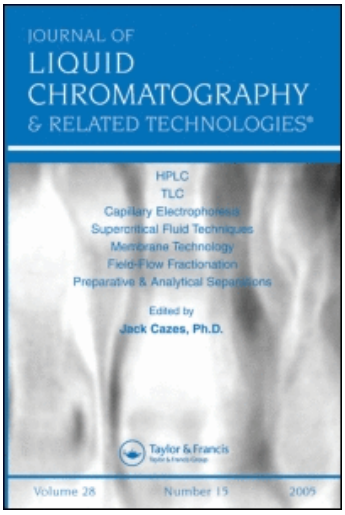

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Modification of a Conventional High Performance Liquid Chromatograph for Use in High Speed Liquid Chromatography

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Abstract: A practical procedure is described for the modification of a conventional HPLC system to a set-up compatible with high speed HPLC requirements. A number of experimental parameters, such as extra-column volume, extra-column band broadening, effectiveness, and dwell volume, were examined to characterize and demonstrate the suitability of the modified system for use in high speed HPLC applications. In addition, an injector bypass is presented as an alternative to reduce the pressure damage suffered by short columns packed with microparticles.

Keywords: High speed HPLC, Extra-column band broadening, Injector bypass

INTRODUCTION

Modern liquid chromatography is a powerful analytical technique that is now included in the restrictive group of total analysis systems (TAS).[1] This technique satisfies most of the requirements demanded of analytical techniques by modern industry and is essential in current organic analysis. For this reason, HPLC is the leading analytical technique in various fields, such as clinical, forensic, environmental, food, and pharmaceutical chemistry. Nevertheless, in some applications such as fermentation

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control, pharmacokinetic studies, and process control in general, classic liquid chromatography may be slow for real time control. This inconvenience may be overcome using high speed liquid chromatography (HSLC), also called fast HPLC, which performs rapid and reliable analyses in seconds or few minutes.

In its more classic version, this technique uses columns of 50 mm × 4.6 mm I.D., 3–5 μm particle size, operated at a flow rate of around 3 mL min^{-1}. In our opinion, the diameter of these columns should be lower than 3.2 mm, as such columns allow the flow rate to be reduced notably. In addition, narrow bore columns increase the effectiveness, sensitivity, and precision of chromatographic methods. If the former columns show bad resolution, it is now possible to acquire high speed columns packed with a 2 or 1.5 μm particle size (porous or non porous), which present higher efficiency per unit length (higher than 150,000 plates/m) and are able to carry out complex and fast separations. Their current price is, however, high. Another modern alternative is the monolithic stationary phase, characterized by its high porosity and permeability. The reduction in analysis time is achieved by increasing the flow rate up to 9 times the usual one in a conventional column. The consumption of solvents is high and the price of the columns remains expensive.

In order to maintain the true efficiency of these columns, some attention must be paid to special requirements of high speed instruments as regards the injection valve, the detector, and the connecting tubes with the aim of reducing the instrumental bandwidth (IBW), also known as extracolumn band broadening or spreading. These effects of instrumental dispersion on separation effectiveness have been studied by a great number of researchers and some equations and methods have been developed for their determination.[2–10] All of these highlight the need to reduce IBW in order to obtain fast separations.

In this paper, we describe the conversion of a conventional HPLC system to a configuration that is compatible with HSLC, based on the previous developed chromatographic theory. For this modification, no more than 2500 $ are needed. The performance of the adapted system was tested with the purpose of enhancing laboratory productivity, increasing mass sensitivity, and reducing solvent consumption, thus reducing both the cost of analyses and environmental pollution.

