The Selection of Optimal Conditions in HPLC
II. The Influence of Column Dimensions and Sample Size on Solute Detection

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Key Words

Liquid chromatography
Dilution and signal enhancement factor
Maximum injection volume
Calculation methods
HPLC small volume columns

Summary

The responding signals of eluted components can be enhanced by using high performance small volume columns with long lengths generating more plates than required for a separation with a preset resolution in combination with instruments which show very small external bandbroadening. The excess number of plates is then consequently erased by maximizing the injection volume of the sample until the preset resolution is reached. Equations, describing the dilution, relative signal enhancement and maximum injection volume as a function of the process parameters are derived and experimentally verified. Theory and experiment are in agreement only if variances of response functions are calculated as their second normalized central moments.

Introduction

The influence of column performance, -dimensions and sample size on solute detection has been investigated theoretically and experimentally by several authors [1–4]. Recent developments in column technology, with emphasis on small volume high efficiency columns, are putting high demands on instruments which are able to cope with this trend [5–27]. These demands are reduction of external band spreading together with appropriate time constants and sampling rates of the analog and digital parts of the data handling system.

In our opinion description of external band spreading in the literature has limited applicability for three reasons:

a) by subdividing the chromatographic system into subsystems and assuming that the latter are independent of each other,

b) by describing the individual subsystems with physical models neglecting, however, border effects which are responsible for serious deviations from these models,

c) by using methods for calculating variances of output functions which are not accurate.

In a previous paper [27] we proposed a system approach to describe external band spreading and deduced the interdependence of the latter with injection volume and flow.

The results are used in this paper to describe the relationship between external band spreading, injection volume, column bandbroadening and solute dilution on small volume columns.

Theoretical

Dilution and Chromatographic Process Parameters

The dilution of a component during its migration through the column directly affects its detectability.

The latter depends on both the sensitivity of the detector (= slope of the calibration curve) and the peak height or maximum concentration of the eluted component at the column outlet, which can be regarded as a meaningful deviation from the base line noise. The minimum peakheight is defined as a multiple of the peak to peak noise [4]. It is therefore obvious that the chromatographer wants to increase the peakheight of eluting components.

The relationship between the maximum concentration of a solute at the column outlet, C_{max}, and the amount of mass, Q, injected is [28].

$$C_{max} = \frac{Q}{\sigma_{v(col)} D_0} \qquad (1)$$

where $\sigma_{v(col)}$ = volume standard deviation of the impulse response of the column and D_0 = normalization factor which accounts for the shape of the elution profile and the method with which its variance is calculated [27].

If mass overloading (affecting column efficiency and peakshape) is to be avoided, the linear part of the distribution isotherm should not be exceeded.

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Moreover, solubility of sample components may limit the initial concentration, C_0.

As

$$Q = C_0 V_{inj} \quad (2)$$

where V_{inj} = injected sample volume, Q can only be increased by injecting a larger sample volume, which in itself has an effect on column efficiency.

The solubility limitation of the sample mainly occurs if complex sample matrices are involved. Combining eqs. (1) and (2) the dilution, DF, of the solute can be described as

$$DF = C_0/C_{max} = \frac{\sigma_{v(col)} D_0}{V_{inj}} \quad (3)$$

[Note that this expression is the reciprocal of the currently accepted definition. Here, DF = 1 (optimum value) if $C_{max} = C_0$ and $DF > 1$ if $C_{max} < C_0$ (sample diluted)].

With

$$\sigma_{v(col)} = \frac{\epsilon_t V_{col} (1 + k')}{N_0^{1/2}} \quad (4)$$

where ϵ_t, V_{col}, k' and N_0 are the total porosity of the column bed, column volume, capacity factor and the number of theoretical plates generated by the column respectively, eq. (3) changes into

$$DF = \frac{\epsilon_t V_{col}(1 + k')D_0}{V_{inj} N_0^{1/2}} \quad (5)$$

Obviously, the dilution of a solute is small for low capacity factors, large plate numbers and small ratios of column and injection volumes.

If column volume, particle size (d_p) and capacity factor are kept constant then the number of plates can only be increased by increasing the column length and decreasing the column diameter.

