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Adaptation of high-performance liquid chromatographic methods to multiple spectral analyses

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Abstract

Adapting well-characterized existing liquid chromatographic separations for compatibility with mass spectrometric detection often requires changes in chemical and/or chromatographic conditions. Non-volatile solvent modifiers, such as phosphates or alkylsulfonates, have been widely used in chromatography because of their buffer or ion-pairing capacities, coupled with advantageous low-UV-wavelength absorption characteristics. Non-volatile components in the eluent stream are generally avoided in mass spectrometry as they quickly decrease the detector response. In addition to issues such as buffer capacity and effective ion-pairing, consideration should be given to the impact of stationary phase and column geometry.

The work presented here illustrates advantages of a new high-purity, high-pore-volume stationary phase used in Waters Symmetry columns when attempting to accommodate a wide range of conditions imposed by different detection schemes. The effect of column geometry under a variety of typical LC–MS conversion situations is also included. The studies include resolution of critical hydrophilic pairs and stationary-phase loading performance relative to converting existing separations for LC–MS analysis while achieving maximum sensitivity and maintaining chromatographic fidelity.

Keywords: Liquid chromatography–mass spectrometry; Stationary phases, LC; Interfaces, LC–MS; Particle beam ionization; Column geometry; Anti-inflammatory drugs

1. Introduction

The current practice of liquid chromatography interfaced with mass spectrometry (LC–MS) benefits from more than a decade of published experience with the three most widely used commercial interfaces, thermospray, particle beam and electrospray. Each requires specific flow-rate and solvent chemistry considerations to achieve maximum sensitivity. Summary reviews have been published in these areas in addition to numerous publications describing work with specific applications [1–3]. In addition to physical aspects of liquid introduction into a vacuum, numerous articles have appeared characterizing the effects of non-volatile modifiers, mobile-phase suppression of ion emission and other artifacts

derived from efforts to introduce compounds for analysis that were not previously amenable to on-line sample introduction for mass spectrometry [4–6].

Particle-beam LC–MS, by removal of the solvent following its use in chromatography, introduces solute to the high-vacuum source region for electron-impact (EI) ionization. The resulting spectra are similar to EI spectra generated by analytes commonly amenable to GC–MS analysis. Particle-beam LC–MS is capable of producing widely understood, library searchable spectra from each peak separated in any given analysis but exhibits generally less efficiency than other detection techniques. Other techniques which generate “soft” ionization, or more intense molecular-ion formations and less fragmentation spectra require techniques such as

MS–MS for structural elucidation. Ionization and transport efficiencies for each technique places increased importance on dispersion efficiency, separation quality and absolute amounts of sample injected.

Achieving and maintaining low dispersion is important in a multispectral system. Since the ultimate sensitivity and quality of the mass spectra are enhanced by improved signal-to-noise, loss of peak height for a given chromatographic band due to inadequate system design or the mechanics of construction is to be avoided. In addition to dispersion losses from each union and fitting, interfacing a second detector contributes unique losses of chromatographic band fidelity. For example, particularly volatile analytes can be lost during conversion from condensed phase to vapor phase as they are transported through the momentum separator region of the particle beam [7,8].

Adapting an existing chromatographic separation developed at flow-rates typically higher than practicable for LC–MS requires that decisions be made in areas of both kinetic and thermodynamic variables. Kinetic variables in general, such as flow-rate, have no effect on selectivity and retention and a relatively small effect on efficiency and are therefore often overlooked in favor of factors that influence sample migration when the method is initially developed. Flow-rate, and the consequential vapor load on the vacuum chamber, can be accommodated when adapting existing separations through use of a smaller diameter column with the same packing material. Theoretically, maintaining an equivalent linear velocity through reduction of the flow-rate by the inverse square relationship of flow-rate to column diameter preserves chromatographic fidelity. The flow-rate reduction produces a nearly equivalent separation without loss of efficiency for an equivalent chromatographic band. Practical limits exist however when an existing gradient is applied with only a flow-rate conversion.