EXPERIMENTAL

Standards and Mobile Phase

The efficiency experiments were carried out using a standard reversed-phase test mixture containing naphthalene (100 mg L^{-1}), acenaphthene (200 mg L^{-1}), and anthracene (4 mg L^{-1}), whereas the extracolumn band

broadening studies were performed with anthracene (5 mg L^{-1}) as the test probe. All polyaromatic hydrocarbons were obtained from Supelco (Bellefonte, PA, USA). The mobile phase consisted of water-CH_3CN (35 : 65), except for dwell volume determination, where methanol was employed. HPLC gradient quality acetonitrile and methanol were purchased from Romil (Loughborough, UK) and Milli-Q water from Millipore (Milford, MA, USA). Standard solutions were prepared in acetonitrile and filtered through a 0.22 μm PVDF syringe filter (Lida, Kenosha, WI, USA).

Instrumentation

All the LC experiments were performed on a Shimadzu HPLC system (Duisburg, Germany) equipped with an on-line DGU-14A vacuum degasser, two LC-10ADvp pumps, a SIL-10ADvp autosampler, a CTO-10Avp oven, and a UV-Vis SPD-M10Avp photodiode array detector. This standard equipment was purchased with 60 cm connecting tubes of 305 μm internal diameter and a detector flow cell of 10 μL and 10 mm path length. The modified equipment has tubing of 127 μm I.D. and 60 cm length, and a detector micro flow cell of 2.5 μL and 5 mm path length. The column used was a Kromasil 100 C_{18} (50 mm × 2.1 mm I.D., 3.5 μm) (Teknokroma, Barcelona, Spain). The chromatographic experiments were carried out at 30°C, 254 nm, and 80 ms of response time.

Injector bypass was carried out using two Valco union tees (stainless steel 1/16 in o.d. tubing), three connecting tubes, and the appropriate fittings. More details are given in Figure 2. Union tees, Valco male nuts, and ferrules were purchased from Supelco. Rheodyne male nuts and ferrules were obtained from Upchurch Scientific (Oak Harbor, WA, USA).

All connecting tubing was stainless steel 1/16 in o.d. Tubing and polyether ether ketone (PEEK) finger tight fittings were supplied by Upchurch Scientific. Chromatographic data were collected and processed using Shidmadzu CLASS-VP Version 5.032 software.

The efficiency of the Kromasil column was calculated by using the peak width at half-height method. Extra-column volume, extra-column band broadening and dwell volume were measured by replacing the column with a Valco zero dead-volume union acquired from Supelco. The retention time of anthracene (5 mg L^{-1}) was multiplied by the flow rate to calculate the extra-column volume. IBW values were obtained by means of peak widths at half-height and multiplying by the flow rate and by a factor of 1.7, according to the Tangents Method. Dwell volume was determined graphically from a run instantaneous step change from methanol to methanol containing 10 mg L^{-1} anthracene. An S-shaped detector trace is created and the time delay is measured from the point at which the gradient has started to the point where half the height of the step is reached. The gradient delay or dwell volume is, thus, obtained by multiplying this time by the flow rate.

All results were the mean of at least four injections. Asymmetry was measured at 10% of peak height and was always found to be lower than 1.3.

RESULTS AND DISCUSSION

The overall performance of a chromatographic system is given by the performance of the column itself and by the instrumental contribution to band broadening. Assuming a Gaussian peak shape and that all contributions to peak variance are independent, the variance is an additive property and the system variance (σ^2_{sys}) may be broken down into contributions from the column variance (σ^2_{col}) and the extracolumn variance (σ^2_{ex}).[11]

$$\sigma^2_{sys} = \sigma^2_{col} + \sigma^2_{ex}$$

The extracolumn variance consists of the variance originating from the injection process (σ^2_{inj}), the connecting tubes (σ^2_{tub}), the detector (σ^2_{det}), and other contributions (σ^2_{other}) from the remaining components, for example switching devices and connectors.

$$\sigma^2_{ex} = \sigma^2_{inj} + \sigma^2_{tub} + \sigma^2_{det} + \sigma^2_{other}$$

Critical, in this respect, is the ratio of the extracolumn volume to the column volume itself. The smaller the column used, the stronger the influence of the extracolumn volume of the instrument. Extracolumn band broadening effects must, thus, be minimized so as to obtain the maximum performance from the column, especially for narrow bore columns employed for fast separations (fast gradients and early eluting peaks). The loss in plate number due to IBW effects should not exceed 10%.[12]

