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Choosing sample volume to achieve maximum detection sensitivity and resolution with high-performance liquid chromatography columns of 1.0, 2.1 and 4.6 mm I.D.

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Abstract

The relationship between sample volume and the chromatographic performance factors of detection sensitivity and resolution were explored for high-performance liquid chromatography (HPLC) columns of 1.0, 2.1 and 4.6 mm I.D. Performance was evaluated for isocratic and gradient elution in systems having low, intermediate and high extracolumn dispersion. Sample volumes ranged from 0.4 μl to 2 ml. This paper provides guidelines for selecting sample injection parameters so that the performance of microbore columns is not needlessly sacrificed by using too small or too large a sample volume. The guidelines are extracted from the works of others and are modified by our own experimental results. Comments are also made on the relative merits of various injection techniques.

Keywords: Sample volume; Column diameter; Detection, LC; Resolution; Injection methods

1. Introduction

A wide range of HPLC column sizes are now in common use. Although lengths of 200 to 300 mm and inner diameters of 4 to 5 mm are still the mainstay in many laboratories, columns of smaller size are being used with increasing frequency [1–3]. As is well known, small columns produce peaks that are eluted in small volumes, and which place greater demands on detectors, connecting tubes and injectors, in order that extracolumn dispersion (instrument band broadening) does not excessively degrade column performance [4–6]. Both length and diameter have been reduced.

Short columns usually have as their purpose the achievement of fast analysis. These "high-speed" columns have lengths of between 10 and 100 mm and inner diameters of typically ≥3 mm, although diameters as small as 1 mm are sometimes used [7]. Most use a small-particle packing, which also contributes to smaller peak volumes [8–10].

Small-diameter columns usually have as their purpose the achievement of one or more of the following benefits [11–13]: (i) The volume of mobile phase used per analysis is small, providing economic and environmental value [14], (ii) the flow-rate is low, providing enhanced performance with some detectors, such as some electrochemical detectors and mass spectrometers operating in certain ionization modes [15–17], or (iii) the mass sensitivity is high, i.e., the detection sensitivity and resolution are improved, when the volume of sample available for injection is small [18–20].

Because eluted peak volume decreases linearly with reduced column length, but decreases with the square of reduced column diameter, injection into

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small-diameter columns is the more problematic [21–23] and is the subject of this report. The experimental work is limited to manual sample injection into 1, 2.1 and 4.6 mm I.D. columns. However, many of the principles, insights and guidelines apply to injection by autosampler and to injection into packed columns with diameters that are less than 1 mm. The terminology of small-diameter columns has varied over time. In this report, the term microbore refers to 1 and 2.1 mm columns, in agreement with some recent practices [3,24]. The term small-bore will be used to indicate all columns with diameters of less than 4.6 mm.

The migration to small-bore columns was previously inhibited because, when used in a chromatograph designed for conventional columns, the sensitivity and resolution were compromised. In isocratic analysis, flow channels in the injector, connecting tubes and the detector caused significant dispersion, which reduced the resolution and sensitivity that would otherwise be available from the column. Even when the detector volume was reduced, to lower the dispersion for the sake of resolution, optical effects resulted in loss of sensitivity [13,25]. In gradient analysis, the same limitations existed, except that demands on the injector were not as severe, since most analytes were concentrated at the top of the column during injection.

These limitations have been significantly reduced. Newer, general-purpose, instruments often perform adequately with 2.1 mm columns, and can use 1.0 mm columns if some performance compromises are accepted or special accessories are used. Some instruments are designed specifically for use with small-bore columns. Nonetheless, it is our impression from discussions with chromatographers that, when using small-bore columns in HPLC, performance is sometimes needlessly sacrificed by using inappropriate injection parameters — injecting too small or too large a sample volume or selecting an injection technique that is poorly suited to the application.

We therefore thought it timely to report on sample injector performance when used with HPLC columns with diameters of 1.0, 2.1 and 4.6 mm. The study provides guidelines for selecting injection volume so that the performance factors most important for the problem at hand can be maximized. The guidelines result from an integration of our own experimental results and insights obtained from work previously reported by others. Many articles and books present equations that describe the relationships between sample volume and performance. In this report, information is in the form of chromatogram comparisons, rather than equations, in order to illustrate the relationships visually. In most cases, the measurements were made using three chromatographic systems, differing in their instrumental band broadening (dispersion) characteristics.

1.1. Chromatographic performance factors, injection variables and conditions

The performance factors, injection variables and conditions were as follows: Sensitivity was indicated by peak height at constant noise levels. Resolution was indicated by the height of the valley between two partially resolved, weakly retained ($k' \approx 0.5$) peaks. This represents almost the worst case, since, ordinarily, conditions should be chosen that produce greater retention of the analytes of interest. Resolution is discussed concurrently with sensitivity, since they are linked by a common dependence on dispersion when using concentration detectors; the peak area represents mass and an increase in peak width must be accompanied by a corresponding decrease in peak height, when other variables are constant.

Sample volumes from 0.4 μl to 2 ml were injected, depending on the column and the elution mode. Analyte concentrations were kept below those that would cause non-linear isotherms (mass overload) or that would exceed the linear dynamic range of the detector. The partial-fill injection technique was used in these experiments.

The following techniques are discussed: (i) partial fill, in which the volume injected is determined by the amount dispensed from the loading syringe, (ii) complete fill, in which the volume injected is determined by the size of a sample chamber, which has been overfilled to mostly or completely displace the mobile phase, (iii) timed injection, in which the volume injected is determined by the duration of time the injector is left in the inject position and (iv) various on-column and precolumn enrichment techniques.

Data and discussion of injection technique refers to use of a manual sample injector (though at times it may be electrically or pneumatically actuated), not an autosampler, unless noted otherwise. A sample chamber can be either a flow channel machined into an element of the injector, or it can be an actual length of internal or external tubing, a sample loop. The term "loop" in this report will usually refer to both.

2. Experimental

2.1. Columns

Two sets of 150 mm columns were used, one set for isocratic analyses and the other for gradient analyses. The isocratic columns were Vydac 201HS 5 μm C_{18} bonded spherical silica, 90 Å pore size (Separations Group, Hesperia, CA, USA). The gradient columns were HAIsil 5 μm C_{18} bonded spherical silica, 100 Å pore size (Higgins Analytical, Mountain View, CA, USA). Refer to Table 1 for column specifications. The hold up time, t_0, used to calculate k', was the retention time of uracil dissolved in the mobile phase.

2.2. Instrumentation

The liquid chromatograph consisted of an HP 1100 binary pump, an HP 1100 thermostated column compartment set at 30°C and an HP 1100 variable-wavelength detector (Hewlett-Packard, Little Falls, PA, USA). Flow cells used with the HP 1100 detector were a G1314A#020 micro flow cell (Hewlett-Packard) and a UZ-HP11-CAP flow cell (LC Packings, San Francisco, CA, USA). For experiments without a column, the HP detector was modified by replacing the cell outlet tube with a 13-in. long, 0.0025-in. bore polyether ether ketone (PEEK) tube (1 in.=2.54 cm), and using it as the inlet, thus bypassing the heat exchanger of the standard cell. For some applications, a Jasco UVIDEC-100-V UV detector (Japan Spectroscopic, Tokyo, Japan) was used. The flow cell used with the Jasco detector was a standard 8 μl cell that had been modified by removing the heat exchanger tubing. See Table 2 for cell specifications and applications. The mobile phases were degassed by helium sparging.