When components of interest are less than 1% of the total the practitioner must choose to preconcentrate the sample or simply inject more onto the column at the existing concentration in order to produce effective spectra by particle beam. If the critical pairs for a separation have been established, the preferred means of analysis would be to inject

more material directly without changing the separation. In fact, selectivity may be such, that no sufficient changes can be made in selectivity without downgrading the quality of the overall separation.

1.1. Chromatographic kinetic factors

Column efficiency performance varies with linear velocity proportional to the actual flow-rate and dependent on the geometry. Fig. 1 illustrates linear velocity calculated for three column diameters as:

$$u = L/t_m$$

where u is the linear velocity in cm/s, L is the column length and t_m is the time required for an unretained peak to elute at the specified flow-rate. A reduction in flow-rate can be extrapolated to the next smaller diameter column to achieve the same linear velocity.

Resolution (R_s) can be expressed as the relationship between the average number of plates for two bands of interest (N), the selectivity for each peak (α) and the average retention factor (k') for the two peaks.

$$R_s = 1/4N^{1/2}(\alpha - 1)(k'/[1 + k'])$$

R_s increases proportional to k' without appreciable loss of peak height until approximately $k' = 5$.

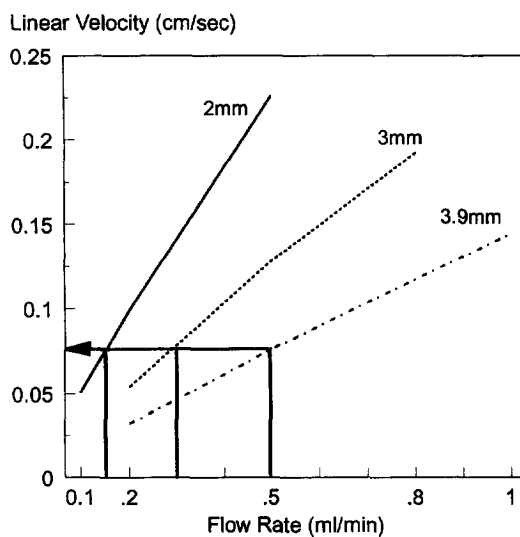


Fig. 1. Linear velocity response for various column geometries.

Therefore a separation dependent on solvent strength can achieve increased resolution (increased k') and consequently the ability to load more sample on the column but only by decreasing the stronger eluting solvent. Consequently the study was designed to maintain $2 < k' < 5$ to avoid enhanced resolution at the expense of peak height. If the peaks elute at the hydrophilic region decreasing solvent strength will have limited effectiveness. Doubling the column length, a common means of increasing N , increases N by only 40%. Changing the diameter on the other hand has no effect on N if the flow-rate is scaled to maintain the same linear velocity [9].

1.2. Resolution

Baseline resolution is defined as the peak-to-peak separation of two adjacent peaks in ratio to their average peak width:

$$R_s = \frac{T_{\text{Peak A}} - T_{\text{Peak B}}}{1/2(T_{\text{Peak width A}} + T_{\text{Peak width B}})}$$

An $R_s > 2$ reduces column-to-column deviations and aging effects although baseline resolution with as little as 1% overlap can be equivalent to $R_s > 1$ when bands are of approximately equal volume.

1.3. Sample and volume overload

Determining sample and volume overload limiting factors for methods development have been described in detail by Snyder and Kirkland [10], Knox [11] and others. Isocratic separations, when the sample is dissolved in the mobile phase, allow approximately 10% of the peak volume as the maximum injection volume before the adsorption isotherm is exceeded. Similarly, the packing material theoretically accepts between 10^{-3} and 10^{-4} grams of solute per gram of packing material before overloading. Using calculations based on the most restrictive case the expected load ability would be 50–500 μg of solute without adverse effect for a single component if the (nominal) 150×2 mm column contains 0.5 grams of packing material. A maximum injection volume of 20 μl was chosen, assuming a moderate column efficiency ($N = 6000$)

for a given column with $V_0 = 204 \mu\text{l}$ and an average of 70% interstitial column volume:

$$\text{Volume} = \frac{V_0 \cdot (1 + k') \cdot 4}{\sqrt{N}}$$

2. Experimental

2.1. Equipment

A Waters Integrity System, incorporating a photodiode array UV detector in series with a particle beam interfaced mass spectrometer as an integrated system was used for all measurements. The computations for resolution and correlation of results was achieved using the Waters Millennium 2010 Chromatography Manager software on the DEC 590 Celebris Pentium platform.