Solvent Delivery System

The flow rate (F) at which a chromatographic column can work may be calculated by the expression:

$$F = u\pi d_c^2 \varepsilon/4$$

where u is the linear velocity of the mobile phase, d_c the column diameter, and ε the column porosity (0.7).[13,14] The modern pumps employed in HPLC could work between $1\,\mu L\,min^{-1}$ in isocratic mode (or $10\,\mu L\,min^{-1}$ in gradient mode) and $5\,mL\,min^{-1}$. These specifications are able to provide a precise and constant flow of the mobile phase to operate with conventional (3.2–4.6 mm I.D.) and narrow bore (1–2.1 mm I.D.) columns, now employed in HSLC, though not with the conventional monolithic columns that could work at $9\,mL\,min^{-1}$.

The reduction in column diameter from conventional (4.6 mm) to narrow bore (2.1 mm) scale causes an approximately five fold reduction in void volume, since the column volume is proportional to the cross sectional area. This down scaling is inevitable in order to maintain the performance of the system and should be applied to all components of the HSLC system.

Injection System

The maximum injection volume that can be injected into a chromatographic column may be expressed by the equation:

$$V_{max} = \theta K \pi \varepsilon d_c^2 L(k+1)/N^{1/2}$$

where θ is the tolerated fractional loss of efficacy, K is an injection system constant (injection profile), L is the column length, k is the retention factor, and N is the theoretical plate number.

Allowing a typical value of 5% for plate number lost, and column porosity $\varepsilon = 0.7$, assuming that the injection profile is almost an ideal rectangular plug ($K = 4$), a retention factor $k = 1$ for the first retained peak, and that the columns present good efficiency with reduced plate height $h = 2$, and substituting N by L/hd_p, where d_p is the particle size of the stationary phase, the following equation is obtained:

$$V_{max} = 1.14 d_c^2 (L d_p)^{1/2}$$

For conventional (4.6 mm I.D.) and narrow bore (2.1 mm I.D.) short ($L = 50$ mm) columns, packed with 3 μm particle size, this equation provides an estimation of the allowable maximum value of 10 μL and 2 μL, respectively.

As can be seen in Figure 1, an increase in the injection precision and a loss in effectiveness in the separation are observed when the injected volume into the chromatographic column increases. Since, in many applications, instrumental repeatability should be lower than 2%, in pharmaceutical analysis, for example, injection volumes should be higher than 1 μL. Moreover, for reasons of effective loss, it is not convenient to exceed 2 μL of injected volume. These results match predicted theoretical values.

The use of columns of a smaller diameter forces the injection of smaller sample volumes for which the repeatability of an automatic injection system is not adapted. In this situation, the autosampler must be modified, changing the standard injection valve for a microvalve in line with Simpson's proposal.[15]

Another problem that may occur at high flow rates are pressure pulses associated with the interruption of flow during the sample injection process, which may result in a reduction of the lifetime of short columns. This effect depends on the speed at which the injection valve rotates, being more