2.3. Sample injectors

All sample injectors were standard Rheodyne injector valves, except for the special model 7410 injectors, which were modified with smaller stator

Table 1
Characteristics of HPLC columns used in experiments

Column diameter	4.6 mm	2.1 mm	1.0 mm
Column cross-sectional area, normalized	21.16	4.41	1.00
Flow-rate used in experiments[a]	1690 μl/min	350 μl/min	80 μl/min
Particle size of packing	5 μm	5 μm	5 μm
Column efficiency, theoretical, N[b]	10 000	10 000	10 000
Peak variance, $k'=1$, theoretical[c]	1050 μl^2	46 μl^2	2.3 μl^2
Peak variance, $k'=5$, theoretical[c]	9450 μl^2	410 μl^2	21 μl^2

[a] The flow-rate producing maximum efficiency was determined by varying the flow-rate over a range from half to twice the flow-rate predicted from theory, assuming the reduced plate height $h=2d_p$ and determining the minimum in the H vs. u curve. Since the slopes of the curves at flow-rates above the minimum were small, the flow-rates chosen for use in the experiments were typically about 50% higher than the minimum. An 80 μl/min flow-rate was chosen for the 1 mm I.D. column. The flow-rates for the larger diameters were then scaled up from these, in proportion to the cross-sectional areas, so that the linear velocities were equal. This resulted in a reduction in plates from the optimum, but the analysis speed was increased significantly.

[b] The theoretical column efficiency describes the efficiency only in terms of geometric factors. It was calculated as L/H, where $L=150$ mm and H is assumed to be equal to three particle diameters, $3d_p$.

[c] The theoretical variance of a peak with $k'=1$ was calculated according to the equations in Ref. [37]. For this purpose, the column porosity is assumed to be 0.65, H is assumed to be equal to $3d_p$. Observed variances were larger, due, in part, to the extracolumn dispersion of the system.

Table 2
Characteristics of the three chromatographic systems

System designation	Injector[a] (internal volume)	Sample loop size installed on the injector used with each column[b] (extracolumn dispersion as measured by direct connection without a column)[b]			Column inlet tube from injector (length) (bore) (volume)	Column outlet tube to detector (length) (bore) (volume)	Detector optical cell[c] (illuminated volume) (path length)	Detector heat exchanger tube (length) (bore) (volume)
		Column diameter and flow-rate						
		1.0 mm 80 μl/min	2.1 mm 353 μl/min	4.6 mm 1690 μl/min				
System A, conventional	Model 7725 (3.2 μl)	10–50 μl ($114\ \mu l^2$)	20–200 μl ($234\ \mu l^2$)	50 μl–1 ml ($205\ \mu l^2$)	300 mm length 0.254 mm I.D. 18.2 μl[d]	250 mm length 0.254 mm I.D. 12.7 μl	Jasco UVIDEC-100-V 8 μl 10 mm	None
System B low dispersion	Model 8125 (0.79 μl)	10–50 μl ($12\ \mu l^2$)	20–200 μl ($23\ \mu l^2$)	50 μl–1 ml ($60\ \mu l^2$)	200 mm length 0.127 mm I.D.	250 mm length 0.127 mm I.D. 2.5 μl 3.2 μl	HP 1100 micro 1 μl 5 mm	555 mm length 0.1 mm I.D. 4.6 μl[e]
System C, very low dispersion	Model 8125 (0.79 μl)	10–50 μl ($1.3\ \mu l^2$)	20–200 μl ($3.2\ \mu l^2$)	N/A[f]	80 mm length 0.0635 mm I.D. 0.25 μl	310 mm length (fused-silica) 0.06 mm I.D. 1.46 μl	LC Packings UZ-HP11-CAP 35 nl 8 mm[g]	None

Three systems were used, differing in their injector, detector and connection tubing. In some experiments, additional injectors and detectors were used, as noted in the text. In these cases, reference to one of these systems is still valid by substituting the appropriate injector and/or detector.

[a] See Table 4 for the characteristics of the sample injectors. The internal volume is that through which both the leading and trailing end of the sample zone must pass in order to leave the injector. In these injectors it is comprised of rotor seal grooves, two stator face passages, and two stator port passages.

[b] All injections were made using the partial-loading technique. The loop was always at least twice the volume of the injection. The extracolumn dispersion of the three systems is included only because it varies with the column diameter used, as did the sample loops. There is no relationship between the loops and the extracolumn dispersion.

[c] All cells were used with the HP1100 variable wavelength detector at $\lambda = 250$ nm and the fastest response time (0.06 s), except System A which used a Jasco UVIDEC-100-V detector at $\lambda = 250$ nm and a time constant of approximately 0.1 s.

[d] The volume for the conventional system includes a 3-μl solvent pre-heater.

[e] The heat exchanger was removed for experiments in which the injector was connected directly to the detector.

[f] The 4.6 mm column was not used with System C, due to the excessive back pressure generated by the small-bore passages at the 1690 μl/min flow-rate.

[g] The 8 mm path of the 35-nl detector cell did not give a signal that was 1.6 times as high as that of the 5 mm path of the 1 μl cell, nor was the linear dynamic range as high. The excellent dispersion characteristics of the 35-nl cell come at a price of a lower signal-to-noise ratio and a limited linear dynamic range.

passages, to reduce dispersion, in timed injection experiments for characterizing the instrumental band broadening of the various systems. For most experiments, the injectors were electronically controlled using pneumatic or motor actuation to minimize timing uncertainties occurring with manual actuation. In this case, the last digit of the model number in Table 2 is a six instead of a five, i.e., model 8126 not 8125. The flow passages and all aspects of performance, except for actuation speed, are identical.

2.4. Data acquisition

Data was acquired using Chrom Perfect Direct and a DT2802 chromatography board (Justice Innovations, Mountain View, CA, USA). A program written in Microsoft Visual Basic was used for statistical moment calculation of peak variances. A model 2000-001 Validator (Axxiom Chromatography, Moorpark, CA, USA) was used to calibrate both programs.

2.5. Chemicals

HPLC-grade acetonitrile (Aldrich, Milwaukee, WI, USA) and water (EM Science, Gibbstown, NJ, USA) were premixed for isocratic runs at an acetonitrile–water ratio of 60:40 (v/v). The isocratic sample consisted of uracil, benzyl alcohol, phenethyl alcohol, acetophenone, propiophenone, butyrophenone, valerophenone and hexanophenone. The alkyphenones were purified using preparative chromatography on a 10-mm I.D. C_{18} column. The gradient sample was identical to the isocratic sample,

except for the addition of heptanophenone and octanophenone. All sample components were purchased from Aldrich.