2.2. Chromatography and chemicals

Gradient flow-rate scaling was performed with a mixture of anti-inflammatory compounds, oxyphenbutazone, ketoprofen, phenylbutazone, flunixin, meclofenamic acid and naproxen obtained commercially (Sigma, St. Louis, MO, USA) at a concentration of 10 mg/l each. A water–methanol–acetonitrile gradient from 30:50:20 to 20:30:50 over a 10-min period was used for elution. Selectivity and resolution were determined to be adequate at the van Deemter optimum flow-rate of 0.8 ml/min using a 150×3.9 mm Waters NovaPak C_{18} column. Comparisons were performed with the same packing material in 150×3 mm and 150×2.1 mm columns. Columns are commercially available although the 3.0-mm NovaPak column is a custom order. Linear velocity was established using acetone to determine V_0 .

Chromatographic performance determinations for resolution and linearity were performed with a mixture of relatively neutral analytes, caffeine (at 100 ng/ μl) and 4-acetamidophenol (at 200 ng/ μl) obtained commercially (Sigma). The diluent was either 100% water, 85% water or 50% water with the balance (v:v) acetonitrile. Accurate resolution of both analytes for a series of column geometries can be controlled using only the strength of the organic

component. Isocratic measurements were performed using reagent grade water–acetonitrile (85:15, v/v) at the van Deemter optimum flow-rates for each column, determined to be 0.2 ml/min for the 2.1-mm column, 0.5 ml/min for the 3.0-mm column and 0.8 ml/min for the 3.9-mm column [12]. Columns used were all 150 mm in length; Waters NovaPak C₁₈ stationary phase, 60 Å pore-size by nitrogen sorption, 4 μm particles, pore volume=0.35 cm³/g at a 7% nominal carbon load in the 2-mm, 3-mm and 3.9-mm diameter columns; Waters Symmetry C₁₈ stationary phase, 90 Å pore-size by nitrogen sorption, 5 μm particles, pore volume=0.9 cm³/g at a 20% nominal carbon load in the 2.1-mm, 3-mm and 3.9-mm diameter columns.

Peak height was used to determine improvements in performance, rather than peak area, since the goal was to optimize the mass spectrometer response while maintaining chromatographic fidelity. Since peak height is proportional to concentration, peak height can also be used more reliably than peak area to indicate a quantitative change in the “flux” of ions over time. An improvement in peak height indicates an improvement, chromatographically, in the signal-to-noise ratio of the mass spectrometer. Therefore, control of the chromatographic response is required before optimizing the signal-to-noise ratio and other performance parameters in the mass spectrometer.

3. Results and discussion

3.1. Gradient scaling

Theory indicates that gradient separation, unlike isocratic separation, results in a significant loss of fidelity when transferred to a much smaller-bore column. The change in internal volume, even with a relatively shallow gradient, significantly alters the column elution rate (k'). Since the system (or extra-column) volume is constant, k' changes as the system volume becomes larger relative to the column volume. A separation developed for anti-inflammatory drugs on a 150×3.9 mm C₁₈ column at the optimum flow-rate (0.8 ml/min) was reproduced at a reduced flow-rate of 0.5 ml/min (approximately a 37% reduction) to achieve minimum acceptable

chamber pressure in the mass spectrometer (approximately between 5×10^{-5} and 8×10^{-5} Torr). Adequate resolution was maintained with a slight loss of peak height due to dispersion in the hydrophilic region between critical pairs ketoprofen and naproxen (Fig. 2) as expected for a loss of efficiency incurred with the reduction of flow-rate. Transferred directly to a 150×3.0 mm column of the same stationary phase at the same flow-rate, the peak height improved for the same amount injected by an average factor of 25% (Fig. 3). The same separation either at the optimum for the 2.0-mm column or appropriately scaled for flow-rate/linear velocity at the reduced flow-rate was significantly degraded (Table 1) and would require redeveloping the separation rather than adopting the existing method. Currently, computer-assisted development software such as Drylab is available (LC Resources, Walnut Creek, CA, USA) but numerous interrelated factors affecting selectivity and limitations inherent in the predictive quality of these programs (such as inability to model ternary gradients effectively) do not guarantee equivalence.

