration, various articles report anywhere from three to 20 column volumes can be used.[2-3]

For example, a 19 x 50 mm column has a volume of about 12 mL. Two column volumes or 24 mL of 95% B were used to flush the column, and 60 mL of 5% B were used to re-equilibrate the column. With the gradient flow of 25 mL/min, the flush takes about 1 minute, and the equilibration takes about 2.5 minutes.

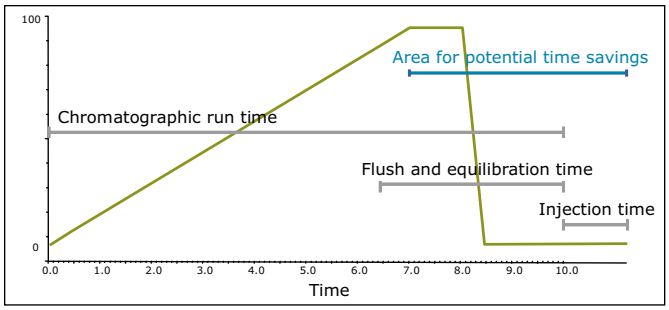


Figure 5. Illustration of an injection cycle with chromatographic analysis time, equilibration and flush time, and injection cycle for next injections time displayed. The area where time could potentially be saved is noted.

However, the flow rate can be elevated above optimal chromatographic conditions (30 mL/min for 5 µm packing), so long as the system can withstand the overall pressure increase. We found that the flow could be increased to 40 mL/min, only generating an additional 1300 psi of backpressure, reducing the flush time to 0.6 min and the re-equilibration time to 1.5 min, for a 1.5-minute savings.

Off-line regeneration

To increase throughput, a regeneration pump can be used to flush and re-equilibrate the first column off-line, while the next sample is running on a second column.

In this method, the run is terminated at 2.5 min for the narrow gradients, or 7 min for the generic and the next injection started. The first column is switched off-line and its flush started, while the second column is put in-line to receive the next sample. As mentioned earlier, the time required for the injection to be performed is 2 min.

The run-time savings for a generic preparative saw a reduction of 3 min per sample, for a reduction in the total run time from 120 to 90 minutes, or a 1.2-fold savings.

The run-time savings for a narrow gradient was more significant. Injection-to-injection time was reduced from 12 min with the generic method to 4.5 min using narrow gradients and off-line column regeneration. This reduced the total run time from 120 to 45 minutes, a 2.7-fold savings.

Early termination

To further reduce the time required for analysis, a software tool can be used to automatically end the run after the target has been collected. The throughput improvements of this feature will be illustrated for both generic and narrow gradients.

For either gradient approach used, once the target has finished collecting, the gradient will stop and flush with 95% B to wash the remaining material off the column. After a defined time of rinsing, the column will then be re-equilibrated with the initial gradient solvent. (Note: 2 minutes of equilibration time is performed between injections.)

Sample	Generic Run Time	Generic with Regeneration	Narrow Run Time	Narrow with Regeneration
1	4.03	3.43	4.23	3.63
2	8.05	7.45	4.50	3.90
3	4.20	3.50	4.59	3.99
4	7.52	6.92	4.79	3.19
5	6.03	5.43	4.60	4.00
6	5.40	4.80	4.75	4.15
7	5.26	4.66	4.80	4.20
8	7.91	7.31	5.19	4.59
9	4.97	4.27	4.15	3.55
10	5.48	4.88	4.93	4.33
Total Run Time	58.75 min = 2.0 Fold Increased Throughput	52.75 min = 2.3 Fold Increased Throughput	46.53 min = 2.6 Fold Increased Throughput	40.53 min = 3.0 Fold Increased Throughput

Table 5. The overall throughput improvement using the run termination function can range from a two- to three-fold increase, depending on what additional tools are used. Using the regeneration pumps saves 0.6 min per injection when compared to a single column method. This corresponds to the time required to rinse the column. The re-equilibration time is incorporated into the 2 min to make an injection.

Optimized injection routine

Throughput can be further improved by reducing the time between injections. The injection cycle can be divided into three segments:

- Aspiration of the sample into the needle
- Dispensing the sample into the loop
- Washing the assembly

Optimizing the speed of the aspiration enables the sample to be quickly drawn into the needle and holding loop. Care must be taken to ensure the increased syringe speed does not create air bubbles in the system.

Once the sample has been drawn into the holding loop, it is dispensed at an optimized flow rate. Care must again be taken to ensure that a high-pressure condition does not occur by operating the syringe too quickly.

Two options are available for positioning the sample in the loop. The default setting is to center the sample in the loop, but the sample centering can be disabled to allow the sample to be more quickly loaded onto the front of the sample loop.

When sample centering is removed, it is possible to operate with only one wash solvent and to be able to perform this wash at the beginning of the injection sequence, decreasing the injection time.

Cumulative time-savings

The time required to inject and rinse was reduced from 2 min with the standard partial loop injection to 0.4 min with the new settings. Table 6 shows the throughput possible by combining optimized injection settings with the various other tools.

Tool	Original Total Run Time	Optimized Injection Total Run Time	Default Injection Routine	Overall Increase with Optimized Injection Routine
Generic	120	104	—	1.2
Generic + End Run	58.75	53.75	2.0	2.2
Generic + End Run + Regeneration	52.75	36.75	2.3	3.3-Fold Increased Throughput
Narrow	70	54	1.7	2.2
Narrow + End Run	46.53	41.63	2.6	2.9
Narrow + End Run + Regeneration	40.53	24.53	3.0	4.9-Fold Increased Throughput

Table 6. Using optimized injection routines can improve the overall throughput. The improved injection routine has a greater impact when using regeneration because the 2 min for the normal injection is used to re-equilibrate with a single column. But with regeneration, the re-equilibration is done off-line and the injection time is dead time.

CONCLUSION

Throughput can be increased by about five-fold using a combination of narrow gradients, early run termination, off-line column regeneration, and an optimized injection routine. This correlates to an 80 percent decrease in run time.

- Narrow gradients can be used to improve throughput, but require additional information about the target.
- Off-line column regeneration has a greater impact on throughput as the run time is reduced.
- Early run termination improves throughput and reduces the amount of consumed solvent saving both time and money.
- Optimizing the wash sequence and adjusting when it is performed will save additional time between injections.
- Various combinations of throughput-enhancing tools can be used based on the specific requirements.

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3. UD Neue, American Laboratory. 1997; March.

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34 Maple Street
Milford, MA 01757 U.S.A.
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F: 1 508 872 1990
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Mass-Directed Purification of Glucoraphanin from Broccoli Seeds using Preparative-Scale Hydrophilic-Interaction Chromatography (HILIC)

Andrew Aubin
Waters Corporation, Milford, MA, USA

INTRODUCTION

Broccoli has long been considered a healthy food choice. Recent research and news reports have stated that eating the sprouts of broccoli may reduce the risk of stroke, high blood pressure, and cardiovascular disease.

The phytochemical compound most often associated with these health benefits is glucoraphanin (4-methylsulfinylbutyl glucosinolate, Figure 1). Glucoraphanin is the most predominant glucosinolate in broccoli and has been thought to have cancer-prevention qualities, along with the other aforementioned health benefits.

As researchers continue to investigate the health effects of glucoraphanin, there is a need to have on hand suitable quantities of pure glucoraphanin reference material to accurately determine amounts of glucoraphanin in various vegetables, and for investigations for the use of glucoraphanin as an active compound.

Broccoli seeds have been shown to be rich in glucoraphanin (20 to 50 mg/g) and are easy to obtain, making them a good choice for the isolation of glucoraphanin on a preparative scale. This application describes the mass-directed purification of glucoraphanin from broccoli seeds using the Waters® AutoPurification™ System with the 3100 Mass Detector (Figure 2) and an Atlantis® Hydrophilic-Interaction Chromatography (HILIC) Silica Column.

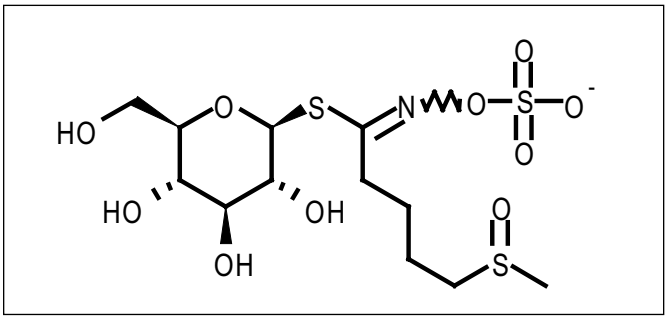

Figure 1. Glucoraphanin $C_{12}H_{22}NO_{10}S_3$.

Figure 2. Mass-directed AutoPurification System, with the 3100 Mass Detector.

EXPERIMENTAL

Glucoraphanin was extracted from broccoli seeds using a method similar to that of Rochfort et al.[1] Broccoli seeds (25 g) were boiled in deionized water (250 mL) for five minutes and then homogenized to a slurry. Following sonication for 30 minutes, 25 g of Celite 545 was added to the mix and the entire slurry passed through a glass fiber filter. The clear extracts were reduced in volume by 10 times prior to chromatography.

The isocratic separation method was first developed on an analytical scale (4.6 x 150 mm Atlantis HILIC Silica Column, 5 µm at 2 mL/min). Separation conditions were scaled up to the preparative column using the following calculations.

Scaling injection volume

$$Vol_{PREP} = Vol_{HPLC} \bullet \frac{D^2{}_{PREP}}{D^2{}_{HPLC}} \bullet \frac{L_{PREP}}{L_{HPLC}}$$

Here, Vol is the injection volume (µL), D is the inner diameter of the column (mm), and L is the column length (mm). A 5-µL injection on a 4.6 x 150 mm HPLC column corresponds to a 57-µL injection on a 19 x 100 mm preparative column.

Experiments showed that an injection volume of up to 100 µL could be used on the prep column without degradation of the chromatographic peak shape. Higher injection volumes negatively impacted peak shapes primarily due to the high solvent strength of the sample diluent.

Scaling flow rate

Based on column dimensions, the following equation is used to geometrically scale flow rate:

$$F_{PREP} = F_{HPLC} \bullet \frac{D^2{}_{PREP}}{D^2{}_{HPLC}}$$

Here, F is flow rate (mL/min) and D is the diameter of the columns (cm). A 2.0 mL/min flow rate on a 4.6 mm I.D. column equates to a 34.1 mL/min flow rate on a 19 mm I.D. column. The Waters Prep Calculator (Figure 3) was used to convert the analytical separation method to the preparatory separation method.

Figure 3. The Prep Calculator entry screen.

Elution of glucoraphanin from the preparatory column was monitored by mass spectrometry in electrospray negative mode and the fraction collection was triggered based on mass. The glucoraphanin fractions from each injection were combined in a single collection vessel. Collected fractions were reduced to dryness under vacuum and subsequently analyzed using a conventional HPLC system (detection by UV at 220 nm) to estimate the purity of the isolated glucoraphanin.

An exact mass measurement of the isolated material was performed using the Waters LCT Premier™ Mass Spectrometer. The measured m/z of 436.0409 was within 0.7 ppm of the theoretical value of 436.0406 for glucoraphanin.

When performing elemental composition analysis, the i-FIT™ algorithm was used. This algorithm compares the theoretical isotopic distributions of proposed elemental compositions against the experimentally-measured isotopic distributions. For each proposed chemical formula a numerical value is calculated that reflects the "goodness of fit" between the measured and theoretical distributions. The measured value for glucoraphanin was the top hit, with the next possibility giving an i-FIT value 32 times higher.