3. Results and discussion

A few comments about column diameter are in order. The relationships between column diameter, sample volume, sensitivity and resolution have often been described [5,18,11,13,26–28]. When the mass of sample available is unlimited, such as when the sample exists in a given concentration and any volume is available for injection, there is no inherent difference in the sensitivity (limit of detection) and resolution provided by well-packed columns with diameters varying from 1.0 to 4.6 mm. This is because the volume injected can be scaled to the column volume. For example, for isocratic analysis using columns identical except for their diameters, a 1-μl injection of an unretained peak into a 1.0-mm I.D. column occupies the same 0.5 mm of column length (assuming an ideal injection and 65% column void volume) as does a 4-μl injection into a 2.0-mm I.D. column. Indeed, when the amount of sample available is sufficient to inject an optimum volume onto a conventional column, the use of a column with a smaller diameter sometimes reduces sensitivity and resolution, if the extracolumn dispersion is large, and reduces sensitivity if a detector is used, which sacrifices signal-to-noise ratio for the sake of reduced cell volume.

However, when the mass of sample available for injection is limited, such as when it exists in a given concentration in a given volume, and when that volume is smaller than could be injected into the larger column without compromising resolution, then the smaller column will produce greater sensitivity in terms of the mass limit of detection (MLOD). For example, for the aforementioned columns and, assuming that 1 μl is all the sample that is available, then the larger column produces a peak that is one-fourth as high and four times wider, i.e., one-fourth the concentration at peak maximum and in an eluted volume that is four times larger. This generalization assumes that the sample mass injected does not overload the sorptive capacity of the column and that the detector is within its linear dynamic range.

Thus, the theoretical relative MLOD of different column diameters is simply the ratio of the reciprocal squares of their diameters. However, the observed relative MLOD can be different, depending on the amount of extracolumn dispersion in the system and on the characteristics of the injectors and injection techniques used. This report concerns these second-order effects.

3.1. Characterization of injector dispersion

A theoretical discussion of injector dispersion, and measurements of the performance of various injectors used or mentioned here, is beyond the scope of this paper. The remarks below are qualitative descriptions intended to aid in understanding the general performance differences between injection techniques.

The performance factors of sensitivity and resolution depend on dispersion (band broadening) phenomena. The general theory of intra-column and extracolumn dispersion has been well documented by others. A review of some salient points follows.

3.1.1. Use of variance to characterize dispersion

The amount of performance loss due to dispersion from the injector depends on the magnitude of its dispersion relative to the magnitude of the dispersion caused by the column and the other components [18,25,29]. Dispersion is a measure of the extent of spreading of the concentration distribution about its center of gravity.

One way of quantifying dispersion uses a graphical determination of peak width, which is accurate only for Gaussian peaks [30]. It expresses the peak width, w, in microliters, as that which contains four standard deviations, 4σ. It is approximated by the width transected at the baseline when tangents are drawn to the peak's inflection points. One standard deviation, σ, is a quarter peak width. The variance is the square of the standard deviation.

Another way of quantifying dispersion uses the second statistical moment of the concentration profile. It is accurate for all peak shapes, including the asymmetrical ones that emerge from an injector, and is used most frequently in this report. The second statistical moment is the peak's variance, σ^2, in μl^2 [31,32]. It is the most useful description of disper-

sion because, in addition to its accuracy with all peak shapes, it allows the variance contributions of a series of components to be added to determine the total variance of the system [33,34,30]. The additivity of variances applies only when the dispersion due to each component is independent of the others. It is not clearly established that this independence is always the case, but the additivity of variances is a useful assumption in this work.

The total variance of a peak, as observed by the detector, is the sum of variances produced by the column (intra-column dispersion) and by components external to the column (extracolumn dispersion).

$$\sigma^2_{tot} = \sigma^2_{col} + \sigma^2_{ext} \quad (1)$$

3.1.2. Intra-column dispersion

The equations that describe the dependence of the variance of a particular peak (with a particular k') on a column's geometric factors, i.e., diameter, length, packing porosity and packing particle size are well known [35,36]. One can calculate the variance of a peak with a particular k', making certain assumptions about the relationship between particle diameter and plate height and about solute diffusion coefficients. The peak variance values in Table 1 were calculated according to the equations and assumptions in Ref. [37].

3.1.3. Extracolumn dispersion

The extracolumn variance is the sum of all non-column contributions to the peak variance and represents the instrumental contribution to band broadening. Several methods have been proposed for its determination [38–40,30,41–46]. The following method was used here to characterize the relative amount of extracolumn dispersion produced by the three systems. The column was removed and the tubes were connected by a drilled-through union, making a tube-to-tube direct connection. A 5-μl timed injection (see below) was made. The theoretical variance of this plug injection (2.08 μl^2) was subtracted from the observed variance. The result is the extracolumn variance of the system minus the injector. The values are given in Table 2.

Eq. (1) can be expanded to include a term for each component,

$$\sigma^2_{tot} = \sigma^2_{col} + \sigma^2_{inj} + \sigma^2_{tub} + \sigma^2_{det} + \sigma^2_{time} \quad (2)$$

i.e., the column, injector, tubes, detector, junctions and electronic time constant distortions.

3.1.4. Injector dispersion

Injector dispersion has been discussed by many workers, among them [33,35,36,47–55]. Most have considered sample injection to occur largely by displacement of a sample plug from the loop, accompanied by various contributions from exponential dilution (mixing). Injector dispersion has been considered as being too complex to be described by a simple mathematical model. Instead, performance has been assumed to be proportional to the square of the sample volume, with a constant of proportionality representing an observed performance factor:

$$\sigma^2_{inj} = V^2_{inj}/K \quad (3)$$

K is expected to vary between one and twelve, with typical values being between two and eight. The limiting values come from considering the injector as (i) operating at a theoretical optimum, whereby it introduces a true plug of undiluted solute into the column, which is not dispersed at all during transit out of the injector, in which case, $K=12$, or (ii) operating in a high-dispersion mode, as is produced by a mixing chamber, in which case, $K=1$, and the expression is that for the exponential dilution of a mixing chamber.

In any case, the injected sample enters the column in a finite volume, usually larger than the volume of the sample because of dispersion within the injector and connections to the column. The resulting solute zone formed at the column head is usually less than the volume as it enters the column, due to retention. In general, there is not one zone width but several, one for each analyte that has a unique amount of retention. The elution strength of the injection solvent and the effect of solvent dilution with the column's mobile phase will determine each of these initial zone widths. In the case of an unretained analyte, there is no focusing of the zone and all dispersion associated with the injection is manifested in the chromatogram.

3.1.5. Dispersion of a plug injection

The ideal injected sample enters the column as a plug, a volume element of uniform concentration [33,56,57]. It would result, for example, if a loop was filled completely with sample of uniform concentration and if, during travel of the plug to the column, (i) the rear boundary (with the incoming mobile phase) did not disperse as it passed through the loop, (ii) the front and rear boundaries did not disperse as they passed through the channels of the injector stator and (iii) there was no dispersion of either boundary during transit through the column's connecting tube. A plug has a variance which has an exponential dependence on volume, as defined by the central statistical moment of a square peak [33,58,59]:

$$\sigma^2_{plug} = V^2_{plug}/12 \quad (4)$$

Plug variance represents the lowest possible contribution from an injector. Ideal injector performance is closely approached only in the timed injection technique, discussed subsequently. In the other techniques, dispersion does take place, and the dispersion caused by an injector is the sum of the plug variance, which depends only on the sample volume and on the variance caused by dispersion of the sample zone as it passes through and out of the injector.