Dispersion losses at flow-rates well below the van Deemter optimum for a given column also result in loss of peak height as illustrated in Fig. 2 where decreased peak height is seen when the 3.9-mm column is operated at 500 μl/min. The 2.0-mm (nominal) column geometry allows the least range of control by flow-rate. Optimum plate heights are achieved at 100–200 μl/min. Plate height (N) is reduced 10% (approximate) at 300 μl/min and 15% at 400 μl/min. Lower flow-rates are not compatible with dispersion losses in most chromatographic systems and are not useful in the current particle-beam design. The 3.0-mm column produces <3% reduction of N for flow-rates between 200 μl/min and 700 μl/min. Dispersion, and/or efficiency losses, produce a 12% reduction in N at 200 μl/min and 6% at 700 μl/min [12].

3.2. Isocratic scaling

The response of columns with different geometries and packing materials to situations encountered in converting methods was determined from the hydrophilic peak chromatographic performance while

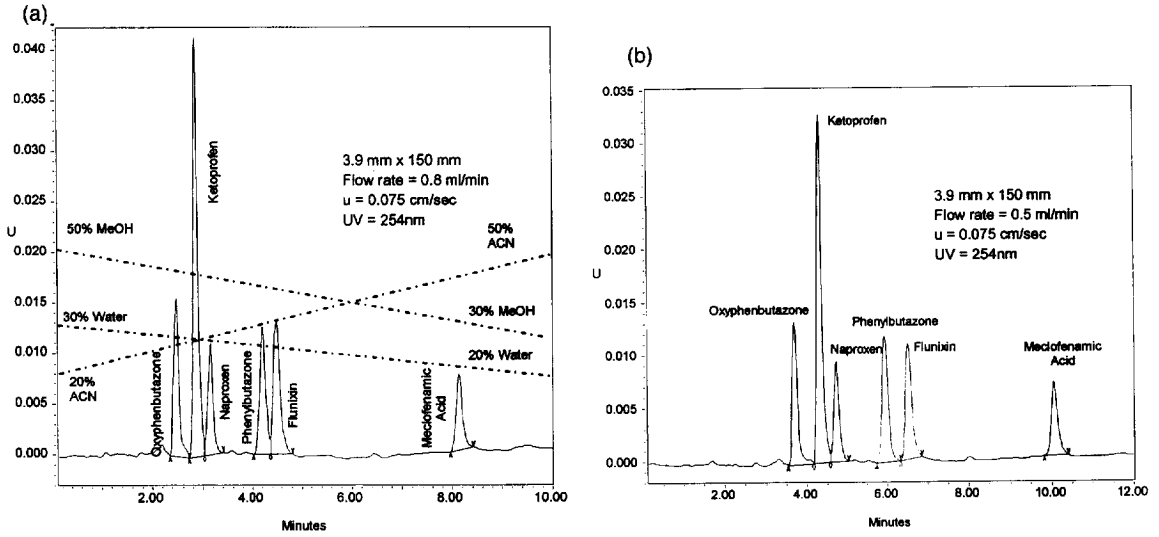


Fig. 2. Comparison of a separation of anti-inflammatory drugs at (a) original conditions and (b) reduced flow-rate on a 3.9-mm column.