Analytical LC conditions

LC system:	Alliance® HPLC System
Detector:	2996 PDA Detector
Column:	Atlantis HILIC, 5 µm 4.6 x 150 mm
Column temp.:	25 °C
Flow rate:	2 mL/min (Isocractic 7% A, 93% B)
Mobile phase A:	50 mM Ammonium formate, pH 6.5
Mobile phase B:	Acetonitrile

Preparative LC conditions

LC system:	AutoPurification System
Pump:	1545 Binary Gradient Module
Injector/collector:	2767 Sample Manager
Detector:	3100 Mass Detector and 2996 PDA Detector
Fluidics:	System Fluidics Organizer
Column:	Atlantis HILIC Optimum Bed Density (OBD™), 5 µm 19 x 100 mm

Column temp.:	Ambient
Flow rate:	34 mL/min (Isocractic 7% A, 93% B)
Mobile phase A:	50 mM Ammonium formate, pH 6.5
Mobile phase B:	Acetonitrile

MS conditions

MS system:	3100 Mass Detector
Ionization mode:	ESI Negative
Capillary voltage:	3400 V
Cone voltage:	25 V
Desolvation temp.:	500 °C
Desolvation gas:	800 L/Hr
Source temp.:	150 °C
Acquisition range:	150 to 650 *m/z*

RESULTS AND DISCUSSION

Glucoraphanin is a relatively polar substance. Separation of glucoraphanin from other related glucosinolates and other polar extractables by reverse phase chromatography proved be difficult, with glucoraphanin eluting close to the void volume. Hydrophilic-Interaction Chromatography (HILIC) was evaluated and subsequently used for this separation.

HILIC is a separation technique that can retain and separate polar, water-soluble organic compounds. HILIC is often described as a variation of normal-phase chromatography. In normal-phase chromatography, the mobile phase is 100% organic, however using HILIC, water (usually <20%) is added to the organic mobile phase (typically an aprotic solvent like acetonitrile) making it possible to retain, separate, and elute polar compounds.

Water, a very polar solvent, competes effectively with polar analytes for the stationary phase. Polar compounds that are initially attracted to the polar packing material are eluted by modifying and increasing the polarity of the mobile phase by adding more water. Analytes are eluted in order of increasing hydrophilicity, i.e. chromatographic polarity relative to water. In the case of the broccoli seed extract, glucoraphanin is the most polar glucosinolate leaving it highly retained using HILIC.

A further advantage to using HILIC is its ability to reduce the time required to dry down samples since fractions are eluted in predominantly organic solvent. This makes evaporation and dry down significantly faster compared to reverse-phase methods where fractions are often predominantly water. Column backpressures are typically lower, due to the lower viscosity of the highly organic mobile phases, allowing for higher flow rates that in turn lead to higher sample throughput.

As was described previously, the analytical separation method was transferred to a preparatory scale. Fraction collection was triggered based on mass. In this technique (referred to as mass-directed purification), a small portion of the column effluent is spilt off and directed to the mass spectrometer. Following the split, a make-up pump is used to increase the flow rate of the splitter effluent to ensure that the data from the mass spectrometer can be processed by MassLynx™ Software in time to divert the column flow to the fraction collector.

Collection is triggered when the mass spectrometer detects the user-defined mass (in the case of glucoraphanin, *m/z* 436). This technique allows very precise collection control, as fraction collection is only triggered by a peak containing the mass (or masses) of interest, ultimately resulting in fewer fractions of higher quality. An example of the mass spectrometer output can be seen in Figure 4. The pink shaded area represents the collected peak fraction.

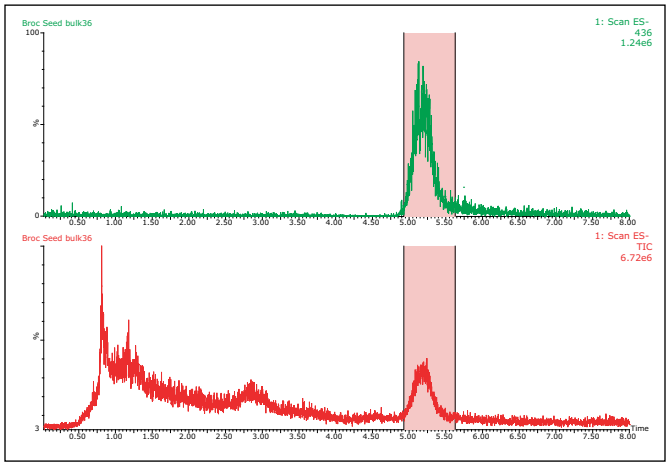

Figure 4. Mass-directed purification of broccoli seed extract. The shaded area represents the collected peak fraction.

In this purification example, a series of thirty 100-µL injections of concentrated aqueous-based broccoli extract were performed. Each injection liberated a fraction containing glucoraphanin in approximately 23 mL of mobile phase. All fractions from all injections were collected into a single container. The combined fractions were reduced to dryness with ~139 mg of dry material remaining. The identity of liberated crystal material was confirmed to be glucoraphanin using an exact mass time-of-flight mass spectrometer.

Purity of the material was evaluated using the analytical HPLC method with UV detection at 220 nm and was found to be 95% (area percent basis). The raw starting material evaluated for purity using the same technique was found to be ~ 53%. Before and after chromatograms are pictured in Figure 5.

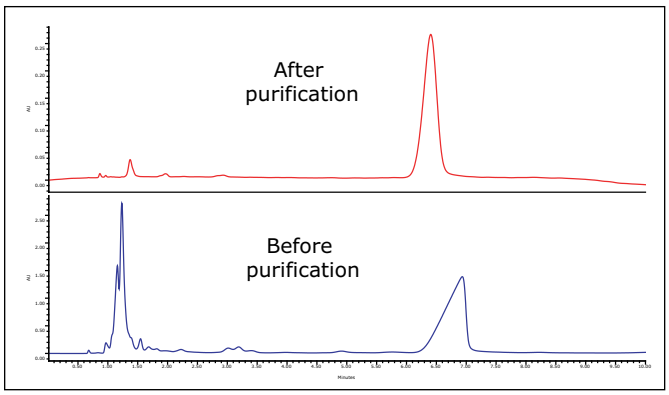

Figure 5. Broccoli extract before (bottom) and after (top) purification.

CONCLUSIONS

- Preparatory-Scale Hydrophilic-Interaction Chromatography (HILIC) provided a useful separation technique for the isolation of the polar glucoraphanin. Higher throughput is possible due to reduced fraction dry down times and the opportunity to use higher flow rates.
- An increase in purity from 53% to 95% was accomplished using mass-directed purification for the isolation of glucoraphanin from broccoli seeds.
- A total of 139 mg of glucoraphanin was isolated in this example. This amount is consistent with published values.
- Exact mass TOF-MS analysis of the purified fraction confirmed the presence of glucoraphanin.

References

1. Rochfort et al. Journal of Chromatography A. 2006; 1120: 205-210.

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Effective Use of Temperature Control in Compound Isolation

Jo-Ann M. Jablonski, Thomas E. Wheat, and Diane M. Diehl
Waters Corporation, Milford, MA, USA

INTRODUCTION

All chemical processes are temperature sensitive and chromatography illustrates this point quite dramatically. Generally, as the temperature rises, viscosity and pressure decrease, retention decreases, and the selectivity of the separation may change. Temperature control in analytical chromatography is routine, but temperature is seldom controlled or used as a parameter for manipulating chromatography at the preparative scale. The reason for this is two-fold. First, large diameter columns cannot be effectively heated from the outside. Second, high flow rate separations actually occur at the temperature of the incoming solvent. Electric blankets and column ovens, while satisfactory for small scale chromatography, cannot heat a large column uniformly from its inlet to its outlet or across the diameter of the column. A temperature gradient also forms from the wall to the center of the column, which impacts the chromatography. The variable temperature inside the column results in the preparative separation not being comparable to the analytical separation. This work illustrates an effective solution for controlling temperature of large columns at high flow rates.

EXPERIMENTAL APPROACH

- Measure time for system pressure stabilization at RT
- Measure time for system pressure stabilization at 60 °C
- Determine optimal configuration for preheating solvent
- Ensure scalability with temperature control

EXPERIMENTAL CONDITIONS

Instrumentation

LC system:	Waters 2525 Binary Gradient Module, 2767 Sample Manager, Column Fluidics Organizer, 2996 Photodiode Array Detector, ZQ™ Mass Spectrometer, Water Bath
Column:	XBridge™ Prep OBD™ C_{18} Column 19 x 150 mm, 5 µm (Part Number 186002979)
Flow rate:	25 mL/min
Mobile phase A:	0.1% TFA in water
Mobile phase B:	0.08% TFA in acetonitrile

RESULTS AND DISCUSSION

Pressure stabilization at room temperature

The observed system pressure changes only as a result of the change in column pressure and in the viscosity of the solvent upon heating. As the solvent is heated, the viscosity decreases and the column pressure decreases. The pressure of the system without the column is constant. The column pressure can then be calculated using the following equation:

Column pressure = system pressure with column – system pressure without column

Therefore, temperature is measured indirectly by noting the column pressure. A stable column pressure indicates that the column is at equilibrium. This does not demonstrate, however, that the temperature is uniform throughout the column.

Run 100% water through the column at room temperature. Record the observed pressure and the length of time required for the pressure to stabilize. Replace the column with a union and repeat the experiment to establish the pressure attributed to the system. It takes approximately one minute to achieve stable pressure at 25 mL/min. The column pressure is ~1100 psi. Results are shown in Figure 1.

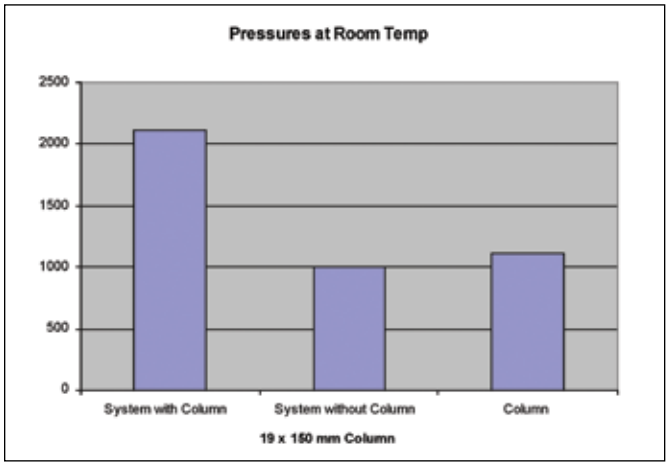

Figure 1. System pressure comparisons at room temperature.

Proposed solution for temperature control

After submerging the column in an equilibrated water bath at 60 °C, as shown in Figure 2, it took approximately two minutes for the pressure to stabilize. A 19 x 150 mm column has a volume of about 36 mL and the flow rate is maintained at 25 mL/min, so this amount of time for pressure stabilization is quite reasonable.

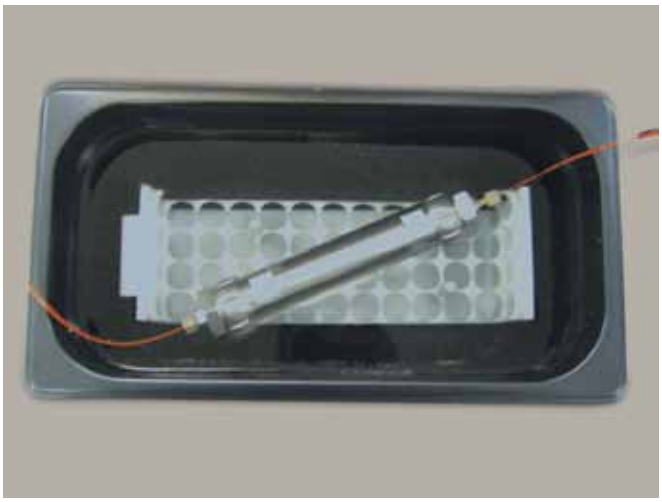

Figure 2. Approximately 2 min is needed to achieve stable pressure at 60 °C with the column submerged in an equilibrated water.

As shown in Figure 3, a 5 mL loop plumbed at the head of the column serves as a solvent preheater. Continuously introducing the solvent at 60 °C brings the column to equilibrium internally while the water bath stabilizes the external column environment. In this case, it takes about 2.5 minutes for the column to come to stable pressure due to the extra volume contributed by the 5 mL loop and because the column is coming to equilibrium. Adding a second 5 mL loop for extended solvent preheating does not change the amount of time for the pressure to stabilize; therefore, one 5 mL loop is sufficient for heating the column uniformly. As shown in Figure 4, the 1100 psi attributed to the column at room temperature drops to 650 psi by putting the column into the water bath. The pressure drops another 100 psi when the solvent is heated before reaching the column. A 100 psi reflects a change in viscosity of about 15%. An additional 5 mL solvent preheating loop has no effect on the column or system pressure.