3.2. Sensitivity and resolution versus sample volume

In addition to the previous references, others have remarked on the dependence of sample volume on performance [27,51,60–63]. It is generally accepted that sensitivity is increased by injecting more mass (more volume at a given sample concentration), but that a loss of resolution occurs as the variance of the sample zone becomes significant compared to the variance contribution of the column and other system components. The following figures illustrate these relationships visually.

3.2.1. Isocratic elution.

Fig. 1 is a chromatogram of the sample used for the isocratic runs. It contains an unretained peak; two partially resolved, relatively unretained peaks, $k' \approx 0.5$ (hereafter referred to as the doublet) and an homologous series of five peaks with retention varying from $k' = 1$ to 5.

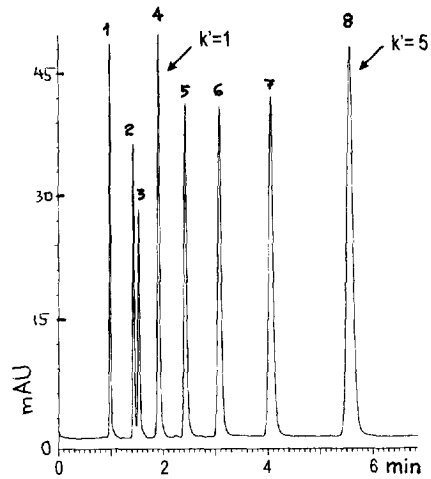


Fig. 1. Test mixture for isocratic analysis on all three column diameters. This chromatogram was made on the low dispersion system (system B of Table 2). UV detector at 250 nm and 62.25 mA.U.F.S. 150×2.1 mm I.D. Vydac 201HS 5 μm C_{18}. Flow-rate, 350 μl/min. Mobile phase, acetonitrile–water (60:40, v/v). Temperature, 30°C. Sample components: 1 = uracil (unretained), 2 = benzyl alcohol, 3 = phenethyl alcohol, 4 = acetophenone, 5 = propiophenone, 6 = butyrophenone, 7 = valerophenone, 8 = hexanophenone. The k' of peak 4 is about 1.0 on the 4.6 and 1.0 mm columns. It is 0.9 on this 2.1 mm column.

The height of the valley between the unresolved peaks is a convenient indicator of the loss of efficiency due to excessive sample volume and/or extracolumn dispersion. The height of all peaks is an indicator of sensitivity and also of resolution (the efficiency factor of resolution, the number of theoretical plates, N), since with a concentration detector, the peak area represents mass, and a decrease in peak height must be accompanied by a corresponding increase in peak width.

Figs. 2–4 are the results from injections into systems with progressively lower extracolumn dispersion; the conventional, low dispersion and very low dispersion systems (A, B and C in Table 2), respectively. They show how sensitivity and resolution are affected by sample volume. For each figure (each system), there is a set of fifteen chromatograms, five chromatograms for each of the three column diameters. The five chromatograms (vertical columns in the figure) are for five different volumetric loadings, expressed as a percentage of the

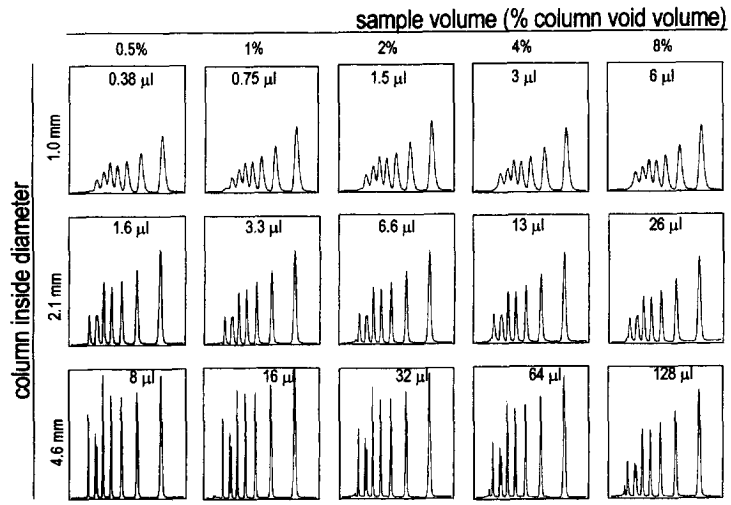


Fig. 2. Sensitivity and resolution versus sample volume in isocratic analysis on the conventional system (system A of Table 2). The volumetric loading of sample is listed in the column headings. It is expressed as the percentage of the column void volume. The actual volumes injected are listed with each chromatogram. See Fig. 1 for conditions.

column void volume; 0.5, 1%, etc., each successive one doubling the previous amount and therefore covering a sixteen-fold range of loading. Each successive volume contained sample at half the concentration of the previous volume. Thus, the total sample mass injected, for any one column diameter, across the range of five sample volumes, remained constant. This allowed the detector sensitivity, in terms of milliabsorbance units full scale (mA.U.F.S.), to be set at a constant level throughout the entire matrix of experiments, so it is easy to observe the changes in peak heights as representing changes in system sensitivity or concentration limit of detection (CLOD). For example, if a particular compound in a particular column has a peak height of 400 mA.U. with a 1-µl injection and has the same

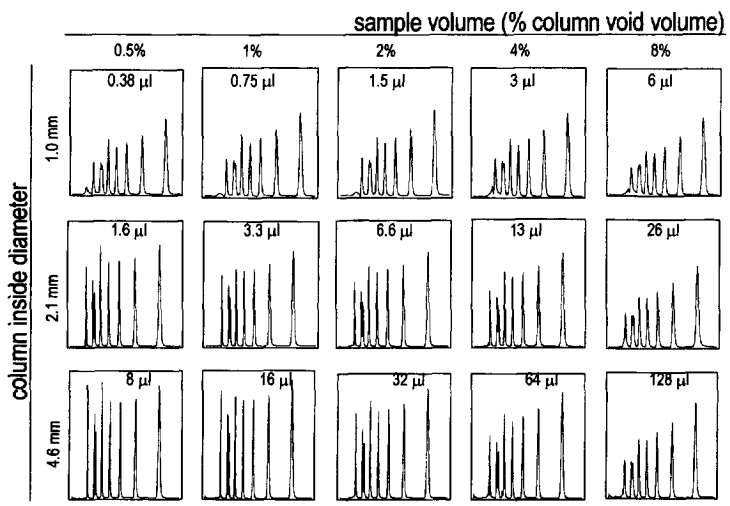


Fig. 3. Sensitivity and resolution versus sample volume in isocratic analysis on the low dispersion system (system B of Table 2). Same data as Fig. 2, but on the low dispersion system and at a detector sensitivity of 22.5 mA.U.F.S.