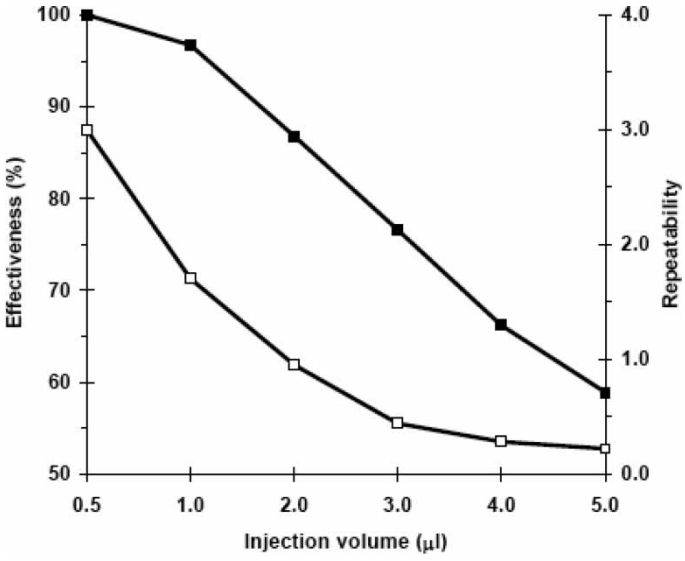

Figure 1. Effectiveness (■) and repeatability (□)–expressed as relative standard deviation–shown by the HPLC system adapted to HSLC requirements with injector bypass A (split ratio of 2.73). Column: 50 mm × 2.1 mm I.D., 3.5 μm, Kromasil 100 C_{18}. Mobile phase: water and acetonitrile (35 : 65) at 0.2 mL min^{-1}. Temperature: 30°C. Detection UV-Vis at 254 nm. Response time: 80 ms. Test probe: anthracene (4 mg L^{-1}).

important when the rotation is slow, as a higher pressure drop strikes the top of the column. For this reason, this drawback can be serious when manual injectors are used, which depend on operative ability, whereas this problem is less significant for modern automatic injectors. With the aim of avoiding this detrimental effect, we used an injector bypass in accordance with DiCesare et al. suggestions,[16] although other alternatives have also been published.[5,17] The bypass split ratio may be accomplished with capillary tubes of different inner diameters and lengths by making use of the Hagen-Poiseuille relationship.[18]

$$F = (\pi \Delta P r^4)/(8L\eta)$$

where F is the flow rate, ΔP is the pressure drop, r and L are the radius and length of the capillary, respectively, and η is the viscosity.

The split ratio (S_R) can be calculated as flow ratio through the capillaries.

$$S_R = F_1/F_2 = (r_1/r_2)^4(L_2/L_1)$$

An injector bypass must be built so as to eliminate pressure pulses, yet without substantially contributing to additional extra-column band broadening. Two union tees, appropriate capillary tubes, and fittings were used

(Fig. 2). Different split ratios were obtained by changing the length of the L_2 capillary (Table 1).

For the automatic injector employed in this work, the pressure pulses are only approximately 10% of the working pressure (Fig. 3). It should be kept in mind that, for some applications of fast HPLC in routines where many analyses per hour are required working at elevated pressures, this phenomenon has an accumulative effect. Some high speed, short columns are very sensitive under these conditions, which may reduce their lifetime. In particular, columns packed with 3 μm particles are more sensitive to pressure damage than conventional ones with 5 μm particles.[19] Furthermore, it should be realized, that a little pressure damage for a short column represents a higher percentage of decreasing effectiveness than for a longer column. In these cases, an injector bypass would be recommendable whenever the IBW constraints can be accepted.

As can be seen in Figures 3, 4, and 5, when the split ratio of the bypass decreases, a lower pressure pulse and higher IBW are obtained. Moreover, the sample takes more time to pass through the injector. Thus, a compromise must be reached in order to enhance column lifetime without losing effectiveness. Bypass A reduces the pressure pulse to less than a half without significantly altering the rest of the chromatographic parameters. For this reason, it was selected by us for our HSLC application,[20] successfully increasing the column lifetime fourfold.

Detection System

Another source of band broadening within a chromatographic system is the detector cell, which must have small volumetric and temporal dispersions if the inherent resolving power of the column is to be preserved. Kirkland et al. have shown that the detector cell volume should be no greater than one-tenth of the peak volume.[21] In our instrumental device, a non-retained

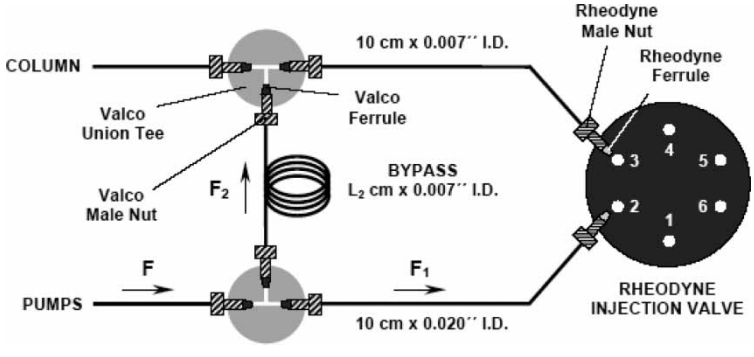

Figure 2. Scheme of the injector bypass construction (drawing not to scale).

