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Fast Gradients for RP-HPLC

Under pressure to increase throughput and decrease development time, many chromatographers strive to develop faster HPLC separations. HPLC methods have traditionally been developed using columns measuring 4.6 mm ID and 15 to 25 cm in length. At the same time, isocratic methods are generally preferred in order to eliminate the time for re-equilibration required by gradient methods. Gradient methods have been employed only when necessary to achieve a separation that cannot be done by isocratic means.

Short columns (5 cm or less in length) are now available packed with 5 µm particles at high efficiencies. Using these short, efficient columns, gradient methods can be used to achieve rapid analysis of samples with a wide range of analyte polarity. Shorter columns create lower backpressures, allowing higher flow rates and shorter run times. In fact, run times (including re-equilibration) can become quite favorable in comparison to isocratic methods. This can be particularly useful for high-throughput analyses where a large number of samples need to be analyzed in as little time as possible.

The utility of fast gradients is shown in Figure 1. Six parabens were chromatographed under gradient conditions on Discovery C18 . Panel A of Figure 1 shows the gradient elution of the compounds when employing traditional conditions. Baseline resolution results for the six well-spaced peaks.

Panel B displays the results when the column is shortened to 5 cm, and the gradient volume proportionately reduced. While the run time is reduced by a factor of 3, the peaks remain baseline resolved. This has been achieved by only reducing the column length. Since column length has been reduced, the corresponding lower backpressure permits application of higher flow rates as well.

If the flow rate is quadrupled, while maintaining the same gradient volume (Panel C) as the previous run with the 5 cm column, the run time is reduced to less than 2 minutes. Though the higher flow rates may compromise resolution (peaks 1 & 2), band spacing can still remain adequate for the separation. If needed, the flow rate, gradient volume and/or shape can be further optimized.

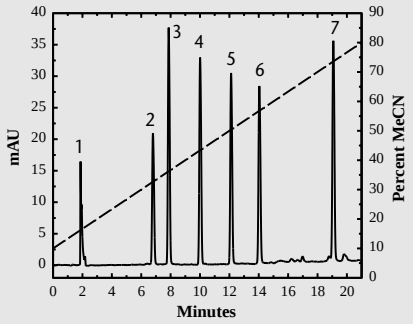
To demonstrate this further, Figure 2 shows the separation of the three components of Excedrin® on Discovery C8. All three active ingredients are baseline resolved within 1.5 minutes!

(continued on page 3)

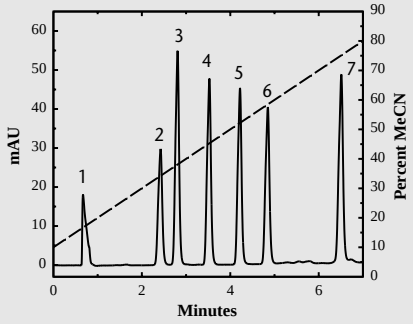
Figure 1. Fast Gradient Development

Conditions for A, B & C differ only in column length, flow and gradient.

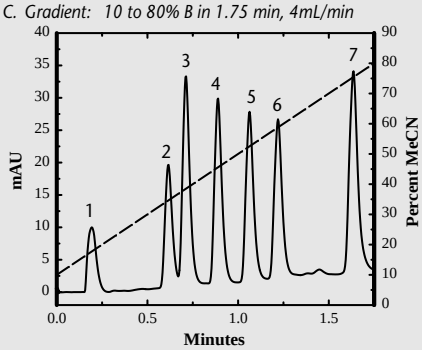
Panel A. ***Column: Discovery C18, 15cm x 4.6mm ID, 5µ***
Cat. No.: 504955
Sample: Gradient RP Test Mix (Cat. No. 4-8271)
Mobile Phase A: H_2O
Mobile Phase B: MeCN
Gradient: 10 to 80% B in 21 min
Flow Rate: 1 mL/min
Temperature: 35° C
Detection: 254 nm
Injection: 10 µL
1. *Uracil*
2. *Phenol*
3. *Methyl paraben*
4. *Ethyl paraben*
5. *Propyl paraben*
6. *Butyl paraben*
7. *Heptyl paraben*



B & C: ***Column: Discovery C18, 5cm x 4.6mm ID, 5µ***
Cat. No.: 504947
Panel B: Gradient: 10 to 80% B in 7 min, 1mL/min