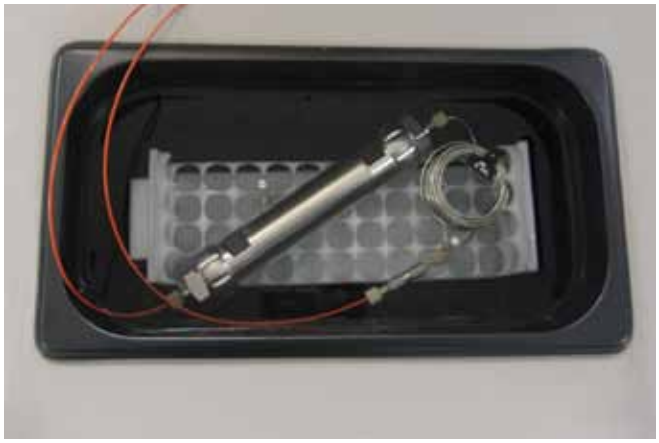

Figure 3. A 5 mL loop plumbed at the head of the column and submerged in the water bath acts as a solvent preheater. Approximately 2.5 min are required to achieve stable pressure at 60 °C.

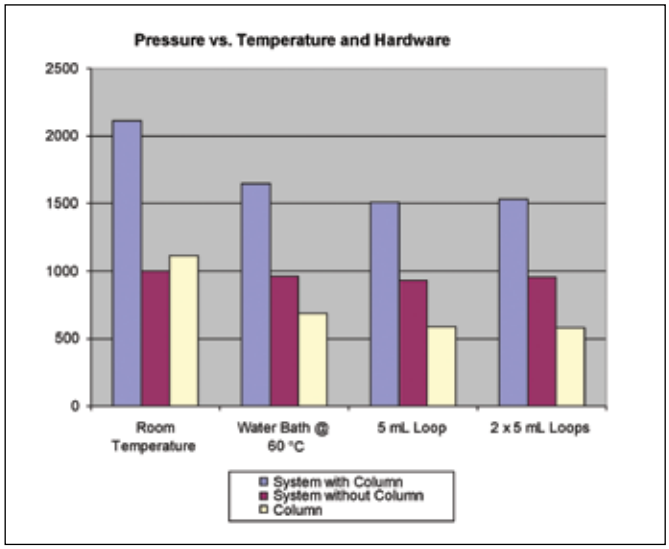

Figure 4. System and column pressure comparison at room temperature and at 60 °C.

Figure 5 shows a comparison of the system equilibration time as it relates to temperature. A column at room temperature takes about a minute to equilibrate to stable pressure; whereas, increasing the temperature of the column with solvent preheating requires the longest equilibration time to ensure equilibrium conditions within the column interior.

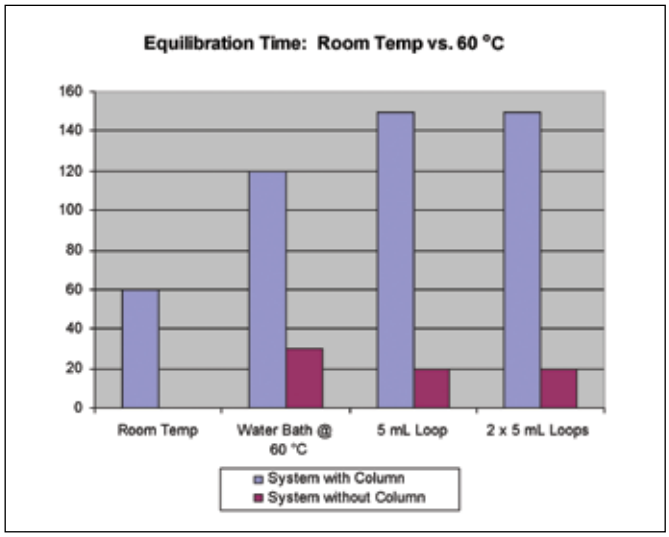

Figure 5. Equilibration time at room temperature and at 60 °C.

Scalability of temperature control

For those instances when pilot scale chromatography will also be performed, the solvent should have the same amount of preheating before entering the analytical column as the solvent does before entering the preparative column. This ensures that the chromatography at both scales will be identical, assuming that the separation is otherwise scaled properly.

To calculate how long the mobile phase is heated at the prep scale, multiply the volume of the preheater loop by the flow rate. A 5 mL loop on a system running at a flow rate of 25 mL/min provides 0.2 min or 12 seconds of residence time for the solvent in the preheater loop. Scaling from 25 mL/min down to 1.46 mL/min, the flow rate at which the pilot scale chromatography will be conducted, is a 17-fold reduction in flow. By proportion, a 12-second residence time for the prep scale calculates to 0.7 second heating time for the pilot scale. At a flow rate of 1.46 mL/min, the volume of the preheating loop needs to be about 17 µL. A 0.010" I.D. tubing has a volume of about 0.4 µL per cm. Therefore, the length of the tubing required to make the pilot scale preheating loop is 42.5 cm. Make the small scale preheating loop with stainless steel tubing to maximize heat transfer. Install the preheating loop at the head of the analytical column and place both the loop and the column in the water bath.

The amount of band broadening attributed to a solvent preheating loop is negligible because the loop is made with narrow inside diameter tubing. Furthermore, temperature control is used most often with gradient methods which reconstitute the sample at the head of the column.

Effect of temperature in compound isolation

Temperature control is most often used to ensure reproducible separations. In isolation and purification it is also valuable for those separations where sample solubility is less than ideal, and in those cases where the mobile phase viscosity is high, resulting in higher system pressure. As shown in Figure 6, a compound mixture with poor resolution at 40 °C has better resolution at 60 °C.

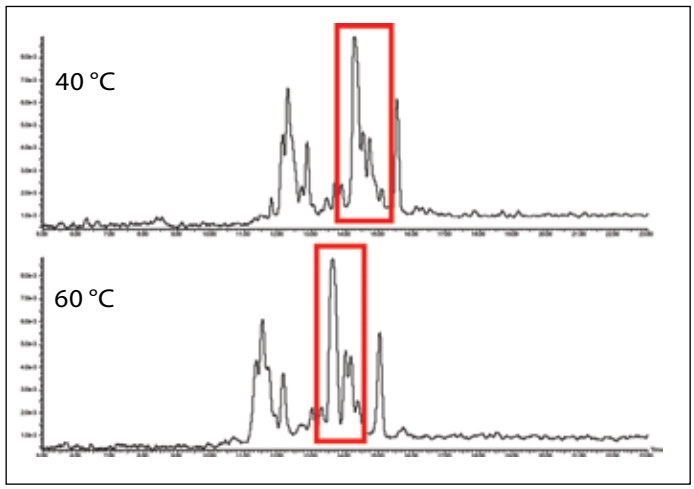

Figure 6. Temperature effect on sample mixture with poor resolution at 40 °C and better resolution at 60 °C.

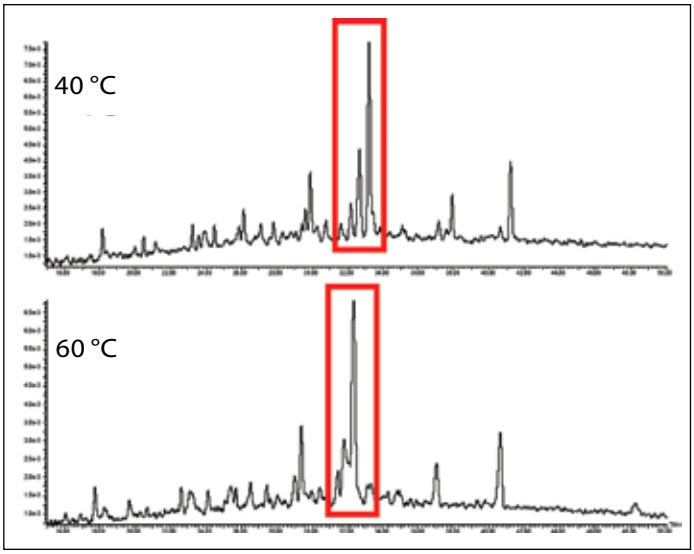

Figure 7. Temperature effect on sample mixture with poor resolution at 60 °C and better resolution at 40 °C.

Some samples give better separations at lower temperature, as shown in Figure 7. These examples illustrate the effectiveness of temperature as a method development tool. An optimized separation makes purification easier and leads to higher product recovery.

CONCLUSIONS

- High flow rate separations occur at the temperature of the incoming solvent.
- Large diameter columns can be effectively heated by using a solvent preheating loop.
- Additional benefits to using higher temperatures include increasing the solubility of hydrophobic samples and improving chromatographic peak shape.
- Improved peak shape leads to higher product purity and recovery, making compound isolation faster and more efficient.

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Impurity Isolation and Scale-up from UPLC Methodology: Analysis of an Unknown Degradant Found in Quetiapine Fumarate

Michael D. Jones and Andrew Aubin
Waters Corporation, Milford, MA, USA

INTRODUCTION

A company that manufactures drug product or drug substance has a vested interest in determining the impurities associated with their compounds. The analysis of impurities can be a very labor-intensive task involving method development, impurity synthesis, isolation techniques, and various analytical approaches to determine the unambiguous identification of the impurity of interest. The lack of a pure impurity can delay a drug development program. Techniques concerning procurement of a targeted impurity are generally based on the project timelines. Impurity synthesis can be a time-consuming process requiring skilled synthetic chemists.

However, purification provides a rapid alternative to chemical synthesis that is appropriately suited to the skill set of an analytical chemist. The purification of an impurity can help with the structural elucidation by providing sufficient material for experiments such as 2D NMR. Also, collection of impurities via purification leads to reference standards of high purity.

UltraPerformance LC® provides a rapid, high-resolution approach to impurity identification and profiling. The process of scaling a UPLC® analytical method to a preparatory method can be a difficult task. Traditionally, scaling from analytical UPLC to preparatory HPLC involves calculations that transfer the flow rate and gradients associated with the original column/particle dimensions to that of the new column/particle dimensions.[1]

In this application note, a strategic approach utilizing high-resolution chromatographic theory and a forced degradation study was applied to maximize the yield of a targeted impurity of the drug substance quetiapine fumarate, an antipsychotic drug. A degradant with *m/z* 402 was found under acid stress conditions (0.1 N HCl) and chosen as the primary target for isolation. Mass-directed purification facilitated this isolation by fractionation collection of the targeted unknown impurity.