In comparing two of these columns, A and B, B being the one with the longer length, and assuming identical output profiles, then it follows from eq. (5) that

$$DF^B = (V_{inj}^A/V_{inj}^B)(N_0^A/N_0^B)^{1/2} DF^A \quad (6)$$

Under these conditions several conclusions can be drawn from eq. (6)

1. plate number (N_0) on each column is not affected by sample size (hypothetically small injection volumes):
   - if $V_{inj}^B = V_{inj}^A$ then dilution on column B is smaller than on column A ($DF^B < DF^A$)
   - to achieve equal dilution on both columns ($DF^B = DF^A$), a smaller sample volume on column B must be injected ($V_{inj}^B < V_{inj}^A$)
2. plate number on column B (N_0^B) is affected by sample size (large injection volumes) so that $N_0^B \Rightarrow N_s^B = N_0^A$ (where N_s^B = number of theoretical plates generated by the chromatographic system with column B under these conditions
   - dilution on column B will be much smaller than on column A. This is described in more detail in the next subsection.

Dilution and External Band Spreading

Up to now external band spreading was neglected in our discussion. It emerges by describing the volume variance of the output function, $\sigma_{v(tot)}^2$, as [27, 79]

$$\sigma_{v(tot)}^2 = \left(\frac{V_{inj}}{D_i} + \sigma_{v(0)}\right)^2 + \sigma_{v(col)}^2 \quad (7)$$

where $(V_{inj}/D_i)^2$ = volume variance of the injected sample, D_i = normalization factor depending on the shape of the input profile and the calculation method [27], $\sigma_{v(0)}^2$ = volume variance of the impulse response of the chromatographic system (instrument only) and the crossterm $(2 V_{inj} \sigma_{v(0)}/D_i)$ reflects the additional variance caused by interdependence of instrumental broadening and the width of the input function.

Replacing $\sigma_{v(col)}$ by $\sigma_{v(tot)}$ in eqs. (1) and (3) and combining with (7) results in

$$DF = C_0/C_{max} = \frac{D_0}{V_{inj}} \left[\left(\frac{V_{inj}}{D_i} + \sigma_{v(0)}\right)^2 + \sigma_{v(col)}^2\right]^{1/2} \quad (8)$$

So, in addition to the statements mentioned before (see eq. 5) dilution is further minimized if $\sigma_{v(0)}$ is kept as small as possible. However, the absolute values of $\sigma_{v(col)}$ and V_{inj} are limited by the boundary conditions for the separation of a component pair.

The Relative Signal Enhancement Factor (E)

For a certain component i this factor, E_i, emerges from the comparison of columns A and B (see eq. (6)) and applying eq. (8); the result is

$$E_i = C_{i,max}^B/C_{i,max}^A = \frac{h_i^B}{h_i^A} = \frac{V_{inj}^B \sigma_{v,i(tot)}^A D_0^A}{V_{inj}^A \sigma_{v,i(tot)}^B D_0^B} \quad (9)$$

where h_i^B and h_i^A are the peakheights corresponding with the injected sample volumes V_{inj}^A and V_{inj}^B on column B and A respectively.

If $V_{inj}^A = (V_{inj}^A)_R^{max}$, which means that for this sample volume the preset resolution, R, for a component pair separated on column A is achieved (or $N_s^A = N_{req}$); then

$$\sigma_{v,i(tot)}^A = V_R/N_{req}^{1/2} \quad (10a)$$

where N_s^A = number of theoretical plates generated by the chromatographic system with column A; N_{req} = required plate number to achieve the preset resolution on column A for a certain α and k' and V_R = retention volume of the investigated component.

It is obvious that the same plate number, N_{req}, will suffice on column B to achieve the same resolution as on column A (under the afore mentioned restrictions on α and k'). As column B, according to its longer length, has intrinsically more plates available than column A if the same sample size, $V_{inj}^B = (V_{inj}^A)_R^{max}$, is injected then its excess number of plates, $N_s^B - N_{req}$, can be erased by increasing the sample volume, V_{inj}^B. This in continued until the plate number on column B has decreased to $N_s^B = N_{req}$ and therefore $\sigma_{v,i(tot)}^B = \sigma_{v,i(tot)}^A$ (eq. (10a)). The maximum signal enhancement factor is then given by eq. (11)

$$(E_i)_R^{max} = \frac{(V_{inj}^B)_R^{max} D_0^A}{(V_{inj}^A)_R^{max} D_0^B} \quad (11)$$

Apart from the peakshape normalization factor, D_0, this result is rather trivial. However, by injecting larger sample sizes this factor substantially changes and cannot be neglected for an exact description of dilution.