Panel C. Gradient: 10 to 80% B in 1.75 min, 4mL/min



LC issue

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SUPELCO
a member of the Sigma-Aldrich family

T200006

NEW PRODUCTS

Micro and Low Volume Static Mixer

Micro and Low Volume Static Mixer

- *Increases reaction efficiency in postcolumn derivitization*
- *Improves accuracy in gradient mixing for microbore and narrow bore analyses*

A highly efficient cross-flow shearing mechanism in the ASI static mixer produces vortex mixing over a wide range of flow rates. Use the binary input housing to combine two flowpaths into one, such as in postcolumn or gradient mixing applications. Use the in-line housing when additional mixing is needed in a single flowpath.

Use the Micro-Mixer cartridges only with Micro-Mixer Housings and the Low Volume Mixer cartridges only with the Low Volume Mixer Housings.

Micro-Mixer Cartridge, Stainless Steel

2µL	**56661-U**
5µL	**56662-U**
10µL	**56663-U**
25µL	**56664-U**

Micro-Mixer Housing, Stainless Steel

In-Line	**56665-U**
Binary Input	**56666-U**
Ternary Input	**56667-U**

Low Volume Mixer Cartridge, Stainless Steel

50µL	**57545**
150µL	**57546**
250µL	**57547**

Low Volume Mixer Cartridge, PEEK

50µL	**500445**
150µL	**500453**
250µL	**500461**

Low Volume Housing, Stainless Steel

In-Line	**57548**
Binary Input	**57549**
Ternary Input	**500488**

Low Volume Housing, PEEK

In-Line	**500496**
Binary Input	**500518**
Ternary Input	**500526**

For more information, request T400100

The Discovery Family of Short HPLC Columns

Discovery HPLC Short Columns

Decrease run times by using shorter columns. Discovery columns are available in a variety of reduced lengths to help you get your results faster.

Phase	Length (cm)	ID (mm)	Particle Size (µm)	CAT. NO.
Discovery RP-AmideC16	*12.5*	*4.6*	*5*	**569332-U**
Discovery C18	*12.5*	*4.6*	*5*	**569232-U**
Discovery C8	*12.5*	*4.6*	*5*	**569427-U**
Discovery Cyano	*12.5*	*4.6*	*5*	**569527-U**
Discovery RP-AmideC16	*10*	*4.6*	*5*	**569323-U**
Discovery C18	*10*	*4.6*		**5569223-U**
Discovery C8	*10*	*4.6*	*5*	**569423-U**
Discovery Cyano	*10*	*4.6*	*5*	**569520-U**
Discovery RP-AmideC16	*5*	*4.6*	*5*	**505005**
Discovery C18	*5*	*4.6*	*5*	**504947**
Discovery C8	*5*	*4.6*	*5*	**59352-U**
Discovery Cyano	*5*	*4.6*	*5*	**59355-U**

For more information, request T499126

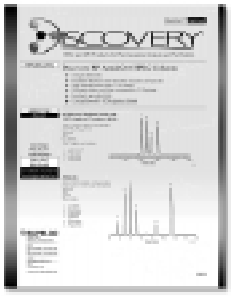
*All literature mentioned in this issue can be obtained from the website, **www.sigma-aldrich.com/TheReporter**, by completing the Literature Request section on the reply card, or by calling our Technical Service Department.*

UPDATED LITERATURE

The Discovery Family of HPLC Columns

Discovery HPLC columns are available in 4 different stationary phases: RP-AmideC16, C8, C18, and Cyano. Request literature on each column chemistry to learn about applications, chemical structures, and quality control data.