EXPERIMENTAL

In transferring the UPLC method to preparative HPLC, three key factors for the new working conditions for scale-up must be considered:

- **Separation efficiency** – L/d_p (column length/particle size) is an indication of the resolving power of the particular column. For example, a 50-mm column with 1.7-µm particles has an L/d_p of 29,411, which is equivalent to a 150-mm preparative column with 5-µm particles and an L/d_p of 30,000.
- **Productivity** – Can a shorter preparative column be utilized? In the separation of impurity *m/z* 402, a 100-mm prep column could still provide enough column efficiency to adequately isolate the impurity.
- **Column volumes** – If each of the gradient segments is scaled appropriately to maintain the equivalent number of column volumes between UPLC and preparative HPLC, the separation profile will be preserved *considering there is no change in stationary phase composition.*

Analytical conditions

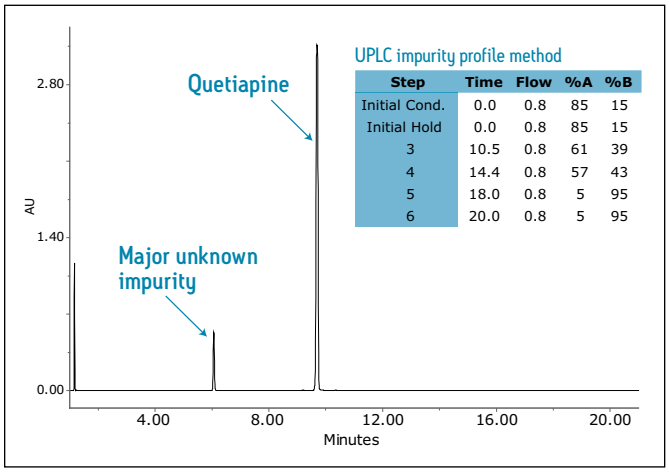
LC system:	ACQUITY UPLC®
Column:	ACQUITY UPLC BEH C_{18} 2.1 x 50 mm, 1.7 µm (optimized)
Column temp.:	Ambient
Mobile phase A:	10 mm Ammonium bicarbonate, pH 9.0
Mobile phase B:	Acetonitrile
Flow rate:	800 µL/min
Gradient (Starting):	See Figure 1
MS system:	ACQUITY® SQD
Ionization mode:	ESI positive
Capillary voltage:	1500 V
Cone voltage:	35 V
Desolvation temp.:	450 °C
Desolvation gas:	900 L/Hr
Source temp.:	150 °C
Acquisition range:	50 to 600 *m/z*

Preparative conditions

LC/MS system:	AutoPurification™ MS
Pump:	2545 Binary Gradient Module
Injector/Collector:	2767 Sample Manager
UV Detector:	2998 Photodiode Array Detector
MS Detector:	3100 Mass Detector
Column:	19 x 100 mm XBridge,™ 5 µm
Solvent A:	10 mm Ammonium bicarbonate, pH 9.0
Solvent B:	Acetonitrile
Flow rate:	25 mL/min
Gradient:	5% to 60% B over 10.5 min, flushed for approx 5 min 95% organic
Data management:	FractionLynx™ Application Manager for MassLynx™ Software

RESULTS AND DISCUSSION

A forced degradation was performed on the drug substance quetiapine. During the study, major degradants were formed for each of the various stress conditions. An impurity profile utilizing UPLC, optimized to produce maximum resolution with a 2.1 x 100 mm, 1.7 µm ACQUITY UPLC BEH C_{18} Column, was used to search for the presence of any degradants (Figure 1). Each of the major degradants was assessed by its *m/z* ratio as reported by the ACQUITY SQD single quadrupole mass detector. Peaks with masses of particular interest needed to be isolated and characterized.



Step	Time	Flow	%A	%B
Initial Cond.	0.0	0.8	85	15
Initial Hold	0.0	0.8	85	15
3	10.5	0.8	61	39
4	14.4	0.8	57	43
5	18.0	0.8	5	95
6	20.0	0.8	5	95

Figure 1. Acid hydrolysis results utilizing a highly-specific UPLC impurity profile that is optimized for maximized resolution, using a 2.1 x 100 mm, 1.7 µm ACQUITY UPLC BEH C_{18} Column.

Importance of an efficient impurity isolation process

The method was developed on an ACQUITY UPLC BEH C_{18} Column and allowed for seamless transfer to an XBridge C_{18} preparatory HPLC Column. Both XBridge HPLC particles and 1.7-µm ACQUITY UPLC BEH particles feature the same BEH Technology.

The mathematical calculations for scaling the impurity profile's UPLC methodology to preparatory conditions did not allow for an efficient isolation procedure. The L/d_p geometric scaling of the column used in the original UPLC impurity profile method resulted in a 300-mm preparatory column, as represented by the calculation:

For original UPLC quetiapine impurity profile:

$$L/d_p = 100 \text{ mm}/1.7 \text{ µm} = 58{,}823$$

Transfer to prep to maintain resolving power:

$$L/d_p = (X) \text{ mm}/5 \text{ µm} = 58, 823$$

$$X = 294 \text{ mm} \approx 300 \text{ mm column}$$

Based on this L/d_p calculation, applying the geometrically-scaled run time would result in a 60-minute preparative method – which would be excessive when considering the number of injections required to isolate milligrams of the target compound from the enriched sample preparation, thus wasting considerable time and solvent. In addition, the geometrically-scaled flow rate of 65.5 mL/min for a 19 x 300 mm column would overpressure the instrument (Figure 2). Therefore, it was necessary to make adjustments to the original UPLC impurity profile to enable a seamless scale-up.

UPLC impurity profile method

Step	Time	Flow	%A	%B
Initial Cond.	0.0	0.8	85	15
Initial Hold	0.0	0.8	85	15
3	10.5	0.8	61	39
4	14.4	0.8	57	43
5	18.0	0.8	5	95
6	20.0	0.8	5	95

Geometrically-scaled method

Step	Time	Flow	%A	%B
Initial Cond.	0.0	65.488	85	15
Initial Hold	0.3	65.488	85	15
2	31.8	65.488	61	39
3	43.5	65.488	57	43
4	54.3	65.488	5	95
5	60.3	65.488	5	95

Figure 2. Geometrically-scaled results for the preparative gradient based on quetiapine's impurity profile under UPLC gradient methodology. Calculations were performed using the Waters OBD™ Prep Calculator.

Re-optimizing for efficiency

The impurity method used to monitor the presence of degradants generated from the forced degradation study was highly specific. The forced degradation resulted in the production of only one major impurity peak with ample resolution from the active pharmaceutical ingredient (API). The decision was made to modify the impurity profile method itself to optimize for speed, while maintaining baseline resolution of the impurity peak of interest.

As stated earlier, impurity procurement is dictated by project timelines. Re-optimizing the impurity profile method to a more generic gradient satisfied analytical needs such as faster run time, lower temperature, and shorter column length (50 mm), while maintaining adequate resolution of the major impurity from quetiapine. By reducing analysis times associated with isolation, we also benefit from decreasing solvent consumption, reducing the amount of waste, and increasing sample production per unit of time.

The resulting re-optimized UPLC method utilized the same method parameters: ammonium bicarbonate at pH 9.0, ACQUITY UPLC BEH C_{18} chemistry, and acetonitrile. The peak purity of the resulting chromatogram (Figure 3) was verified by examining the mass spectral information provided by the ACQUITY SQD.

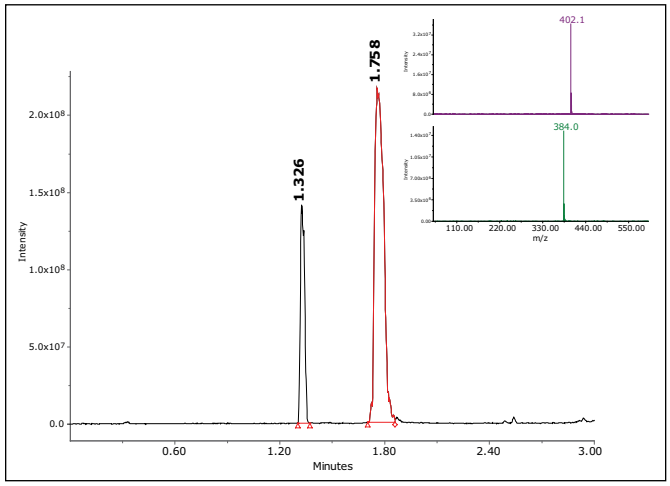


Figure 3. Re-optimized UPLC method for the peaks of interest. The resulting conditions provide an easier and more efficient solution for scaling to preparatory HPLC.

The new UPLC method with a 50-mm column provided rapid determination of the ability to use a shorter preparative column to maintain resolution. The new method resulted in an L/d_p value of 29,411, which would require a preparative column of 150 mm and a 5-µm particle size. However, after reviewing the data the observed resolution is quite large between the peaks of interest. Based on this observation, a shorter 19 x 100 mm preparative column was chosen to further decrease the preparative cycle time.

Optimizing for efficient preparative isolation

The gradient conditions for the preparative isolation analysis were optimized to maintain the same number of column volumes per gradient segment as used with the UPLC methodology. It was observed that the last peak eluted in less than two minutes, resulting in a 60% acetonitrile composition to elute all components in the re-optimized UPLC method. This observation allowed us an approach that would decrease the run time of the preparative method by applying one focused gradient segment. This practice is similar to an approach using multiple focused gradients to facilitate impurity isolation of many closely-resolved peaks.[2,3]

Step-by-step calculations were followed to determine the gradient segment durations needed for use with a 19 x 100 mm column flowing at 25 mL/min. The resulting preparative gradient is represented in Figure 4.

UPLC Column 2.1 x 50 mm, 1.7 µm	Time (min)	Flow Rate (mL/min)	%A	%B	Segment Duration Time (min)	Segment Duration (c.v)
	0.0	0.8	95	5	0	0
	2.0	0.8	40	60	2	9.25
	3.0	0.8	5	95	1	4.62

Calculated migration from UPLC to prep

Prep Column 19 x 100 mm, 5 µm	Time (min)	Flow Rate (mL/min)	%A	%B	Segment Duration Time (min)	Segment Duration (c.v)
	0.0	25	95	5	0	0
	10.5	25	40	60	10.5	9.25
	15.7	25	5	95	5.2	4.62

Figure 4. Final gradient table used to transition from UPLC to preparative analysis.

Mass-directed autopurification

The isolation of the major impurity *m/z* 402 was facilitated both analytically and chemically. In order to maximize production yield of the degradant present during the hydrolysis-forced degradation study, a stock solution of 8 g/mL quetiapine was refluxed in 0.1 N HCl for eight hours to increase the abundance of the impurity. A preparative load study allowed for 5-mL injections on-column. Together, the sample preparation and load study reduced the number of injections needed to isolate a sufficient amount of the impurity substance required for NMR analysis, while still maintaining resolution of the impurity without interferences (Figure 5).

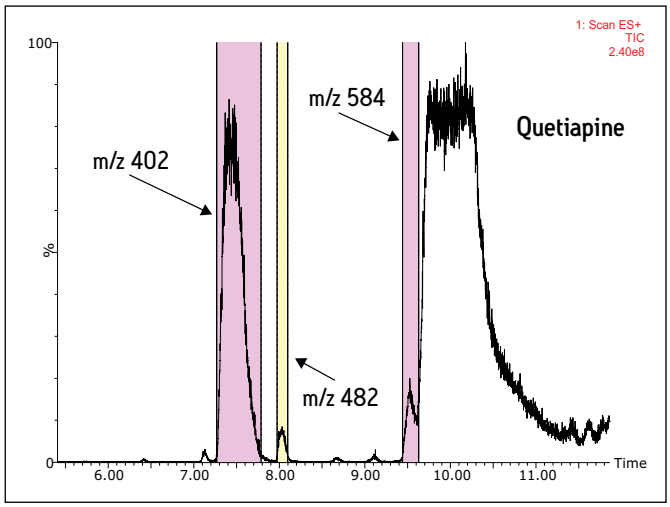


Figure 5. Preparative chromatogram of the forced degradation sample.

Using this instrumentation, automatic isolation was performed by mass triggering using the FractionLynx Application Manager for MassLynx Software. The specificity and purity of the mass triggering process was verified by UPLC/MS using the ACQUITY SQD System for the fraction analysis of the target impurity peak (Figure 6).

Re-optimizing for the impurity of interest via UPLC provided rapid methods for further analysis, such as for confirmation by UPLC/oa-Tof MS and UPLC/MS/MS.[4]

Confirmation of isolation

The isolated fractions collected for *m/z* 402 by the mass-directed purification system were pooled and evaporated to dryness. It was determined that the isolation process yielded 28.6 mg of impurity *m/z* 402. A stock solution was prepared at a concentration of 286 µg/mL, diluted to 2.86 µg/mL in methanol, and injected using the 3-minute UPLC/MS method to determine quality of the resulting isolation (Figure 6).