From the above mentioned limiting conditions the maximum allowable sample size, $(V_{inj}^B)_R^{max}$, can be calculated as follows: As $N_s^B = N_{req}$, therefore

$$\sigma_{v,i(tot)}^B = V_R / N_{req}^{1/2} \quad (10b)$$

and in combination with eq. (7) the result is

$$(V_{inj}^B)_R^{max} = D_i \left[V_R \left(\frac{1}{N_{req}} - \frac{1}{N_0^B} \right)^{1/2} - \sigma_{v(0)} \right] \quad (12)$$

An analogous expression was derived for preparative liquid chromatography in a previous paper [30]. Eqs. (11) and (12) show that the maximum relative signal enhancement for component i increases with increasing number of plates (large N_0) and separations which do not require many plates (small N_{req}).

Moreover, instruments which produce slug type injection profiles (largest D_i) and show small external bandbroadening, $\sigma_{v(0)}$, are strongly favoured.

Experimental

The apparatus was described in a previous paper [27]. Acetonitrile (p.A. grade; E. Merck, FRG) and water (distilled and deionized) were premixed (40/60% v/v respectively) in the eluent bottle. ODS Hypersil® (5 μm) was used as the stationary phase in stainless steel (316) columns of 30 x 4.6 and 144 x 2.1 mm equipped with low dead volume ($< 1.5\ mm^3$) fittings.

The sample consisted of 3,4-dimethylphenol (87 ppm; $\epsilon_M = 1780$) and 2,6-dimethylphenol (103 ppm; $\epsilon_M = 1450$) and was dissolved in the eluent (ϵ_M = molar extinction coefficient).

Results and Discussion

To show the potential of this concept, and the agreement between theory and experiment, columns with the following characteristics were compared (Table I). Platenumbers were calculated by using two different calculation methods:

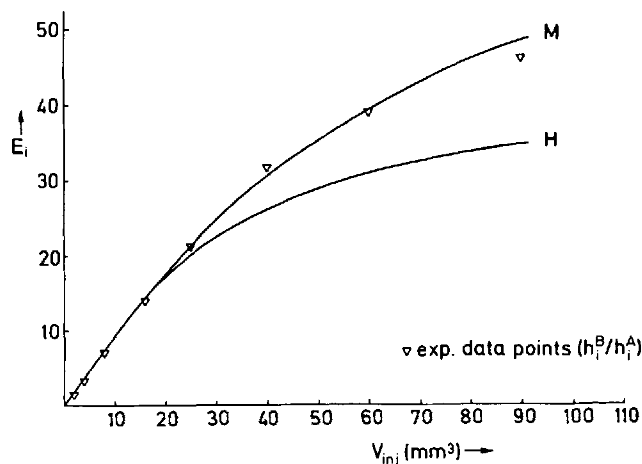

Fig. 1

Comparison of experimental and calculated relative signal enhancement on column B at different injection volumes.

Upper curve, M, calculated with moment method; lower curve, H, with handmethod (eqs. (9) and (10)).

a) from the second normalized central moment (moment method = M)

b) from the width at 0.607 of the peakheight (handmethod = H).

The experimentally observed relative signal enhancement of the second peak of the separated pair on column B was plotted against the injected sample volume and compared with the calculated values according to eq. (9), assuming that the required plate number is achieved on column A for a sample volume of $2\ mm^3$ (see Table I) and $D_0^A = D_0^B$.

The results are summarized in Fig. 1.

This figure shows that the experimental datapoints are in close agreement with the calculated values if the injected sample volume is smaller than $25\ mm^3$. Beyond this volume peaks shapes gradually change and the agreement still holds if and only if statistical moments are applied. This again is a proof that theory and experiment in chromatography are violated if variances of non-gaussian response functions are calculated from the width at 0.607 of the response height.