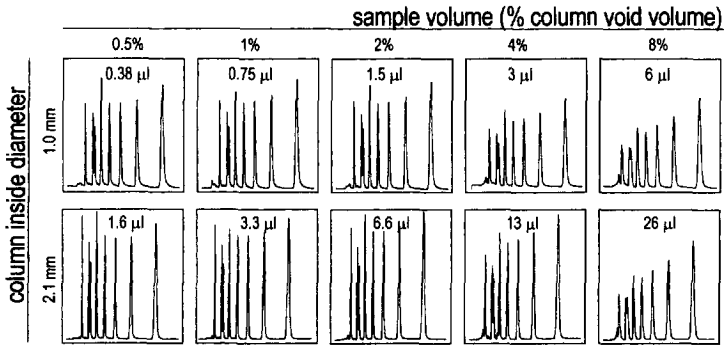


Fig. 4. Sensitivity and resolution versus sample volume in isocratic analysis on the very low dispersion system (system C of Table 2). Same data as Fig. 2, but on the very low dispersion system.

height with a 2-µl injection (at half the sample concentration), then the potential for a doubling of the CLOD is demonstrated; there is no penalty for injecting a larger volume.

Sample volumes were adjusted between the three column diameters so that the five volumetric loadings were the same for each column diameter, i.e., the sample volumes were made proportional to the column volume. The actual injected volume, expressed in microliters, for each experiment is also shown. This set of experiments corresponds to the case where an unlimited amount of sample is available, so that sample volumes can be scaled to the column diameter (see Table 1). The following observations are made.

In the conventional system, Fig. 2, it is clear that its extracolumn dispersion makes it inadequate for separating relatively unretained analytes with a 1.0-mm column. The doublet has fused to a single peak and is unresolved from peak four. Even the 2.1 mm column is a poor performer in this system. With the 4.6 and 2.1 mm columns, the resolution of the early peaks is increasingly compromised, relative to that at the lowest sample volume, as the sample volume is increased. In the case of the 1.0 mm column, if one were forced to use such a system, one might as well use a large sample volume, because the resolution is not noticeably compromised. The dispersion of the system is the dominant source of band broadening and the additional dispersion due to the large sample volume is relatively small. The total variance contributed by the plug and injector dispersion under these conditions, as measured previously in our laboratory, is about 10 μl^2, a small value compared to the 114 μl^2 extracolumn variance caused by the rest of the system (Table 2). If the resolution at the 8% loading is acceptable, it represents a sixteen-fold increase in concentration sensitivity compared to the 0.5% loading.

In the low dispersion system (Fig. 3), it can be seen that the performance of the 2.1 mm column is nearly as good as that of the 4.6 mm column. The same relationships between sample volume, sensitivity and resolution are again seen.

In the very low dispersion system (Fig. 4), the 1.0 mm column performs well. The resolution and the effect of sample volume on resolution is nearly identical to that observed with the 4.6 mm column in the conventional system. However, the sensitivity is not as good as might appear. In Figs. 2 and 3, the maximum on the absorbance scale is 62.25 mA.U. In Fig. 4, it is 22.5 mA.U., the detector sensitivity having been increased in order to produce peaks of adequate height. So, even though the peak heights appear to be identical in the figures, the signal from the 35-nl detector of Fig. 4 is only about one third as high. The noise with the 35-nl cell is somewhat higher also, so the net signal-to-noise ratio is approximately one fifteenth that of the 1-µl detector cell of the low dispersion system.

It should be noted that the 35-nl cell is actually inappropriate for even the 1 mm column. It is designed for use with columns with sub-millimeter diameters. Its volume is less than is necessary to preserve resolution. The more appropriate cell is the 140 nl cell, model UZ-HPII-MIC (LC Packings),

which would have better signal-to-noise characteristics [64]. The 35-nl cell was used for these experiments since it was on hand, having been used for experiments where the injector was connected directly to the detector for study of injector dispersion.

3.2.2. Gradient elution

Large sample volumes can be used in gradient elution because sample concentrates at the column head during the initial conditions. Dispersion of the sample that takes place before it enters the column is often insignificant for all but the early peaks. The enrichment of sample during injection may be viewed as a reduction of the sample–charge volume, while maintaining a constant mass of the analyte in the sample. For example, peptide purification takes advantage of this feature; it is common to inject 10 μl or more onto a 1-mm column [16,65].

Figs. 5 and 6, resulting from low dispersion (system B) and very low dispersion (system C) systems, respectively, show the same type of information as the previous three figures, except that these are under gradient elution conditions. No data were collected for the conventional system (system A). Two additional solutes with higher retention were added to the sample to provide a wide range of retention. The lowest sample volume has been increased ten-fold, to 5% of the void volume. A volume four-fold higher than this is also injected. As with the isocratic runs, the sample concentration is reduced in proportion to the sample volume so that the mass injected (with a given column diameter) is constant. The total time for these analyses (end of the baseline record) is 10 min.

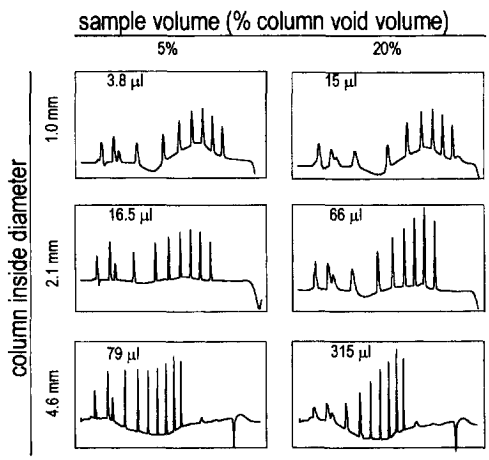


Fig. 5. Sensitivity and resolution versus sample volume in gradient analysis on the low dispersion system (system B of Table 2). This chromatogram was made on the low dispersion system (system B of Table 3). UV detector at 250 nm and 62.25 mA.U.F.S. 150× 2.1 mm I.D. HAIsil 5 μm C_{18}. Flow-rate, 350 μl/min. Gradient program: 40 to 95% acetonitrile in 4 min, hold at 95% for 3 min. Total run time shown on chromatograms is 10 min. Temperature, 30°C. Sample components: 1 = uracil (unretained), 2 = benzyl alcohol, 3 = phenethyl alcohol, 4 = acetophenone, 5 = propiophenone, 6 = butyrophenone, 7 = valerophenone, 8 = hexanophenone. The k' of peak 4 is about 1.0 on the 4.6 and 1.0 mm columns. It is 0.9 on this 2.1 mm column.