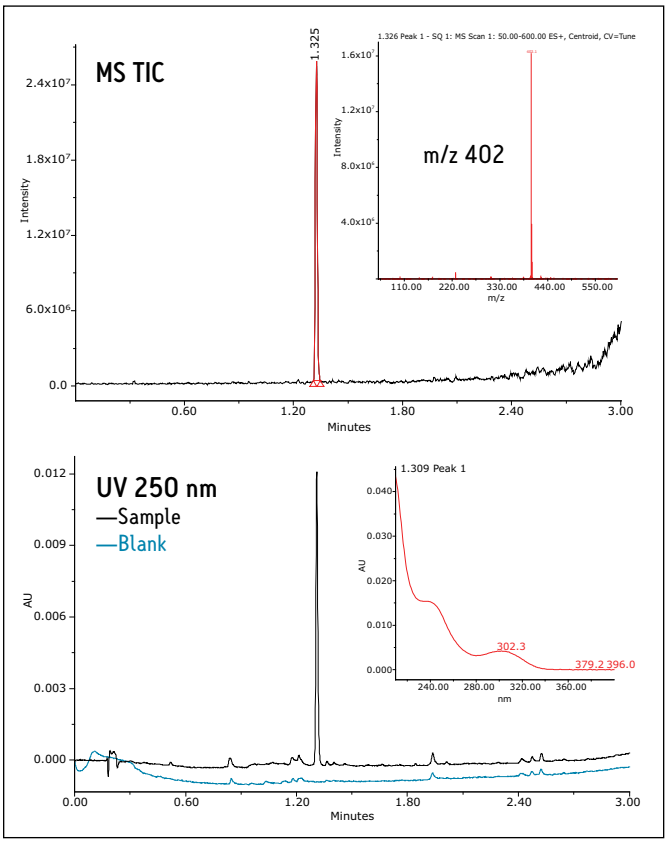


Figure 6. UPLC/MS confirmation of isolated impurity m/z 402. The resulting pooled fraction was determined to be pure, based on the MS and UV spectra that indicated zero presence of foreign substances.

CONCLUSIONS

Analytical benefits

- Maintaining L/d_p ratio was a driving factor to best manage scale-up possibilities.
- The use of a common chemistry platform for analytical-scale UPLC and preparative-scale HPLC is essential for maintaining selectivity.
- Utilizing forced degradation techniques increases production that maximizes yield.
- Mass-directed collection of the fractions assures high purity during impurity collection.
- ACQUITY SQD provides rapid confirmation of the purity composition of the pooled fractions.

Time and fiscal savings

Waters software solutions streamlined data processing and calculations:

- The dedicated FractionLynx browser presents sample and fraction information in a single, interactive location that reduced time needed to decipher data and fraction location.
- The Waters OBD Prep Calculator, a free download, facilitates scaling calculations (**www.waters.com/prepcalculator**).

Re-optimizing the UPLC method prior to preparative-scale fraction collection provided:

- A rapid and highly-specific method that utilizes less solvent and increases confidence in fraction purity.
- A scaled preparative method that requires less analysis time, considerably less solvent consumption, and less waste.
- Significant savings: 30% less time, 60% less solvent.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

A Novel Approach for Reducing Fraction Drydown Time

Paul Lefebvre, Ronan Cleary, Warren Potts, and Robert Plumb
Waters Corporation, Milford, MA, USA

INTRODUCTION

Recent advances in purification technology have shifted the throughput bottleneck from purifying samples to fraction drying. Some of the technologies employed for sample drying include vacuum centrifugation, heated nitrogen blow-down, and lyophilization. However, each one has the same rate limiting factor – the quantity of water present. This quantity is dependant on the separation technique used to generate the fractions. The most commonly used technique is reverse phase- (RP-) HPLC, which can generate fractions with the water content as great as 95%.

One experimental approach is to collect fractions directly onto solid phase extraction (SPE) cartridges. In theory, this method is perfect, but making it automated and rugged has continued to be a challenge. A drawback to this approach is that a very high flow dilution pump is required to trap the compound on the cartridge. This high flow rate requires a large quantity of sorbent with large volume cartridges, and generates large volume fractions. Another problem with collection onto SPE cartridges is the possible change in selectivity that could result in poor trapping or breakthrough of the analyte.

This application note shows the development and optimization of a method that removes the water and reduces the overall volume of the collected fraction. This method works by injecting and trapping the previously collected fraction onto a preparative column. The fraction is trapped by diluting the loading flow with 100% aqueous mobile phase. After the trapping has been completed, 100% organic mobile phase is passed through the column to elute the sample. Collection of the target is triggered by the MS detector and the collected fraction is now in 100% organic mobile.

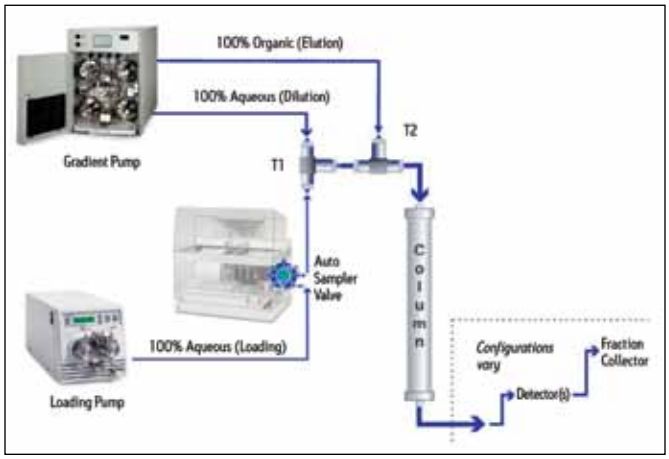


Figure 1. Plumbing diagram for the concentration system. Both fraction collection and concentration was performed on the same mass-directed AutoPurification System. Fraction collection was triggered by MS.

METHOD CONDITIONS

The standard components of the Waters® AutoPurification™ System were used to perform the fraction concentration. In the plumbing diagram shown in Figure 1, the aqueous flow out of the gradient pump is directed into the first tee (T1). This tee acts as a mixer, diluting the organic concentration of the injected fraction, so that it will not break through the trapping column. The organic flow out of the gradient pump is directed to a second tee (T2) and is used to elute sample from the column.

Proof of principle

To establish a baseline performance of the method parameters, 10 drug-like compounds were initially purified. These purified fractions were collected in different concentrations of organic solvent and then used as the samples to evaluate the concentration method. The samples were loaded onto a trapping column and eluted in 100% organic solvent. Once it was determined that the initial method was successful, the process was optimized for minimum fraction volume and maximum throughput. The examples shown have initial fraction volumes as great as 30 mL of aqueous/organic and are reduced to as little as 1.5 mL of organic solvent.

Purification method

- 10 mg sample load
- Generic 5% to 95% gradient with water/ACN/formic acid
- Fraction volume of 5 to 8 mL with recoveries of greater than 95%

	Time (min)	Flow (mL/min)	%A	%B	Curve
1	Initial	20.00	95.0	5.0	Initial
2	1.00	20.00	95.0	5.0	6
3	8.00	20.00	5.0	95.0	6
4	8.50	20.00	95.0	5.0	11
5	10.00	20.00	95.0	5.0	6

Figure 2. Generic 5% to 95% gradient.

Concentration method

The collected fractions were injected onto the same column as was used for purification. The samples were loaded onto the column with a loading pump at 6 mL/min 100% A, and 29 mL/min aqueous from a dilution pump. After 6.5 min, the loading and dilution flow is stopped. Now that the sample is retained on the column, the elution is started at 29 mL/min of 100% B.

RESULTS AND DISCUSSION

Although the remaining sample was purified using the SunFire™ Column, it was not retained on the column during the concentration process. However, because fraction collection was triggered by MS, no sample was lost. Additional work is required to determine why it was not retained.

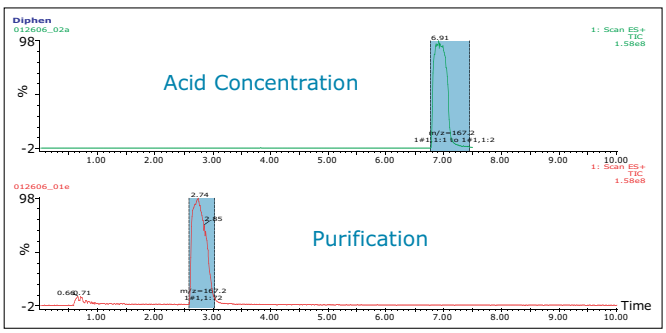


Figure 3. Seven of the 10 were successfully concentrated in the acidic mobile phase in which they were collected. All recoveries were greater than 85%.

Method optimization

Once the trapping method was determined to be successful, we looked into optimizing the conditions. The parameters evaluated included the column dimension and packing, the dilution ratio, and the elution flow rate. An initial fraction of 10 mg of diphenhydramine collected in 8 mL of 60% water was the concentration test sample.

Column dimensions

The column must be able to trap the target fraction and yet give a minimum elution volume for the concentrated fraction.

The maximum flow rate and the minimum loading time were determined to establish a minimum run time. These factors are dependant upon the column I.D., particle size, and injection loop.

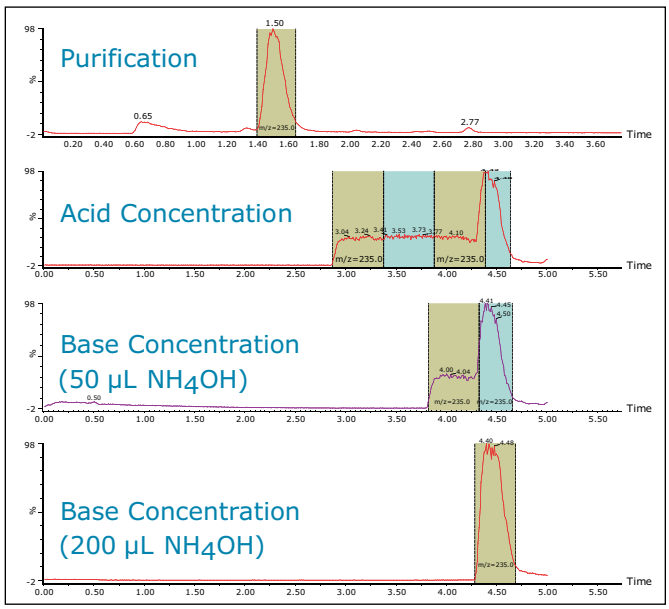


Figure 4. Two of the remaining three were successful after adding base to fraction. This indicates that these samples should have been purified at a basic pH to keep the target neutral.

19 x 50 mm trap column

5 and 10 µm packing gave the same fraction volume. The only difference was the system back pressure.

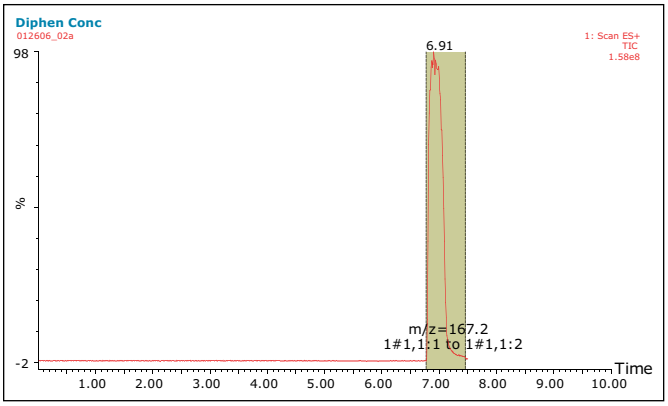


Figure 5. Concentration of the test fraction on a 19 x 50 mm column.

10 x 50 5-µm trap column

The overall flow rate was reduced when the method was transferred to the 10-mm column. By reducing the elution flow rate from 24 to 12 mL/min, the concentrated fraction volume was reduced from 8 mL to 2.9 mL.