Another way to show the potential of the longer column is shown in Fig. 2. Here the sample was diluted 50 times and

Table I. Characteristics of applied columns

	Column Dimensions (mm)	Flow (cm^3/min)	U_0 (mm/s)	V_{inj}^{min} (mm^3)	k'_1	k'_2	α	N_{s2} H	N_{s2} M	R H	R M	DF_2 H	DF_2 M
A	30 x 4.6	2.57	3.68	2	2.3	3.0	1.30	1200	1030	1.82	1.61	50.6	54.6
B	144 x 2.1	0.53	3.63	2	2.3	3.1	1.35	4800	3500	3.84	3.32	25.8	30.8

U_0 linear velocity of an unretained component (calculated with $\epsilon_t = 0.70$)
α k'_2/k'_1; selectivity factor
R resolution between component 1 and 2 (IUPAC definition, see below)
N_{s2} measured plate number of component 2
DF_2 calculated dilution for component 2 with the assumption: $D = \sqrt{2\pi}$
H handmethod
M momentmethod

2 respectively 60 mm^3 were separately injected on column A c.q. B. It reveals that in this way picogram amounts of this sample can be detected on the longer column.

The maximum allowable injection volume at the specified flow (0.53 cm^3/min), on column B can be calculated from eq. (11) by inserting the appropriate values [27]. For the moment method ($D_i = 3.05$; $N_{req} = 1030$; $N_0 = 3500$; $V_R = 1431\,mm^3$; $\sigma_{v(0)} = 6.4\,mm^3$) the result is 95 mm^3 and for the hand method ($D_i = 1.80$; $N_{req} = 1200$; $N_0 = 4800$; $V_R = 1431\,mm^3$; $\sigma_{v(0)} = 5.0\,mm^3$) a value of 56 mm^3 is obtained.

If the values of D_i and $\sigma_{v(0)}$ for a chromatographic system at its optimized working conditions (α, k', U_0) are known, the maximum allowable injection volume for any type of column can easily be calculated from the results of a single small volume injection on that column. This allows to calculate immediately V_R and N_0 for the components of interest. The required plate number, N_{req}, for the investigated components, is then calculated from the obtained resolution, R, as follows:

$$R = \frac{t_{Rj} - t_{Ri}}{2(\sigma_{tj} + \sigma_{ti})} \quad \text{(IUPAC definition for resolution)} \qquad (13)$$

where $t_{Rj,i}$ = retention times of components j and i ($t_{Rj} > t_{Ri}$) and $\sigma_{tj,i}$ = time standard deviations of the respective elution profiles.

Modification of eq. (13) with the expressions $\sigma_{ti} = t_{Ri}/N_i^{1/2}$, $\sigma_{tj} = t_{Rj}/N_j^{1/2}$ and assuming $N_i = N_j = N$ results in