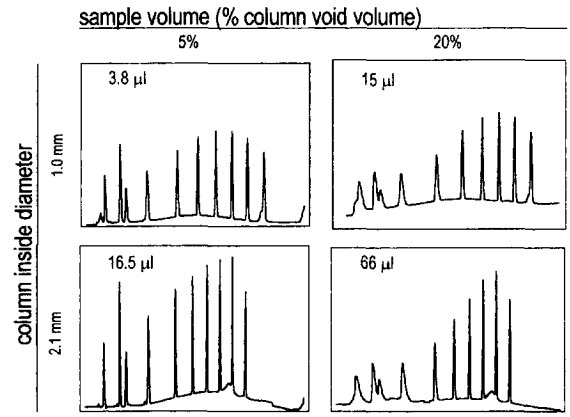


Fig. 6. Sensitivity and resolution versus sample volume in gradient analysis on the very low dispersion system (system C of Table 2). Same data as Fig. 5, but on the very low dispersion system and at a detector sensitivity of 22.5 mA.U.F.S.

In the low dispersion system, sensitivity and resolution are poor for the early peaks on the 1.0 mm column, especially in the 20% volume runs. Note that the first four peaks are affected. This is because the gradient does not reach the column inlet until after the fourth peak emerges, due to the delay (dwell) volume. The time of arrival can be seen from the onset of the negative baseline drift just after the fourth peak, at about 3 min into the run. The 2.1 mm column also had the same dwell time, because, for the 1.0 mm column runs, a splitter was used just before the injector, and the pumps were run at the same 350 μl/min flow-rate as the unsplit flow for the 2.1 mm column. The benefits of peak compression are not experienced by early peaks in gradient work with microbore columns because the delay time

causes the first part of the analysis to be in the isocratic mode.

In the very low dispersion system, the 4.6 mm column could not be run because the high flow-rates produced excessive back pressure in the small bore components. The resolution is improved over the previous system.

3.3. Comments on injection technique

Performance varies slightly among injection techniques because each exposes the sample to the dispersing influences of flow channels in a different way. The following nomenclature will be used: *Loading* (injector in the *load* position) is the low-pressure process of transferring sample into the loop from a syringe, or sample vial in the case of an autosampler. *Injecting* (injector in the *inject* position) is the high-pressure process of transferring sample into the column from the loop. The *injection cycle* is the process of actuating the injector from *load* to *inject* and back to *load*.

The connections of most injectors are made so that sample flows out of the loop during injection in a direction opposite to that during loading, i.e., first in–last out. In the partial-filling technique, this minimizes dispersion, because sample does not pass through the entire length of the loop. Thus, a long loop can be used to accommodate a wide range of sample volumes, without causing undue dispersion on small sample volumes. In this connection scheme, the *near end* of the loop is that into which sample is introduced during loading, and out of which sample flows during injecting; the *far end* of the loop is that from which excess mobile phase and/or sample exits during loading, and into which mobile phase flows from the pump during injecting.

3.3.1. Timed injection technique

Timed injection (also termed temporary, moving and time slice injection) has been described by several workers [66–76]. It has interesting characteristics and is a useful starting point for discussing dispersion phenomena within injectors. It nearly injects an ideal plug, producing injection variances that have a volume dependence that is close to those described by Eq. (4), and providing the best resolution and sensitivity of any injection technique. An excess of sample is loaded in order to flush out mobile phase that was trapped in the loop when the injector was actuated to load. During loading, the leading boundary of sample flowing into the loop disperses, caused primarily by the flow velocity inequalities across the cross-section of the tubing. However, when flushing is thorough enough (requiring many loop volumes if no air segmentation is used), this diffuse zone of sample exits at the far end of the loop and the resulting solute concentration throughout the loop, both axially and radially, is a uniform 100% of the original. The dispersion taking place during loading is therefore of no consequence for the subsequently injected sample.

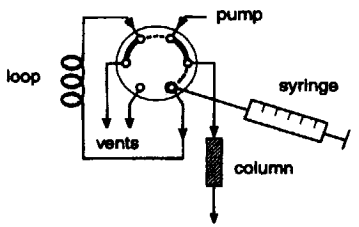
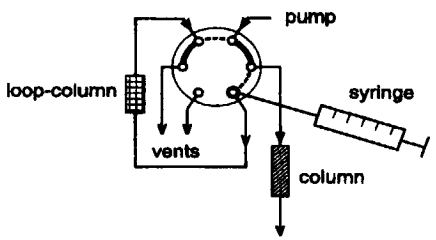
The injector is switched to the inject position and left there only long enough for the mobile phase to transfer the desired volume out of the loop ($s \times \mu l/s = \mu l$ injected). The loop must be long enough so that, when returned to load, sample emerging from the near end of the loop is still at the 100% initial concentration; the dispersing trailing boundary of the sample does not reach the near end of the loop before the loop is cut back off line. This requires a loop that has approximately twice the volume of the sample to be injected, since, as explained previously, the fluid at the tube axis has a velocity that is twice the average velocity, under laminar flow conditions. When the sample loop is a very small chamber, or shaped in such a way that dispersion is greater than that of tubular passages, even less sample can be injected before the disperse trailing boundary reaches the near end. The precise timing necessary to define accurate sample volumes is best achieved with a computer-controlled motor or pneumatic actuator [77–80].

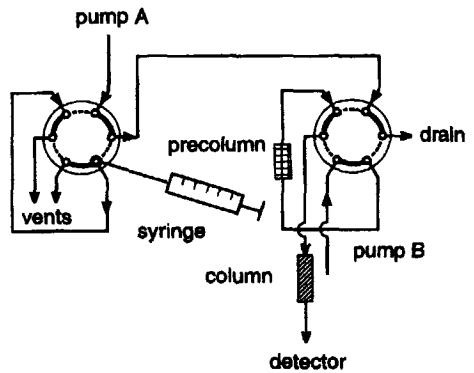
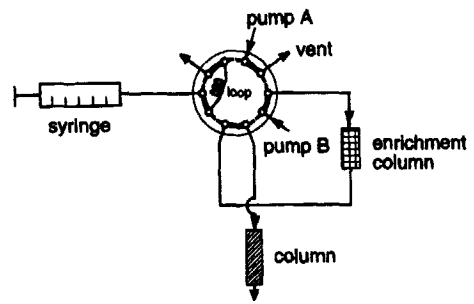
The peak that emerges from the injector has a concentration profile that deviates from a square wave only to the extent that the leading and trailing sample boundaries are dispersed by injector channels that must be traversed even after the return to load, usually one channel in the stator (at the column-connection port) and one rotor channel (a rotor seal groove in Rheodyne injectors). An almost perfect plug profile results when the sample volume is large compared to these channels. When the sample is small, the profile is significantly compromised.

A variation of this technique does not require the loop to be completely loaded, at least not to 100%

Table 3
Sample concentration schemes for reversed-phase HPLC

Sample solvent weaker than mobile phase means one with a higher water content, i.e., one which, if it were the mobile phase, would cause solutes to elute later. All columns are assumed to be reversed-phase columns.