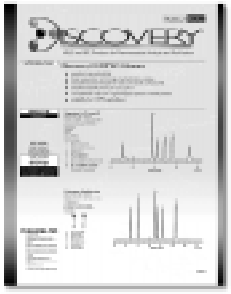
Discovery RP-AmideC16



- Unique selectivity
- Excellent retention and resolution of polar compounds
- Less hydrophobicity than C18 phases
- Different elution profiles compared to C18
- Excellent reproducibility

For more information, request T498019

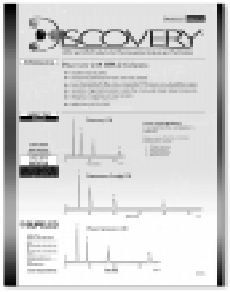
Discovery C8



- Faster separation of strongly hydrophobic analytes
- Exceptional peak shapes for basic and acidic compounds
- Excellent stability from pH2 to pH8 or above
- Compatible with low organic/highly aqueous mobile phases
- Suitable for LC/MS applications
- Excellent reproducibility

For more information, request T499128

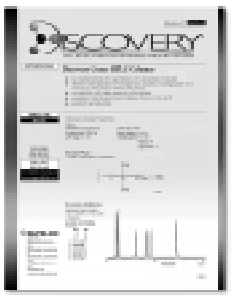
Discovery C18



- Exceptional peak shape for basic and acidic analytes
- Lower hydrophobicity than many comparable C18 columns, providing faster analysis
- Resolution of geometrical isomers and other structurally closely related compounds
- Separation of peptides and small proteins
- Suitable for LC/MS applications
- Excellent reproducibility

For more information, request T497291

Discovery Cyano



- Low hydrophobicity, for rapid elution of hydrophobic molecules
- Retention and separation of strongly basic analytes, including quaternary ammonium salts, with excellent peak shapes
- Compatible with highly aqueous mobile phases
- Exceptional stability and column lifetime, from pH2 to pH8
- Excellent reproducibility

For more information, request T499133.

NEW APPLICATIONS

Rapid Drug Profiling

The utility of fast gradients is illustrated in Figure 3 using an OTC drug formulation with 4 components. Many cough/cold/flu medications contain diverse compounds that are difficult to resolve by isocratic methods, sometimes requiring an hour or more to elute. This example shows an initial organic concentration of 0% acetonitrile, which is necessary to retain the initial sample component. Resolution of all 4 components within a short run time is achieved by applying a segmented gradient: an initial steep gradient to reduce overall run time, and a second more shallow gradient to provide resolution of the later eluting components. Running at 3 ml/min., all 4 analytes are eluted in less than 2 minutes. Column re-equilibration is accomplished in 3-4 minutes, allow rapid cycle times and high throughput.

Protein Separations in Minimal Time

Fast gradients on short columns are not limited to separations of small molecules. Discovery columns are well-suited for separations of peptides or small proteins (<20kDa). While protein/peptide chromatography usually makes use of traditional column lengths (typically 25cm), Figure 4 shows the separation of four proteins on a 5cm Discovery C8 column within 3 min! This has immediate relevance for high-throughput applications. The octyl ligand is preferable to the octadecyl ligand for protein applications since it is sufficiently hydrophobic yet may circumvent disadvantages of the greater hydrophobicity of the octadecyl ligand—broader peaks and longer retention times.

Figure 3. Fast Gradient Analysis of an OTC Formulation

Column: Discovery RP-AmideC16, 5cm x 4.6mm ID, 5µ
Cat. No.: 505005
Mobile Phase A: 0.1% HCO_2H
Mobile Phase B: MeCN
Gradient: 0-5% B in 0.5 min, 20-40% B in 1.5 min
Flow Rate: 3 mL/min
Temperature: 35° C
Detection: 254 nm
1. Phenylpropanolamine
2. Doxylamine
3. Dextromethorphan
4. Acetylsalicylic acid

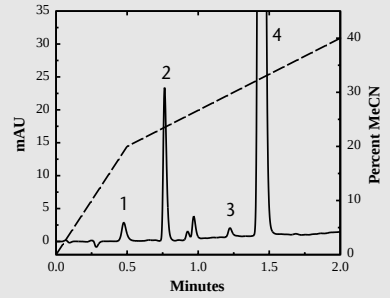
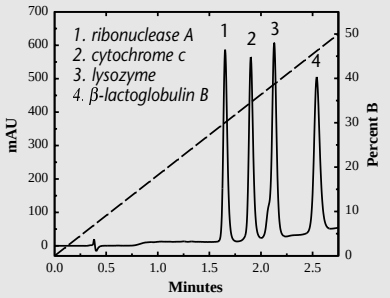