Table 1. Different injector bypasses studied

Bypass	A	B	C
L_2 (cm)	50	25	15
Split ratio (F_1/F_2)	2.73	1.30	0.75
F_1 (%)	73.2	56.6	42.9

peak shows a bandwidth of 27 μL. Consequently, in our experiments, we used a flow cell of 2.5 μL.

Additionally, significant distortions of peak shape may occur if the detector response times are too slow. The response times required for the detector may be determined from the following relation:[22]

$$R_t = \theta t_R / N^{1/2}$$

Thus, for a peak eluting in 1 min with 5% loss in plate number and an efficiency of 1000 plates, a minimum detector response time of less than 100 ms is required. From the standpoint of chromatography, it is desirable to select the fastest sampling rate. However, the detector response time should be kept as long as possible, since baseline noise increases exponentially. Furthermore, the size of the data file increases with the sampling rate, and the hard drive of the data system fills up more quickly. Therefore, a careful compromise

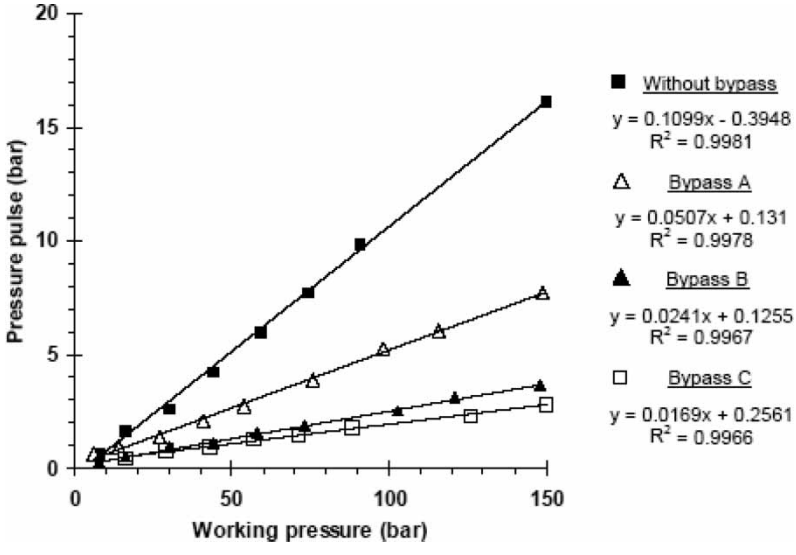

Figure 3. Relationship between pressure pulses, due to the injection process, and working pressure for the modified HPLC system with the different injector bypasses tested. See Table 1.

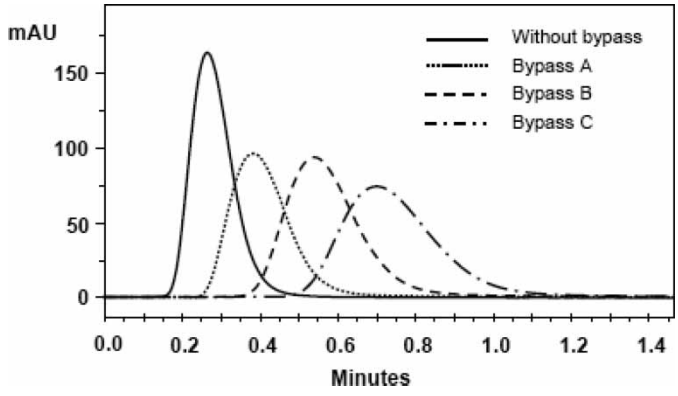

Figure 4. Chromatographic peak obtained for the anthracene ($5\,mg\,L^{-1}$) test probe using the modified HPLC equipment without a bypass and with bypasses A, B, and C (See Table 1). Flow rate: $0.1\,mL\,min^{-1}$. Injected volume: $1\,\mu L$. These experiments were used for the extracolumn volume and band broadening measurements. See Experimental Section for details.

needs to be made between the needs of chromatography and the constraints of the data system. In fact, only 30 points per peak are required to define a peak well and to obtain good reproducibility.[19] In our device, 80 ms response time is sufficient to obtain 30 points per peak width of 0.04 min. Narrower peaks would still be recorded, though they present slight band broadening due to the slowness of the data acquisition system.