METHOD	PROCEDURE	COMMENTS	FLOW DIAGRAM
1. On-column enrichment (injection of a large sample volume directly into the analytical column)			
1A. Volume overload Mobile phase strength is adjusted so analytes of interest are retained enough to avoid excessive dispersion. Sample is dissolved in mobile phase.	• Dissolve sample in mobile phase. • Load sample into loop. • Inject into column.	• Conventional chromatography except that sample volume is very large. • Works best with gradient elution.	
1B. Sample dilution Sample solvent strength is reduced by diluting sample with water. Analytes are initially retained (concentrated) at top of column during injection. On-column concentration more than offsets the dilution.	• Dilute sample with water (buffer) • Load sample into loop. • Inject into column	• Seems counterintuitive, but often works. • Useful when solid phase extraction leaves sample dissolved in solvent stronger than mobile phase; avoids need to take to dryness and reconstitute.	
2. Precolumn enrichment (injection of a large sample volume into a precolumn which is isolated by valving action from the analytical column)			
2A. Loop-column Enrichment column is part of sample loop. Sample is manually loaded into loop column, which has been conditioned to strongly retain analytes.	• Condition loop column with water or weak solvent. • Dissolve sample in solvent strength ≤ conditioning solvent. • Load sample into loop column. • Inject loop column	• Simultaneous sample cleanup is also possible. • Requires time and manual force to precondition loop column and to load it with sample. • Consider using a loop column ≤ 20 mm long.	

2B. Two-valve precolumn Enrichment column is on a separate 6-port switching valve. (requires two pumps)	• Condition enrichment column with water or weak solvent. • Dissolve sample in solvent weaker than conditioning solvent. • Load sample into loop. • Inject into enrichment column. • Switch enrichment column on line with analytical column.	• Simultaneous sample cleanup is also possible. • Requires two pumps, an injector, and a column switching valve. • Use a method-1 procedure when loading loop.	
2C. One-valve precolumn Enrichment column is on a 10-port combination injector/switching valve (requires two pumps)	• Same as 2B, but a single valve serves as both injector and column switching valve.	• Simultaneous sample cleanup is also possible. • Requires two pumps and a 10-port combined injector/switching valve • Use a method-1 procedure when loading loop.	

concentration. Nor does it require a return to load before the diffuse trailing sample boundary reaches the outlet port. The return to load is used just to truncate the dispersing rear boundary, creating a peak with lower dispersion. The precision of the injected mass will not be good, unless sample was loaded under automatic control.

3.3.2. Complete fill technique

In this technique, often called loop injection, loading is achieved as in timed injection. An excess of sample is loaded into the loop and the dispersed leading boundary during loading passes out the far end of the loop and is of no consequence. The dispersion during loading is "discarded" so that the trailing boundary at the far end of the loop starts its journey as a discrete sample–mobile phase boundary. The price of this "fresh start" is sample wastage. The sample volume (and solute mass) injected into the column is then determined solely by the size of the loop [81]. In reality, during loading, the contained mass approaches a maximum value asymptotically, so the final mass depends on the transferred volume relative to the loop volume.

3.3.3. Partial fill technique

This technique was used by the Waters model U6K and was often called universal, or variable, volume injection. In contrast to the previous two techniques, the volume of sample loaded into the loop is less than half of the loop volume, thereby ensuring that none of the in-flowing front boundary reaches the far end of the loop. The sample volume (and solute mass) injected into the column is then determined solely by the volume dispensed from the syringe [82,83], or sucked from the sample vial in the case of many autosampler designs, such as those which open the sample loop to form the sample probe. The in-flowing boundary disperses during its transfer into the loop. During injection, this same boundary, now the out-flowing rear boundary moving in the opposite direction, further disperses during its transfer back out of the loop. Since the far end of this boundary must travel a longer distance than the corresponding boundary, in the complete-fill technique, we might expect this bi-directional flow to cause significantly more dispersion. However, we have observed that partial-fill injection usually produces only twice the dispersion, exclusive of the plug variance, and often makes an imperceptible decrease in sensitivity and resolution [84].

3.3.4. Sample enrichment directly on the analytical column

This is a family of trace enrichment techniques, sometimes call on-column focusing [85] or peak compression [86], for increasing the CLOD. It is useful when the analytes of interest are in very low concentration and there is a sufficient volume of sample available to allow large injection volumes. The eluting strength of the sample solvent and of the mobile phase are adjusted relative to each other, so that the sample solvent is weaker (increased water content in RP-HPLC) than the mobile phase, in order to concentrate analytes of interest at the head of the column during injection, producing a narrow initial sample band. This allows injection of a large volume (large sample mass) to produce tall, narrow peaks.

The following discussion, which refers to Table 3, assumes that reversed-phase chromatography is being used, but the principles can be applied to all separation modes where sorption takes place on the stationary phase.

In the *volume overload* technique (1A), the mobile phase is adjusted to make the analytes of interest be retained sufficiently so that a large injection volume does not excessively degrade resolution. This is the simplest technique, when done in isocratic analysis, but it results in long analysis times. The analysis time can be greatly reduced by programming the mobile phase strength after injection, i.e., by stepwise or gradient elution, as illustrated in the previous figures.

In the *sample dilution* method (1B), the sample is diluted with water or buffer prior to injection. While the large volume is flowing into the column, the analytes are highly retained and focused at the column head, since the chromatography is taking place with a weak mobile phase. After the sample plug has passed into the column, the conditions revert to the mobile phase, effecting what amounts to a step gradient elution. Guinebault et al. [87] and Broquaire and Guinebault [88] showed the usefulness of dissolving an extracted sample in a large volume of solvent that is much weaker than the mobile phase, then injecting all, or part, of this

sample, as an easy means of increasing the sensitivity in reversed-phase and normal-phase [89] chromatography. The use of the weak component (water, in the case of reversed-phase chromatography) of the mobile phase as the sample diluent produced the most reproducible results. They term the sample solvent in such cases as a "non-eluting solvent". Solutions composed of 25% of the mobile phase diluted with the least eluting of its components could be injected without loss of efficiency. Since the analytes concentrate at the head of the column during injection, the peak widths are nearly independent of sample volume and the elution times are increased in proportion to the sample volume. The technique has been applied to microbore columns [90–92]. Neue and Serowik [93] have pointed out that, although it is counter-intuitive to achieve greater sensitivity by diluting the sample, the increased sample volume that can be injected overcompensates for the dilution.

Fig. 7 illustrates the essential features of this method. In this example, the sample was dissolved in a mobile phase of water–acetonitrile (40:60, v/v) and was diluted with water. An approximately sevenfold improvement in the detection limit resulted between the 20 μl injection of original sample (about the limit for the 2.1 mm column used, since larger volumes severely compromise the resolution) and the 2000 μl injection of diluted sample (200 μl of original sample diluted with 1800 μl of water). The improvement would have been even greater had the sample initially been in a solvent that was stronger than the mobile phase, as is common when the sample has been cleaned up off-line by elution from a solid-phase extraction column. In such a case, the volume of undiluted sample that could have been injected without loss of resolution would have been less than 20 μl.

3.3.5. On-line, two-column sample enrichment techniques

This family of techniques uses a precolumn before the analytical column. The sample is injected into the precolumn where enrichment and/or clean-up occurs, then the precolumn is switched on-line with the analytical column. This arrangement allows true multidimensional chromatography, where the two columns use different modes of retention, but this is beyond the scope of this paper.