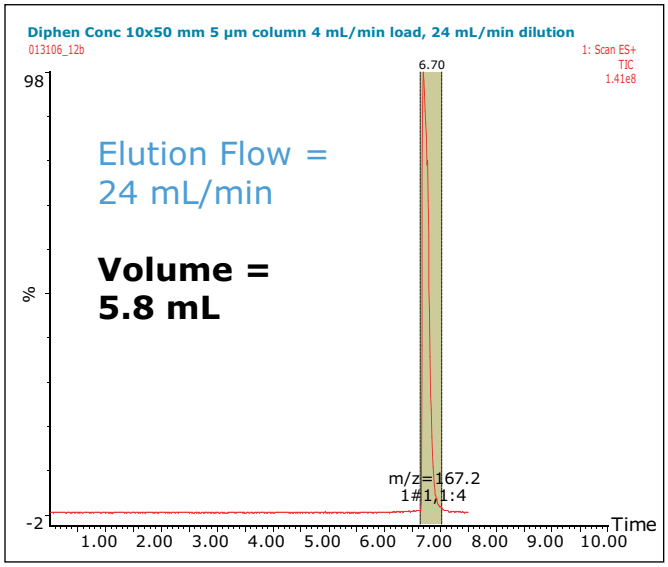
By reducing the flow rate even further to 8 mL/min, the original 8 mL of 60% water was reduced to 1.6 mL of 100% organic solvent.

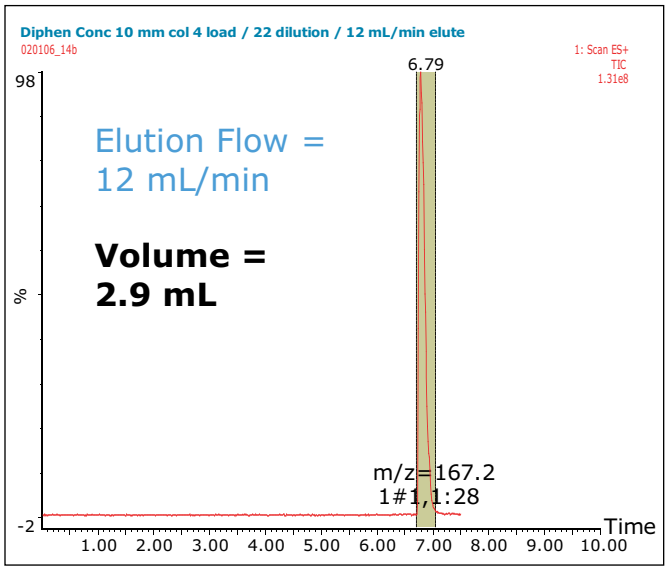
There is minimal loss of the overall speed of the analysis with the reduced elution flow rate. The loading and dilution pumps operate at 24 and 4 mL/min, respectively, until 6.5 min. The flow rate was then reduced to the lower elution flow, accounting for the smaller volume, concentrated fractions.

Improving throughput

Sample loading rules

- The injection volume must be less than half the volume of the sample loop. Because the injection volume was 8 mL, the minimum loop volume was found to be 15 mL.
- Three- to five-times the loop volume is required to clear the sample from the sample loop. The minimum volume found to clear all the sample was 45 mL.





Figures 6 and 7. Elution flow was reduced with minimal adjustment to peak width.

Dilution ratio

The dilution ratio (dilution flow/loading flow) is a critical factor in this method. The dilution ratio is a measure of the amount of aqueous solvent used to dilute the fraction's organic content to allow it to be trapped onto the column. If the dilution ratio is too small, it will cause breakthrough. If it is too large, it will decrease the throughput because of the additional time required to load the sample. Figure 9 shows the effect of the concentration with varying dilution ratios. The results show that, at a ratio of 4.5, there is a jagged breakthrough of the target compound that is not present at a ratio of 5 or higher.

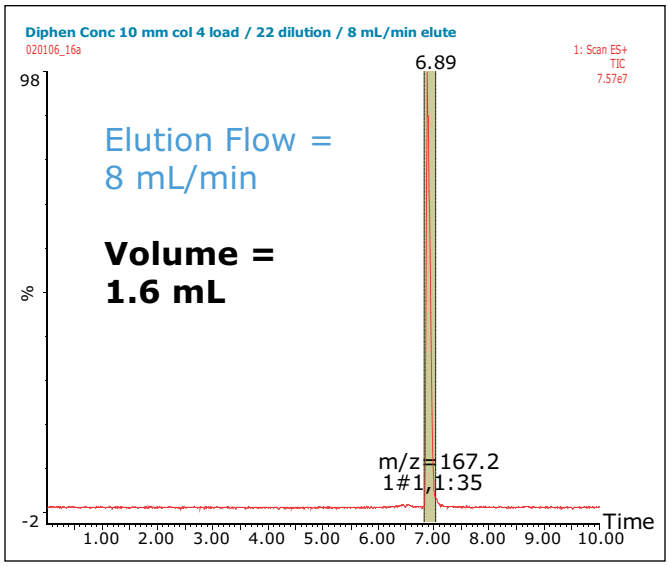


Figure 8. Concentration of the test fraction on a 10 x 50 mm 5 µm column at an elution flow rate of 8 mL/min.

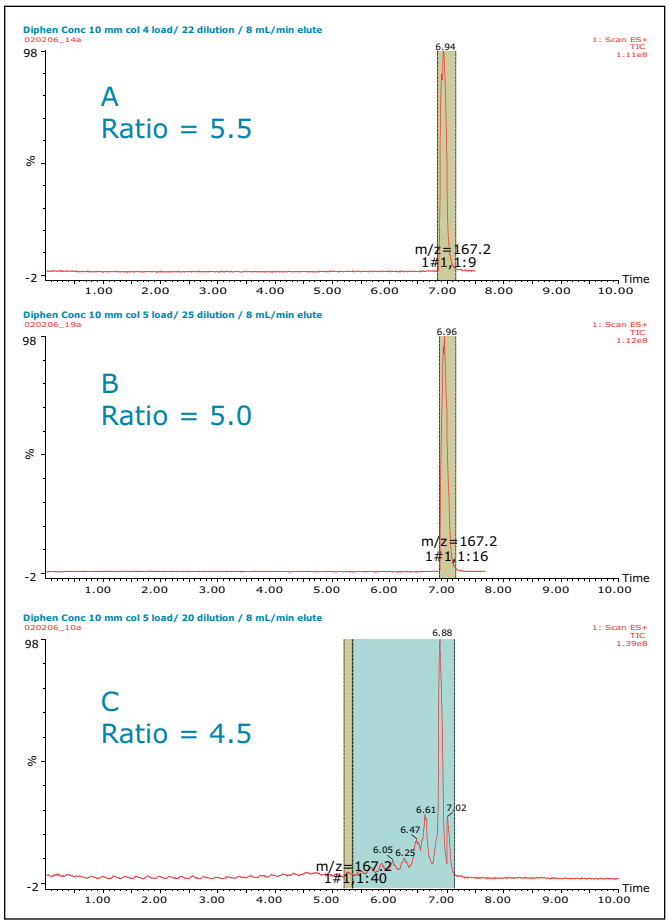


Figure 9 A-C. Concentration of test fraction with varying dilution ratios.

Scaling the method

Based on the minimum loading time and dilution ratio, it is possible to establish the relationship between the loading time and the total flow rate (Table 1).

To reduce the loading time to less than 5 min, the table shows that a loading and dilution flow of 10 and 50 mL/min, respectively, are required. This gives a total flow of 60 mL/min across the column.

Loading Flow (mL/min)	Loading Time (minutes)	Dilution Flow (mL/min)	Total Flow (mL/min)
5	9.0	25	30
6	7.5	30	36
7	6.43	35	42
8	5.63	40	48
9	5.0	45	54
10	4.5	50	60
15	3.0	75	90
20	2.25	100	120

Table 1. Relationship between loading time and total flow.

Handling increased back pressure

- Increase the particle size: a 2x increase equals a quarter of the back pressure
- Waters SunFire Column, 10 x 50 mm, 10 µm
- 60 mL/min only generated 2300 psi

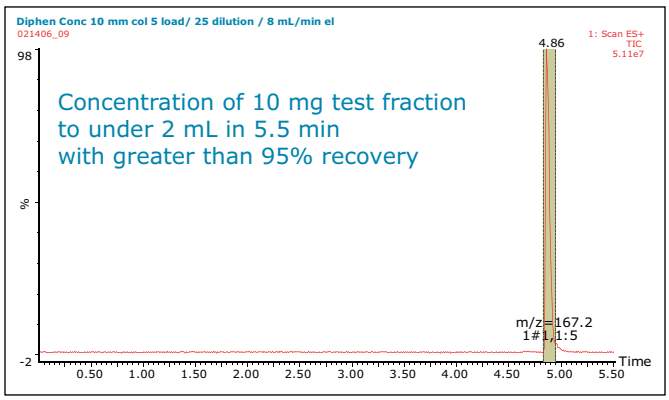


Figure 10. Results from the optimized method.

Mass load

One concern with these optimized parameters is the mass load on the smaller trapping column. To evaluate this, the compounds were purified with increasing mass load on the preparative column until overload conditions were achieved. The collected fractions were concentrated using the optimized method. Two examples are shown.

Example 1: 60 mg of Ketoprofen

The initial purification generated a 10 mL fraction containing about 50% water. The concentration method successfully reduced the volume to 3.6 mL of organic solvent with a recovery greater than 95%.

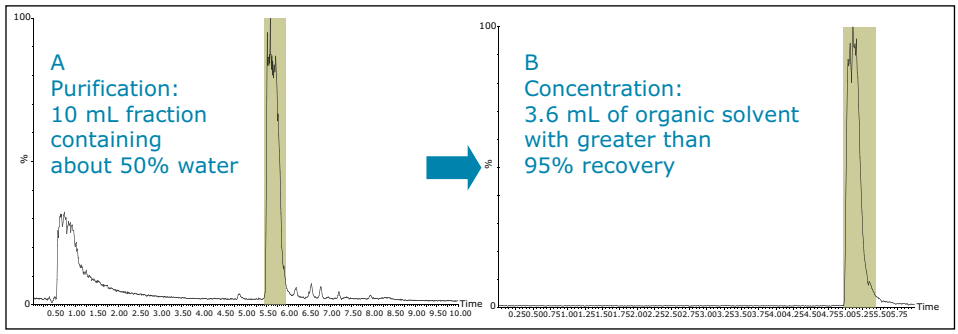


Figure 11 A-B. The purification and concentration of 60 mg of ketoprofen.

Example 2: 20 mg Phenyl-tetrazole

The purification generated two fractions with a total volume of 18 mL containing about 60% water. The concentration successfully reduced the volume to 3.2 mL of organic solvent with the recovery greater than 95%.

When the chromatography begins to overload for the purification on a 19 x 50 mm column, the fraction will not be completely trapped on the 10 x 50 mm column.

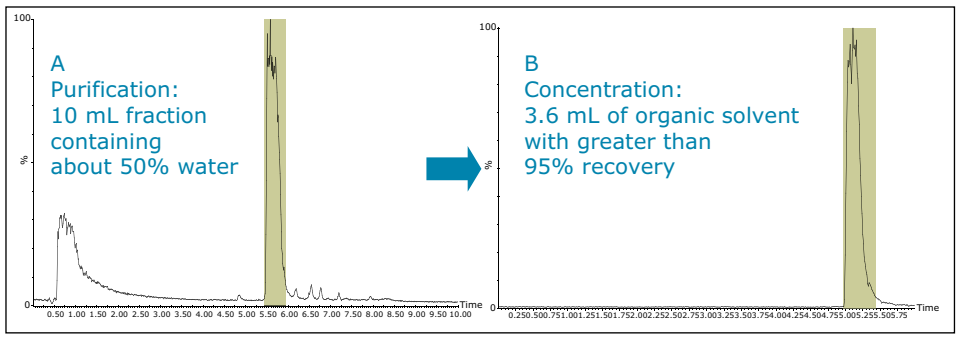


Figure 12 A-B. The purification and concentration of 20 mg of phenyl-tetrazole.

Automatic pooling

Fraction pooling on the trapping column can also increase throughput. In Figure 13, a 3-mL fraction was collected for each of the 10 injections. The fractions were then individually loaded onto the trap column and concentrated. A single 1.5 mL fraction was collected.