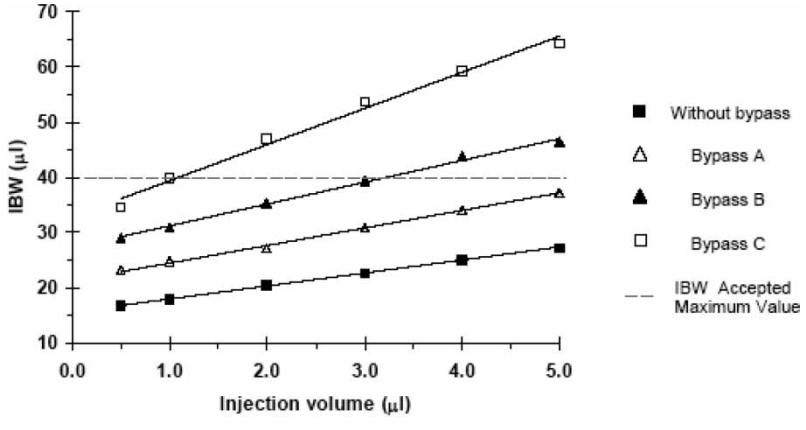

Figure 5. Instrumental bandwidth as a function of the injection volume on the modified HPLC equipment with an injector bypass system. Flow rate: $0.1\,mL\,min^{-1}$. See Experimental Section for details.

Connecting Tubes

A connecting tube is needed between the injector and the column and between the column and the detector. The variance contribution from the connecting tubing used in the system may be calculated using the Taylor-Aris expression,[23] where D_m is the diffusion coefficient of the analyte in the mobile phase:

$$\sigma^2_{tub} = r^4 l \pi F / 384 D_m$$

As can be seen, the radius is the major contributing factor and narrow bore tubing is recommended. In practice, the tubing radius is limited by the maximum operating pressure of the chromatographic system. Bearing in mind, that our instrumental device needs 60 cm of capillary tube to connect the autosampler to the column (placed in a thermostatized oven) and the column to the detector, a capillary tube of 127 μm of inner diameter was selected as a compromise between the pressure drop and the contribution to the variance of the system.

System Evaluation

The performance of the modified system was examined as a function of a number of experimental parameters, such as extra-column volume, IBW, provided effectiveness for a high speed column and dwell volume.

To measure the extra-column volume and extra-column band broadening, the column was replaced by a zero dead volume union, a small volume of sample was then injected and the detector response was recorded (Fig. 4). The extra-column volume is the distance between the point of injection and the peak maximum as a measure of system dead volume. As can be seen in Table 2, the extra-column volume has been reduced more than four-fold for the modified HPLC equipment without an injector bypass. This amount approximately represents the same downscale factor, as occurs when the column internal diameter is reduced from conventional to narrow bore scale. Obviously, the use of an injector bypass increases the system dead volume.

The standard deviation of the peak, shown in Figure 4, may be used as a measurement of the system band spreading (Fig. 5). As is well known, extra-column band broadening increases with injection volume, 40 μL being the tolerated maximum value of IBW for HSLC.[24]

Table 2. Extracolumn volume (μL) for both HPLC equipments

Equipment	Without bypass	Bypass A	Bypass B	Bypass C
Conventional	130	—	—	—
Modified	30	42	57	74

The loss in effectiveness as a result of using an injector bypass was also studied. As shown in Figure 6, the decreasing in the bypass split ratio makes broader and later peaks. Considering efficacy without a bypass, an effective reduction takes place of about 14, 31, and 37%, respectively, for bypasses A, B, and C, with an injection volume of 1 μL. If 2 μL are injected, the efficacy reduction reaches 18, 36, and 47%, respectively. The solute employed by the manufacturer (anthracene) to measure the efficacy of this column was considered for these calculations. These results, together with the previous ones regarding pressure pulses on the injection process, show that injector bypass A is the most suitable bypass device, in the case of it being necessary.