$$N^{1/2} = 2(t_{Rj} + t_{Ri})R/(t_{Rj} - t_{Ri}) \qquad (14)$$

Specifying the desired resolution ($R = R_s$), the required plate number then is

$$N_{req}^{1/2} = 2(t_{Rj} + t_{Ri})R_s/(t_{Rj} - t_{Ri}) \qquad (15)$$

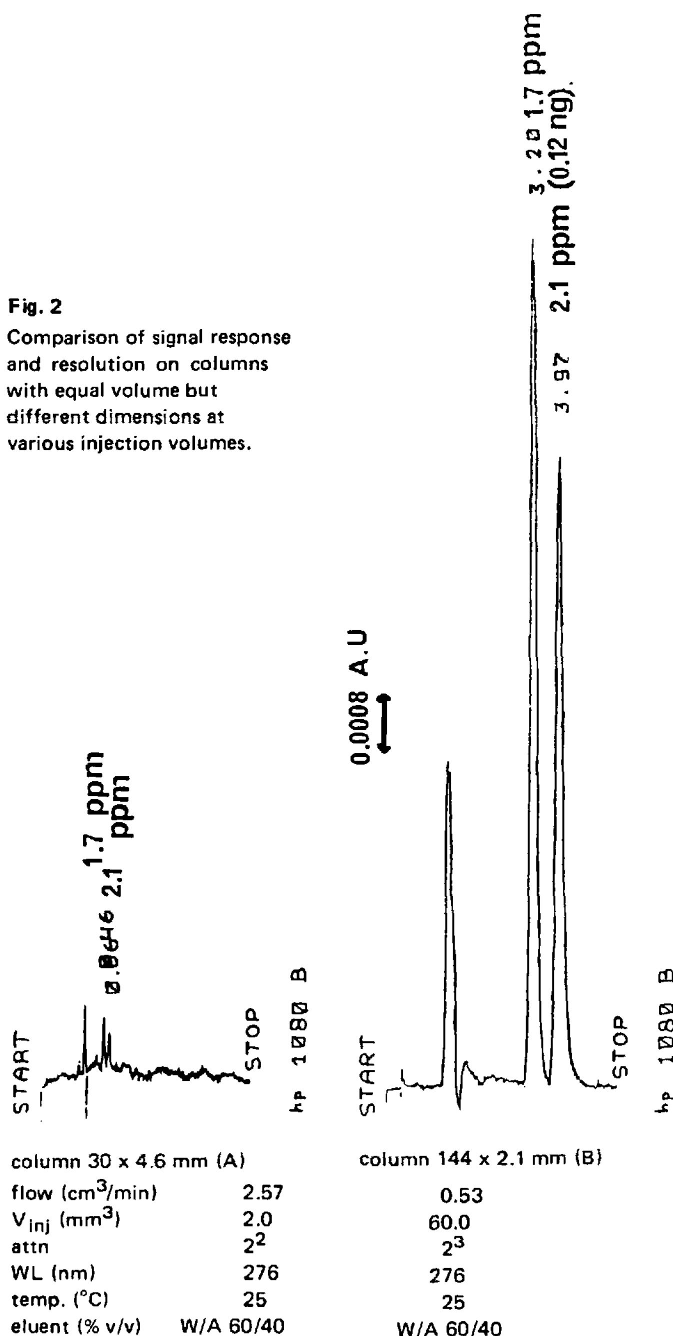

Fig. 2
Comparison of signal response and resolution on columns with equal volume but different dimensions at various injection volumes.

Conclusions

Detection of chromatographed solutes is improved if the dilution during their migration through a column is minimized. This can be achieved by applying small volume, high efficiency columns which generate more plates than required for a particular separation. Optimum results are obtained if the excess number of plates is erased by the maximum allowable injection volume (eq. (11)).

The latter increases with increasing column plate number and decreasing required plate number whereas instruments which produce slug type injection profiles and show very small external bandbroadening further improve this value.

Theory and experiment are in agreement only if variances of response functions are calculated as their second normalized central moments.

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List of Symbols

C_{max}	Maximum concentration of eluted component at column outlet
C_0	Initial concentration of the sample
D_i, D_0	Factor depending on input (i) c.q. output (0) profile and calculation method
$DF^{A,B}$	Dilution factor on column A resp. B
d_p	Mean particle diameter of the stationary phase
E_i	Relative signal enhancement factor of component i

$h_i^{A,B}$ Experimentally observed peakheight on column A or B

k'_1, k'_2 Capacity factor of component 1 resp. 2

N_i, N_j Theoretical plate number of component i resp. j

N_0 Number of theoretical plates generated by the column for an infinitely small injection volume

$N_{0,2}^{H,M}$ N_0 for component 2 calculated by hand-(H) or moment method (M)

N_{req} Number of plates required for a certain resolution

$N_s^{A,B}$ Number of theoretical plates generated by the chromatographic system with column A resp. B

Q Injected amount of mass

R, R_s Any c.q. specified resolution

t_{Ri}, t_{Rj} Retention time of component i c.q. j

U_0 Linear velocity of unretained component

V_{col} Volume of empty column

$V_{inj}^{A,B}$ Volume of injected sample on column A c.q. B

$(V_{inj}^{A,B})_R^{max}$ Maximum volume of injected sample to achieve a preset resolution R on column A or B

V_R Retention volume of investigated component

α Selectivity factor = ratio of capacity factors of a solute pair

ϵ_t Total porosity of the column bed

σ_{ti}, σ_{tj} Standard deviations (time) of the elution profiles for component i resp. j

$\sigma_{v(inj)}$ Volume standard deviation of the injection profile

$\sigma_{v(col)}$ Volume standard deviation of the impulse response of the column

$\sigma_{v(0)}$ Volume standard deviation of the impulse response of the instrument

$\sigma_{v(tot)}$ Volume standard deviation of the elution profile

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