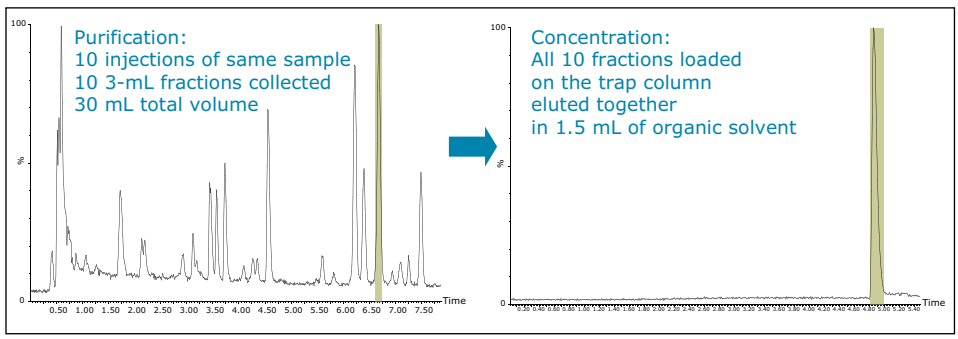


Figure 13. An example of automatic pooling of 10 fraction tubes into a single concentrated fraction.

CONSIDERATIONS

The pKa of the target compound should be considered when performing purification. The target compound should be neutral during the purification. This means that a basic compound should be run in a basic mobile phase and, conversely, an acidic target should be run in an acidic mobile phase.

This will result in better loading and chromatography[1] and will also ensure that the collected fraction is not ionized in solution. By being neutral, it is more likely to be successfully trapped during the concentration process.

The amount of collected material, in both mass and volume, will dictate the required system configuration. The volume of the fraction will determine the size loop required. The mass of collected material will determine the column size. Both the loop and column size will determine the overall throughput of the system.

In the examples shown, all of the concentrated fractions were triggered by MS. However, this was done only for method development purposes. It is possible to collect these fractions by UV or just by time. When collecting by time, each tube has the same volume and organic concentration, so the time required for drying is constant. With typical fractionation, each tube can have a different volume and organic concentration, so the time required for drying is variable. This variability can lead to inefficiency, by either drying too long, or by stopping too early then checking multiple tubes to find that you need to restart for only a few of the tubes.

BENEFITS

Dry-down time

Composition	Volume	Dry Down
Aqueous/Organic	5 to 30 mL	5 to 15 hours
100% organic	1 to 3 mL	less than 30 min

Concentrating the fraction to about 3 mL of organic solvent can be accomplished in 6 min.

Process enhancements

- Shorter drying times equals more efficient use of the driers.
- Automatic pooling of multiple fraction tubes reduces the post-purification sample handling.

Acknowledgements

- Ian Sinclair, AstraZeneca
- Giovanni Gallo, Waters, Manchester, UK
- Paul Rainville, Waters, Milford, MA

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The Application of MS/MS Directed Purification to the Identification of Drug Metabolites in Biological Fluids

Paul Lefebvre, Robert Plumb, Warren Potts, and Ronan Cleary
Waters Corporation, Milford, MA, USA

INTRODUCTION

The identification of drug metabolites following animal or human volunteer studies is essential to drug discovery and development as well as the regulatory submissions process. Traditionally, this has been achieved by the use of liquid or gas chromatography coupled to mass spectrometry.[1,2] More recently, the use of hyphenated techniques such as LC/NMR and LC/NMR/MS have become more commonplace in the drug metabolism laboratory, allowing a more precise identification of the site of metabolism.[3,4]

While LC/NMR and LC/NMR/MS are extremely powerful tools, they are typically low throughput and limited in sensitivity. The capacity of analytical columns restricts the amount of material that can be loaded onto the column before the column exhibits either volume or mass overloading effects and the chromatographic resolution is lost. Thus, LC/NMR is less attractive for the analysis of highly potent compounds dosed at low levels or those compounds that undergo extensive metabolism. In such cases, it is often necessary to perform a pre-concentration step, such as SPE or liquid/liquid extraction, both of which are time consuming and run the risk of losing of valuable information.

The use of MS-directed purification, using semi-preparative scale columns (typically 19 mm I.D.), is now commonplace within the pharmaceutical industry, especially to support lead candidate purification. This approach has also been applied to the isolation of drug metabolites with some success.[5] The extra sensitivity and selectivity of MS/MS mass spectrometry allows for more precise selection of drug metabolites. Furthermore, the use of neutral loss and precursor ion scanning detection modes facilitates the collection of drug metabolites without the need for prior knowledge of compound metabolism.

Figure 1. The Alliance HT System with the Quattro micro Mass Spectrometer.

This application note shows how tandem quadrupole mass spectrometry has been employed for the isolation of the metabolites of common pharmaceuticals from urine. The application also demonstrates different modes of data acquisition, including scan, MRM, constant neutral loss, and precursor ion. We also demonstrate how the use of MS/MS-directed purification facilitates the combination of samples from several chromatographic runs.

EXPERIMENTAL CONDITIONS

A Waters® Alliance® HT System was used with a SunFire™ C_{18} 5 µm 4.6 x 100 mm Column at 40 °C. Eluent flow was split 1:20 with a Valco tee. 95% of the flow passed the 2996 Photodiode Array (PDA) Detector to the Fraction Collector III. The other 5% of the flow was routed directly to the Quattro micro™ Mass Spectrometer equipped with an ESCi® multi-mode ionization source.

METHOD CONDITIONS

Caffeine metabolites methods

Separation

Water/acetonitrile in 0.1% formic acid, 1.25 mL/min total flow gradient. 0 to 5 min: 0%; 5 to 35 min: 0% to 10% B; 35 to 35.5 min: 10% to 95% B; 35.5 to 39.5 min: 95% B; 39.5 to 40 min: 95% to 5% B; 45 minutes end.

MS detection

Electrospray positive, 3 kV capillary voltage, 30 V cone voltage, 20 V collision energy (for MS/MS experiments).

Metabolites of interest

Figure 2 shows a portion of the caffeine metabolism pathway by demethylation.[6] Target metabolites maintain the methyl group in position 1. They also have a common fragment ion, *m/z* 57.

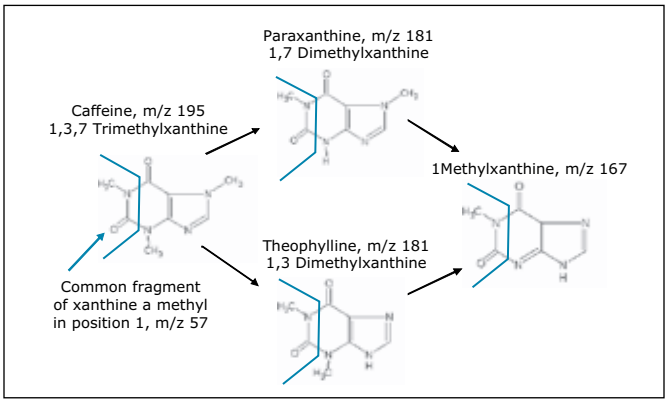

Figure 2. Metabolism of caffeine by demethylation: metabolites that maintain the methyl group in position 1 have a common fragment ion, m/z 57.

Ibuprofen metabolites

Separation

Water/acetonitrile/10 mM ammonium formate, 1.25 mL/min total flow gradient. 0 to 5 min: 5%; 5 to 35 min: 5% to 60% B; 35 to 35.5 min: 60% to 95% B; 35.5 to 39.5 min: 95% B; 39.5 to 40 min: 95% to 5% B; 45 minutes end.

MS detection

Electrospray negative, 3 kV capillary voltage, 30 V cone voltage, 20 V collision energy.

RESULTS AND DISCUSSION

Metabolites of interest

Figure 3 shows the fragmentation patterns of the ibuprofen gluceronide metabolite.[7]

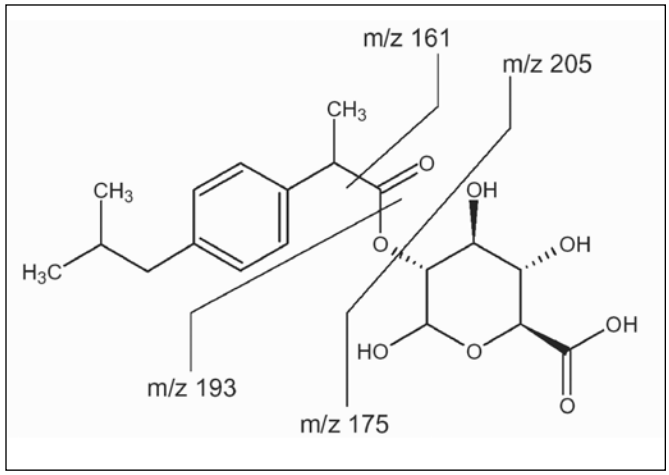

Figure 3. Ibuprofen gluceronide metabolite with a common product ion of m/z 193.

Single quadrupole directed purification

With single quadrupole directed purification, all ions generated in the source are passed through the quadrupole and detected. This is possible on the Quattro micro Mass Spectrometer by using the scan mode of acquisition. Only MS1 is scanned and there is no collision energy or scanning of Q3.

Because all of the ions generated are detected in this mode, complex mixtures can contain numerous isobaric interferences. Consequently, multiple fractions can be generated from a single *m/z* value. Figure 4 shows the collection of the caffeine metabolites with *m/z* 167 and 181 detected using only the first quadrupole. There are eight fractions collected for *m/z* 167 and five fractions collected for *m/z* 181, with additional analysis required to determine the fraction of interest.

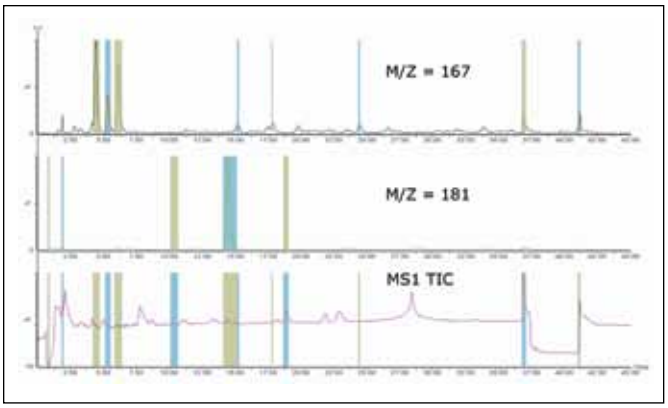

Figure 4. Fractionation based only on scanning the first quadrupole.

Tandem quadrupole directed purification: MRM collection

With multiple reaction monitoring (MRM) data acquisition, MS1 is pre-selected on the precursor mass and MS2 is pre-selected on a specific product ion, as illustrated in Figure 5.

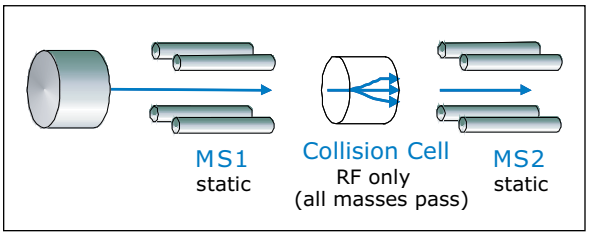

Figure 5. MS/MS MRM data acquisition.

By selectively detecting a product ion, the signal-to-noise ratio is optimized, thus reducing the isobaric interference and allowing only the target to be collected. This mode of acquisition requires previous knowledge of the exact precursor and the exact product ions before purification.

Figure 6 shows the MRM acquisition and collection of the caffeine metabolites. The metabolites of interest for isolation have the transitions of 181 to 134, and 167 to 110.

For a peak to be present in the MRM chromatogram, both the specific precursor and the specific product ion need to be detected. For each target, only one fraction was collected.

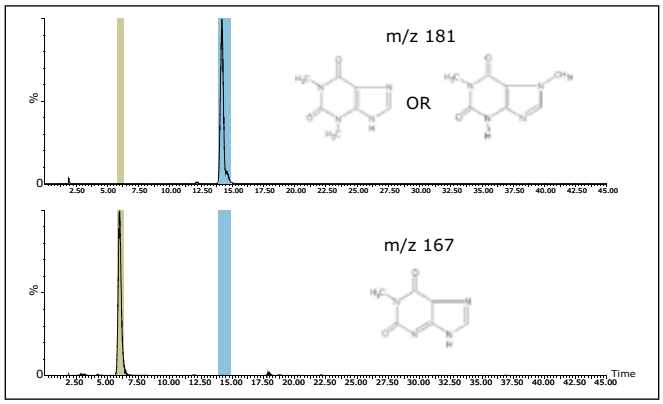

Figure 6. Fractionation based on MRM acquisition.