Figure 4. Fast Protein Chromatography

Column: Discovery C8, 5cm x 4.6mm ID, 5µ
Cat. No.: 59352-U
Sample: (1.67 g/L ea.)
Mobile Phase A: 10% MeCN, 0.1% TFA
Mobile Phase B: MeCN, 0.1% TFA
Gradient: 0 to 50% B in 2.75 min
Flow Rate: 2 mL/min
Temperature: 35° C
Detection: 220 nm
Injection: 5 µL



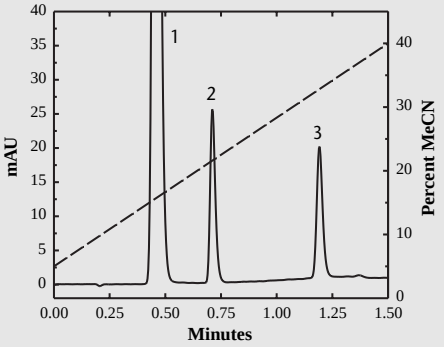
Fast Gradients...

(continued from page 1)

For a quantitative discussion of the higher productivity fast gradient methodology affords (while retaining necessary resolution), see W.K. Goetzinger & J.N. Kyroanos (1998), *American Laboratory* **30**, 27-37, and H.N. Weller et.al. (1997), *Molecular Diversity* **3**, 61-70.

Figure 2. Fast Gradient Analysis of Excedrin

Column: Discovery C8, 5cm x 4.6mm ID, 5µ
Cat. No.: 59352-U
Mobile Phase A: 0.1% HCO_2H
Mobile Phase B: MeCN
Gradient: 5 to 40% B in 1.5 min
Flow Rate: 4 mL/min
Temperature: 35° C
Detection: 254 nm
Injection: 5 µL
1. Acetaminophen
2. Caffeine
3. Acetylsalicylic acid



LC PERFORMANCE TIP

Extracolumn Volume: Minimizing Its Effect on Performance

HPLC system plumbing can have significant effects on resolution and efficiency by directly impacting height and width of a peak. Extra-column volume is defined as that portion of the system through which the sample passes, beginning with the injector and ending with the detector flow cell. Sample diffusion that occurs in transit between the injector and column inlet can contribute to band broadening, but typically the major extracolumn volume effect is post-column. In particular, flow cell geometry can be a major contributor to band broadening. The two parameters of flow cell design that effect measured peak height and width are the internal volume and the optical path length. The smaller the internal volume, the less dilution there is of the eluting sample component, and therefore the greater the sensitivity. Also, the longer the optical path length, the greater the sensitivity. In both instances, greater sensitivity translates to greater peak height. Standard flow cells in UV-Vis detectors typically have internal volumes of 10 to 15µL and optical paths of about 10 mm. Micro-flow cells can have internal volumes as low as 1µL while maintaining optical paths of up to 5 mm. Semi-micro flow cells fall in between these ranges.

The accompanying table gives an example of two different drug compounds (A & B) chromatographed by reversed-phase HPLC, comparing standard (8µL, 10 mm) and semi-micro (2.5µL, 5 mm) flow cells on the same instrument. Their relative peak sharpness is quantitated by peak height divided by peak width at half height. With this system, the semi-micro flow cell affords a 50% improvement in peak sharpness as compared to the standard flow cell.

Flow cell	A, Height/Width	B, Height/Width
Standard	*2813*	*74*
Semi-micro	*4294*	*126*

In general, for analytical HPLC, apparent column efficiency and resolution can be improved by employing the following practices: 1) use of narrow bore tubing (0.005" ID), 2) minimizing tubing lengths between the injector and column inlet, and between the column outlet and detector, and 3) use of semi-micro or micro flow cells in detectors.

CASE STUDY 3

Gradient Method Development: System Dwell Volume

Dwell volume is defined as the volume from (and including) the gradient mixer to the column inlet, and thus represents a delay volume between gradient formation and gradient delivery to the column inlet. Dwell volume represents an initial isocratic segment prior to gradient elution. Since this is a fixed parameter for a system configuration, it becomes significant when comparing different column dimensions to be employed on the instrument. Typical dwell volume for a low-pressure mixing system is about 1 mL. Examining a 5 cm long column, popular with fast gradient methodology, with either 4.6 mm ID or 2.1 mm ID, that 1 mL represents a nearly 5-fold greater relative volume for the 2.1 mm ID as compared to the 4.6 mm ID. The effects of this can be quite dramatic.