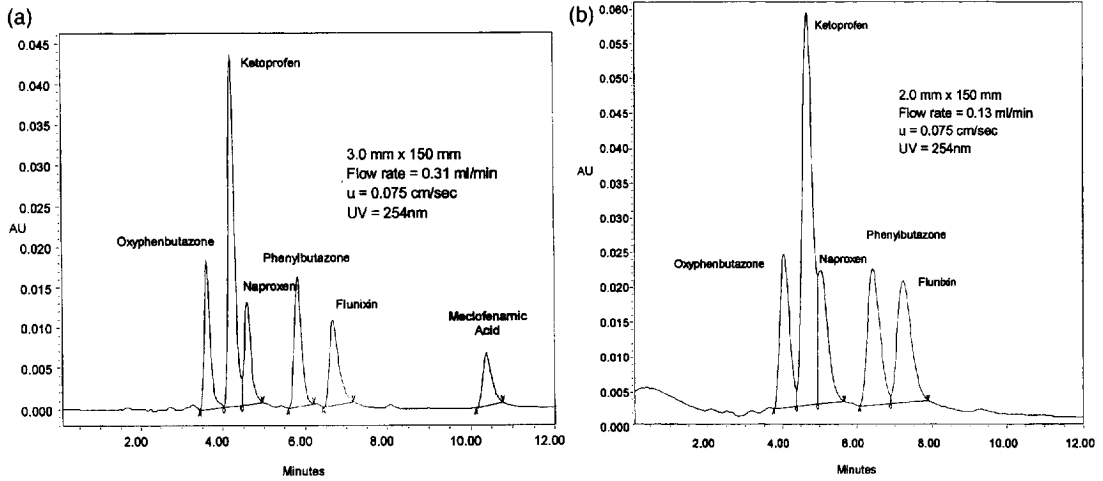


Fig. 3. Comparison of the original separation of anti-inflammatory drugs on (a) 3.0-mm and (b) 2.0-mm columns using scaled flow-rates and linear velocity.

Table 1
Comparison of gradient performance for columns of various diameters^a

Column diameter (flow-rate, ml/min)	Ketoprofen peak height (μV)	Naproxen peak height (μV)	R_s
3.9 (0.8/0.5)	32801	9407	1.48
3.0 (0.31)	43271	12586	0.9
2.0 (0.13)	— ^a	— ^a	— ^a

^a Not resolvable.

limiting the influence of other factors. Minimum statistical sampling was established at $n \geq 9$ for all test cases. Data shown in the figures represents the mean.

Increasing amounts of analyte were injected at the optimum flow-rate for each column geometry under conditions described previously, which ensured a minimum of $R_s > 2$. The larger pore volume Symmetry material gave a higher resolution value than the smaller pore volume NovaPak material, especially as the column diameters increased (Fig. 4). Therefore, the rest of this study of geometry effects was limited to the Symmetry, or 0.9 cm³/g, packing material.

The loss of linearity was most pronounced in the smaller diameter columns. Using the favorable (100% water) diluent system and the 85% water (mobile phase) diluent the 3.0-mm geometry is more resistant to loss of linearity (Fig. 5) than the 2.0-mm (nominal) column. Correlation coefficients of linearity (curve fit) for both the 3.0-mm and 3.9-mm columns, with experiments conducted at the respective optimum flow-rates, were found to be nearly linear (fit equal to 1.000) with the most advantageous diluent (100% water). The 2.0-mm (nominal) column displayed the least linear response for all cases. Resolution was also maintained for higher

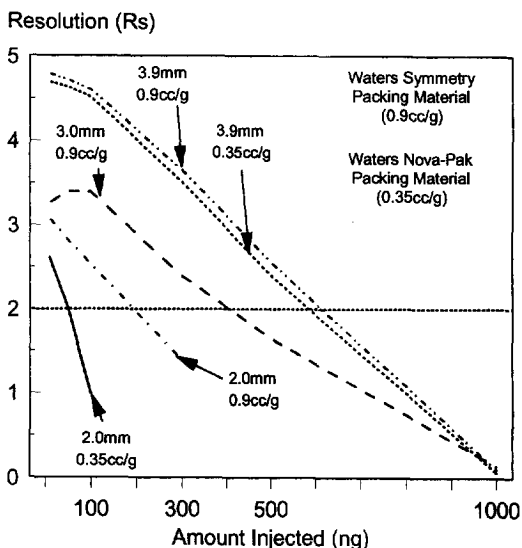


Fig. 4. Hydrophilic model resolution comparison: large and small pore volume packing material.