Constant neutral loss collection

A second possible mode of fraction triggering is from constant neutral loss acquisition. Here both MS1 and MS2 are scanned in synchronization, as illustrated in Figure 7. When MS1 transmits a specific precursor ion, MS2 looks for a product that is the precursor minus the neutral loss value. If the correct product is present, it registers at the detector. The constant neutral loss spectrum shows only the masses of all the precursors that lose the specific mass.

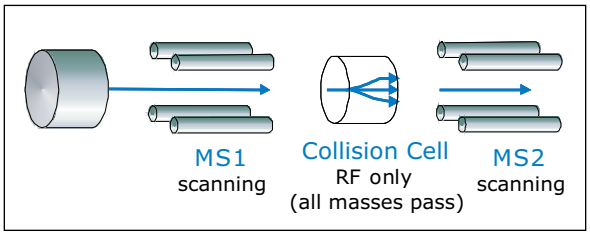

Figure 7. MS/MS constant neutral loss data acquisition.

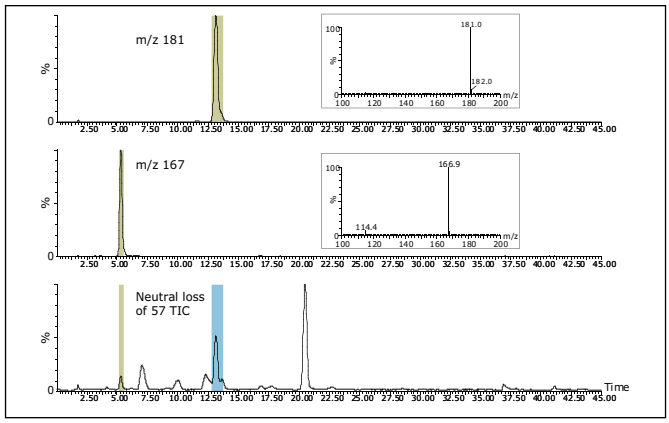

Figure 8. Fractionation based on constant neutral loss acquisition.

Figure 8 displays the constant neutral loss of 57 acquisition and collection of the caffeine metabolites with *m/z* 167 and 181. It shows that two fractions are collected, one for each mass. These fractions contain the target mass and have the specific neutral loss.

Applications for fraction collection from constant neutral loss acquisition

Mass triggered collection

With constant neutral loss acquisition, the only peaks detected are the ones with the loss of the specific mass, in this case, 57. Depending on the specificity of the loss, numerous ions can be detected. This leads to complex total ion chromatograms. Therefore, when triggering by a specific mass, the collected target must contain the precursor of interest and have a specific neutral loss.

Collection triggered on TIC

When using this mode of acquisition and collection, all the peaks with a specific neutral loss are collected. This functionality is valuable when the metabolites have a specific loss related to the drug's structure. It could also be used for isolating a class of metabolites with a generic loss (e.g., sulfates (–80) or glucuronides (–176)). The precursor mass for each fraction can then be extracted and used to aid in the identification of the metabolites.

In the constant neutral loss example shown, collection could also have been triggered from the total ion chromatogram (TIC). All peaks in the –57 TIC would be collected and then additional analysis or data review would be required to find the desired fractions.

Precursor ion collection

A third mode of fraction triggering is from precursor ion acquisition, as illustrated in Figure 9. Here, MS1 is scanning and MS2 is fixed on a specific product ion. If the specific product ion is observed, it is registered at the detector. The spectrum only shows the masses that have that specific product.

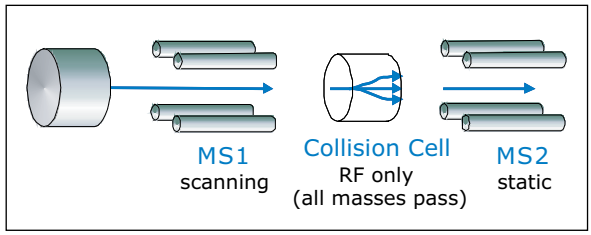

Figure 9. MS/MS precursor ion data acquisition.

Fraction collection from a precursor ion acquisition has to be from the TIC, since the precursor mass is unknown. This mode of fraction collection is valuable when the metabolites are unknown, but there is a common fragment of the core compound that can be detected.

To illustrate the common fragment ion collection capability, Figure 10 shows the glucuronic acid conjugates collected from the ibuprofen urine samples using the precursor ion scan mode of *m/z* 193. There are three fractions that are collected, *m/z* 273 (not drug-related), *m/z* 397 (hydroxyglucuronide conjugate), and *m/z* 381 (glucuronide conjugate).

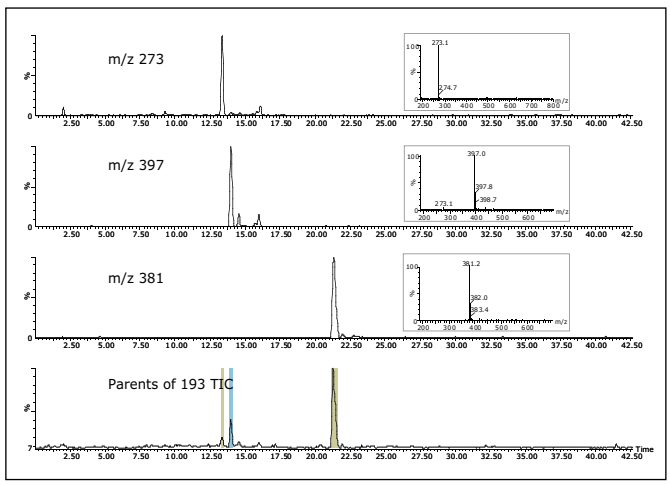

Figure 10. Fractionation based on the precursor ions of the m/z 193 TIC acquisition.

Additional collection options

The ESCi multi-mode ionization source enables both ESI +/- and APCI +/- acquisition to occur within the same run. This allows for fraction collection to be triggered from any of the acquisition channels, thus proving useful if the metabolites require different ionization modes. Prior to this enabling technology, the only options for collection would be to split the sample and run in different modes, or rely upon time-based fractionation and then analyze all the fractions by both modes to determine the targets.

The selectivity of the ESCi-enabled fraction collection process can be further enhanced by the use of mixed triggers. This approach uses Boolean logic strings to trigger collection from multiple data traces (e.g., collection can occur only when Mass A is present and Mass B is not, or a peak has to be present in two different traces at the same time for fractionation).

CONCLUSION

Fraction collection with a tandem quadrupole mass spectrometer is now possible using four different modes of data acquisition: scan, MRM, constant neutral loss, and precursor ion, which enables improved versatility for triggering options.

- Scan mode has the potential to increase the number of isobaric inferences detected and collected.
- MRM mode is the most selective because it only monitors a specific precursor/product ion transition and greatly reduces the isobaric interferences, but requires previous knowledge of the transition.
- Constant neutral loss mode can be used for collecting a class of compounds with a target-specific loss or a generic group loss for a broader study, or can be used as a second filter where the target has to have a specific mass and the neutral loss.
- Collection in precursor ion mode allows for all the precursors with a specific product ion to be collected, which is valuable when the metabolites are unknown, but there is a common fragment of the core compound that can be detected.

Thus, these different modes of collection add value to a wide variety of applications previously accomplished with more laborious, time consuming, and less specific methodologies.

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Waters Corporation
34 Maple Street
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F: 1 508 872 1990
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Keeping Track of Fractions with Barcodes

GOAL

To examine how barcodes can be used with the FractionLynx™ Application Manager for MassLynx™ Software to streamline the purification process. We discuss the introduction of barcodes to the system and how those barcodes can be used after fraction collection.

BACKGROUND

Today's analytical development labs are producing large numbers of samples that often need to be purified by HPLC. Collection of these purified samples, or fractions, can be in single tubes or across multiple tubes, depending on the samples and the technology available to perform the purification. It is necessary to track these fractions through the multiple stages of the purification process, including dry-down and weighing. Barcodes have become increasingly important in this fraction tracking. In many companies, individual fraction tubes are barcoded, along with the plates holding the tubes. Barcoded tubes can be easily weighed by robotic systems before being used, simplifying the weighing of fractions.

Previous versions of software allowed the rack barcode to be carried through the process but not the tube barcode. New software has been developed to allow tube barcodes from robotics systems to be imported into FractionLynx and to export those barcodes from FractionLynx into results reports.

By using barcodes, samples can be traced through the multiple stages of purification. The tube barcoding reader in FractionLynx improves sample tracking, minimizes errors, and improves integration into existing automation systems.

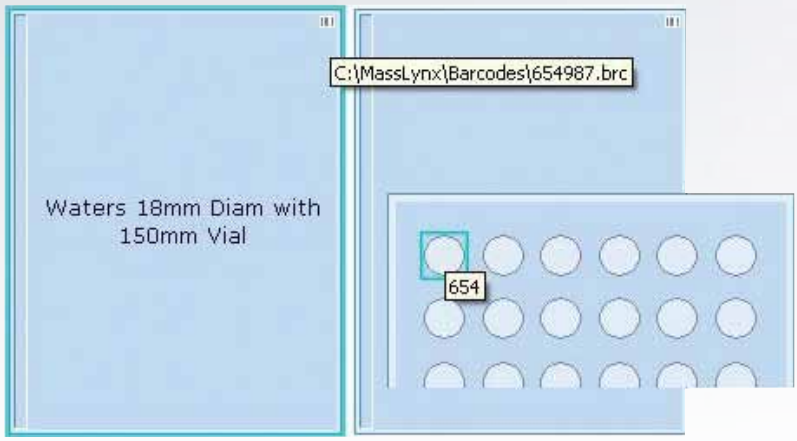

Figure 1. Plate and tube displays showing barcode pop-ups.

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Tube Trigger	Original Target	Ion Mode	Start Time	End Time	Collection Site	Export Tube	Tube Volume	Rack Barcode	Barcode
384.0000	383.0000	Timed	0.50	0.51	1,1:5	NO	0.20	684523	65
533.0000	532.0000	Timed	0.51	0.68	1,1:6	NO	3.40	684523	4

Figure 2. Tube view in fraction collection results pane.

THE SOLUTION

In FractionLynx, barcodes can be used to identify individual collection tubes, an entire collection plate, or both. The use of barcodes is enabled in the FractionLynx Collection Control window. Once enabled, you can assign barcodes to the fraction collector bed whenever the bed is reset.

The barcode generator associates tube barcodes with plate barcodes. By doing so, you only need to scan the plate barcode for all its tube barcodes to also be imported into FractionLynx.

The first step is to define the plate. This tells the software which plate type you are using and how many tubes it holds. The tube barcodes can be entered by importing a text file containing the tube numbers (location) and the tube barcodes. This text file can be generated manually or can come from a robotics system.

Once the fractions have been collected, the plate and tube barcodes can be viewed in two ways. There is a real-time display of barcodes in the Collection Control window. Figure 2 shows how to view the barcodes in this window. Hovering the mouse pointer over a plate in the bed display window will display a tooltip showing the barcode of that plate. Hovering over a fraction tube will display a tooltip showing the barcode for that tube.

The second place the barcodes can be viewed is in the FractionLynx report. Both the rack barcode and the tube barcodes can be seen in the Tube view of the Fraction Collection Results pane (Figure 2). These values can also be included in the printed report and easily exported to liquid-handling systems for dry-down and weighing, or to LIMS systems for reporting and archiving.

SUMMARY

Fraction tubes are barcoded so that they can be traced throughout the multiple stages of purification. Tubes can be weighed before they are used, simplifying the weighing of the collected fractions. Including the tube barcodes in FractionLynx improves sample tracking and minimizes errors. It also improves integration into existing automation systems. Once the sample is dried down, the tube can be weighed to determine how much fraction was collected without transferring the dry sample, because the weight of the barcoded tube is known.

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