The chromatogram of Figure 5 shows the separation of a Tylenol® cold medication. This four component mix was separated on a 5cm x 4.6mm Discovery RP-AmideC16 column, with an instrument having a dwell volume of ~600µL. All components exhibit good peak shape and are eluted within the 2.5 min gradient. When the same method (same linear velocity) is applied to a 5cm x 2.1mm column (Figure 6), the effect of the 5-fold greater relative isocratic segment preceeding the gradient is readily apparent. Peak 1 clearly elutes during the initial isocratic phase, and peak 2 (now much broader because of the greater relative isocratic phase) elutes at only the beginning of the gradient. Again, because of the much greater relative gradient delay, peaks 3 and 4 now elute after completion of the gradient, during the column re-equilibration.

To solve this problem, there must be a means to reduce the dwell volume to an amount proportional to the ratio of column void volumes. (The ratio of void volumes of the two columns is approximated by the ratio of the column total internal volumes, or more simply, the ratio of their cross sectional areas, since they are packed with the same material.) With low-pressure mixing systems there is usually little or nothing that can be done to reduce system dwell volume to the magnitude necessary when going from a 4.6 mm ID to a 2.1 mm ID, approximately a 5-fold reduction. Some volume reduction may come from re-plumbing with smaller ID tubing (0.005" ID) and minimal tubing lengths from the pump, through the autoinjector (or better yet, a manual injector), and to the column, but still the major volume is in the proportioning valve and the pump heads themselves.

The solution to minimize dwell volume is the appropriate choice of a high-pressure mixing system. In this case, the mixing volume can be 5µL or less (see page 2). Plumbed with minimal ID tubing (0.005" ID), the dwell volume can be kept well below 50µL. In the case considered above, the 5-fold reduction of the original dwell volume is 120µL. In such a case, volume must be added back to attain a truly proportional gradient delay! Fortunately, that is convenient by utilizing inline static mixers of varying internal volumes, which has the added potential benefit of improved gradient mixing. Thus, selection of a high-pressure mixing system, plumbed with narrow bore ID tubing, and choice of an appropriate mixer provides for an instrument that is flexible to adapt to a desired dwell volume, making for an ideal gradient HPLC system.

Figure 5. Dwell Volume Effects: Standard Column

Column: ***Discovery RP-AmideC16, 5cm x 4.6mm ID, 5µ***
Cat. No.: ***505005***
Mobile Phase A: *0.1% HCO_2H*
Mobile Phase B: *MeCN*
Gradient: *0 to 25% B in 2.5 min*
Flow Rate: *4 mL/min*
Temperature: *35° C*
Detection: *254 nm*
1. Psuedoephedrine
2. Acetaminophen
3. Guaifenesin
4. Dextromethorphan

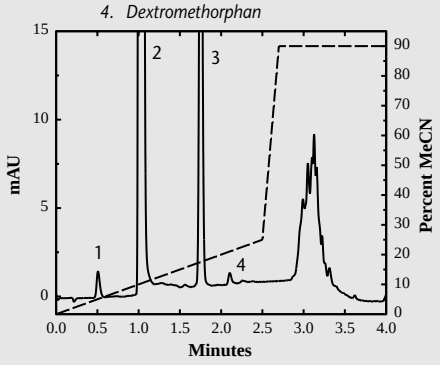
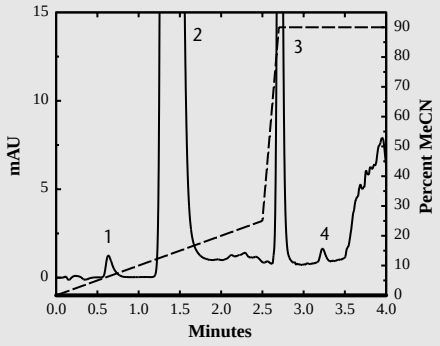


Figure 6. Dwell Volume Effects: Narrow Bore Column

Column: ***Discovery RP-AmideC16, 5cm x 2.1mm ID, 5µ***
Cat. No.: ***50500521***
Mobile Phase A: *0.1% HCO_2H*
Mobile Phase B: *MeCN*
Gradient: *0 to 25% B in 2.5 min*
Flow Rate: *0.834 mL/min*
Temperature: *35° C*
Detection: *254 nm*



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