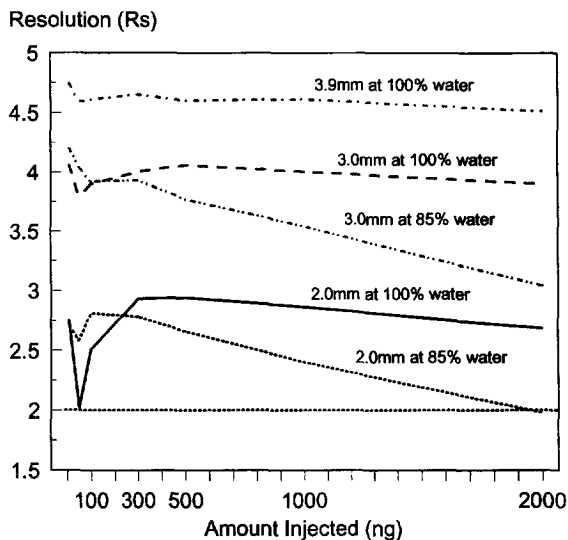


Fig. 5. Hydrophilic model linearity comparison: three column geometries with increasing load.

injected amounts on the 3.0-mm column (Fig. 6) under these conditions. The linear range found for samples dissolved in the high aqueous diluent improved the linear range beyond the 100 ng maximum found with the higher organic diluent for all columns.

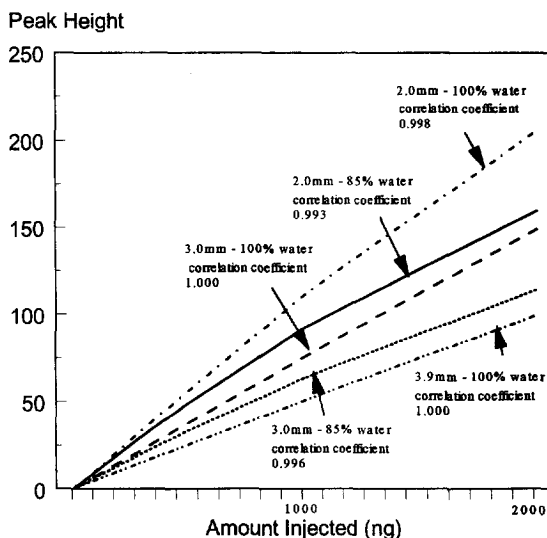


Fig. 6. Hydrophilic model resolution comparison: three column geometries with increasing load.

4. Conclusion

When modifying an existing LC method for LC–MS analysis, column geometry can be used to ensure optimum peak height and signal quality in the LC separation. The effect of the elution strength of the sample diluent, relative to the ability of the packing material to handle solute and solvent loading, has been demonstrated here. With the Symmetry material, resolution and linearity in the hydrophilic region could be maintained under the most adverse sample diluent conditions. This appears to be a function of inter-related characteristics, such as the distribution of the functional groups over the pore volume, the effective surface area and the column geometry.

A well-plumbed conventional LC system, available to most practitioners, can only produce a dispersion efficiency equal to the band dispersion of a 3.0-mm column. Modern well-designed chromatographic pumps may exhibit fixed “leak-rate” errors, or the inability to accurately control flow, in the order of 10–15% at 200 $\mu\text{l}/\text{min}$. This fixed rate would be only of the order of 5% at 500 $\mu\text{l}/\text{min}$ and thus have less influence on the critical eluent composition. Thus, using a 3.0-mm column allows reduction of the mixing volume requirements and further enhances the performance. Also, as demonstrated here, the influence of kinetic effects is minimal when a 3.0-mm column is used when adapting existing gradient separations to accommodate LC–MS flow-rates.

Particle beam and other LC–MS interface techniques requiring flow-rates between 200 and 700 $\mu\text{l}/\text{min}$ should also benefit from using a 3.0-mm column geometry, e.g. flow splitting (a smaller split ratio between the source flow and that delivered

post-split to the analyzer improves quantitation) and post-column addition for either electrospray or thermospray (where a wide optimal range is required to allow flexibility in the addition of the post-column flow). At a flow-rate of 500 $\mu\text{l}/\text{min}$ the column offers the benefits of both low dispersion and a wide sample loading range and is affected least by changes in factors affecting efficiency.

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