In the *loop-column* technique (2A), the precolumn is attached to the injector, in place of the sample loop. The loop-column is conditioned with about two column volumes of water or a mobile phase that is lower in strength than that used for the analytical column, using the same procedure as if loading the

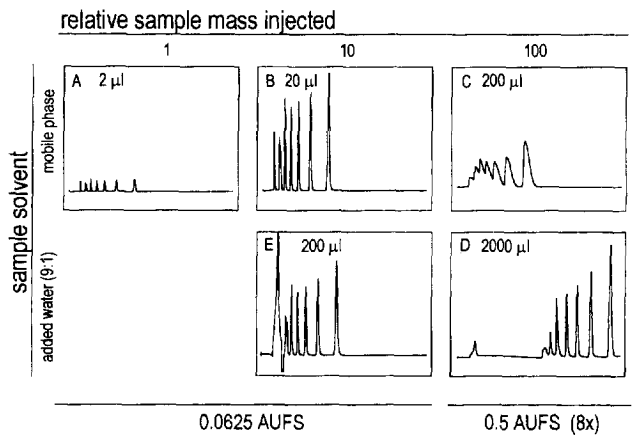


Fig. 7. On column enrichment by sample dilution. This chromatogram was made on the low dispersion system (system B of Table 2). UV detector at 250 nm and 62.25 mA.U.F.S. 150×2.1 mm I.D. Vydac 201HS 5 μm C_{18}. Flow-rate, 350 μl/min. Mobile phase, acetonitrile–water (60:40, v/v). Temperature, 30°C. Sample components: same as Fig. 1. In chromatograms A, B and C, the sample was dissolved in mobile phase. The sample volumes injected are indicated, as are the relative mass amounts (shown as column headings). In chromatograms D and E, water was added to the sample, diluting it ten fold. The detector sensitivity is indicated. The large peak in E is a refractive index peak caused by the added water. It is not so evident in D because of the lower detector sensitivity setting.

sample into a loop, except that the syringe is larger and more force and time are required. Then a large sample volume is manually loaded into the precolumn, the concentrated band of analytes is injected into the analytical column. When connections are made to the injector in the normal manner, the analytes are eluted in a backflush mode from the loop-column, which is usually preferred. Several papers have explored this technique [94–97].

In the *two-valve precolumn* technique (2B), the precolumn is attached to a six-port switching valve connected between the injector and the analytical column. The large volumes are injected onto the precolumn (enrichment column), where they are concentrated. This column is subsequently switched in line with the analytical column and the sorbed analytes are eluted off in a relatively narrow zone. This technique has recently been reviewed [98], and has become commonplace in biological applications. Many articles can be found, especially in the Journal of Chromatography, Biomedical Applications in the last ten years.

The *one-valve precolumn technique* (2C), is identical in function to the above, but uses a single valve to accomplish both injection and switching [80].

4. Conclusions

The chromatograms have illustrated how sample volume can be manipulated to control the trade off between sensitivity and resolution. The discussion provided guidance on choosing an injection technique. To summarize:

(A) To maximize sensitivity

(1) *Inject a large sample volume* under conditions which cause analytes to concentrate at the column inlet. In gradient analysis, use the lowest possible eluting strength for the initial conditions. In isocratic analysis, inject at least 100, 25 and 5 μl into 4.6, 2.1 and 1.0 mm columns, respectively, but many times more if resolution loss of the early peaks can be tolerated. To minimize the loss of resolution caused by the volume overload:

(a) Adjust the mobile phase to cause greater peak retention. However, this will significantly increase the analysis time.

(b) If the sample is in a water–organic mixture, especially if the organic concentration is higher than that of the mobile phase, dilute the sample with water, adding between two and ten parts of water. This will increase the analysis time only by the amount of time it requires to transfer the sample into the column. In the case of a 2.1 mm column, for example, a 2000-μl sample at 300 μl/min mobile phase flow takes about 6.7 min (actually a little longer due to dispersion of the rear sample zone) to pass into the column.

(c) Use a precolumn with one of two enrichment schemes: (I) Use a loop column in place of a sample loop. This requires only a conventional system, i.e., the use of only one pump. But it requires manual loading of the loop column with a syringe, which takes time and effort. (ii) Use a precolumn isolated from the analytical column by a six-port switching valve. This requires a second pump, but allows normal injection into the precolumn. Consider using a ten-port combination injector/switching valve.

(2) *Use the complete-fill injection technique* only if there is plenty of sample to waste. Otherwise, use the partial-fill technique, because this technique usually produces peak heights which are only a few percent lower. The partial-fill technique does not waste any sample. Use an injector designed for conventional systems, such as the Rheodyne Model 7125 or 7725, for isocratic analysis with 2.1 and 4.6 mm columns. These injectors usually also suffice for gradient analysis with 1.0 mm columns, especially when large samples (>5 μl) are injected. Use a low-dispersion injector, such as the Rheodyne Model 8125 or 7410 (see Table 4 for sample volume limitations), for isocratic analysis with 1.0 mm columns, unless one of the volume overload techniques (1b or 1c) is used, in which case there is little observable difference in performance.

Table 4
Characteristics of sample injectors

Model[a]	Type[b]	Loop geometry	Partial-fill volumes (practical range)	Sample loop sizes
7125	Dual mode	External	1 μl–2.5 ml	5 μl to 5 ml
7725	Dual mode	Internal & external[c]	1 μl–2.5 ml	2 μl internal 5 μl to 5 ml external
8125	Dual mode	External	0.1 μl–2.5 ml	5 μl to 5 ml
7410	Single mode	Internal	not practical	0.5, 1, 2, 5 μl
7520	Single mode	Internal	not practical	0.2, 0.5, 1 μl
7750TPMV[d]	Single mode	External	not practical	5 μl to 5 ml

[a] Rheodyne model number.
[b] Rheodyne produces two types of injectors, single mode and dual mode. A dual mode injector can be loaded by two methods, using either partial or complete filling of the sample loop. Sample volume in partial filling is determined by the syringe without wasting sample. With the complete filling technique, the loop is overfilled so that the sample volume is equal to the loop volume. It has seven ports; loading is through the port in the handle. A single mode injector uses only the complete filling technique. It has four to six ports and loading is through a stator port in the rear of the injector.
[c] The model 7725 is identical to the model 7125, except that the 7725 has a 2-μl internal sample loop accessory, continuous flow during switching (MBB, or make-before-break design), a front-end pressure screw and wide port angles.
[d] The 7750TPMV is a two-position electronically driven (motorized) valve.

(B) To maximize resolution

(1) *Inject a small sample volume*, but not less than required for adequate sensitivity. Use the above techniques to allow large sample volumes to be injected while preserving resolution.

(2) Use a 2.1-mm column instead of a 1.0-mm column if enough sample is available, unless the system is designed to handle the smaller column, in order to avoid resolution loss due to instrument band broadening.

(3) Use timed injection when injecting large volumes in isocratic analysis only when even a small improvement in resolution is valuable, since the enhancement is usually marginal.

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