The performance of the optimized HPLC setup was compared with a conventional HPLC system. To compare the IBW of the conventional and modified systems properly, it is necessary to carry out measurements at the same linear velocity. Hence, the flow rate was 0.5 and 0.1 mL min^{-1}, respectively, in accordance with the expression:

$$u = F/\pi r^2$$

where r is the radio of the connecting tubes. The conventional system shows about 113 μL instrumental bandwidth, whereas this value is reduced to 27 μL for the high speed setup. This comparison was performed with an injection volume of 2 μL and without a bypass for the conventional system, but with bypass A for the modified set-up. Once more, the downscale factor is approximately four, though it is five without a bypass.

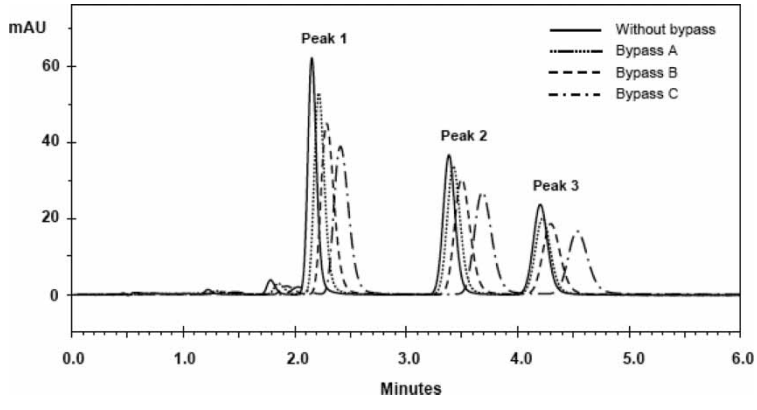

Figure 6. Chromatogram of a test mixture containing naphthalene (100 mg L^{-1}, peak 1), acenaphthene (200 mg L^{-1}, peak 2) and anthracene (4 mg L^{-1}, peak 3) in acetonitrile. The separations were performed on the modified system without a bypass and with bypasses A, B, and C. Column: 50 mm × 2.1 mm I.D., 3.5 μm, Kromasil 100 C_{18}. Mobile phase: water and acetonitrile (35 : 65) at 0.2 mL min^{-1}. Temperature: 30°C. Detection UV-Vis at 254 nm. Injected volume: 1 μL. Response time: 80 ms.

In Figure 7, we can see the improvements obtained using a mixture test as a separation model in the conventional system and the HSLC setup. A higher theoretical plate number is attained that results in an increase in effectiveness of between 59 and 94% when using the HSLC system. At the same time, the increase in effectiveness is higher at shorter times, which represents higher efficacy per unit of time.

When running a fast gradient, special attention must also be paid to the dwell volume of the system. When column dimensions are downscaled, the inconvenient effect is that the separation may be delayed significantly. For example, if we have a system with a total delay volume of 1 mL and we run a gradient at 0.2 mL min^{-1}, it will take 5 minutes for the gradient to reach the top of the column. Therefore, the start of separation is delayed by 5 minutes. Consequently, the split ratio of the gradient delay volume to the column volume should be held constant. This drawback could be overcome by using delayed injection, but this may not be possible on automatic injectors, where the injection triggers the start of the gradient.

With the aim of reducing the dwell volume of the system, special attention must be paid to the head volume of the pump (microheads should be employed), length and internal diameter of connecting tubes from pumps to column, and the mixer chamber dead volume. A dwell volume of 0.22 mL was accordingly found for the adapted equipment to HSLC with

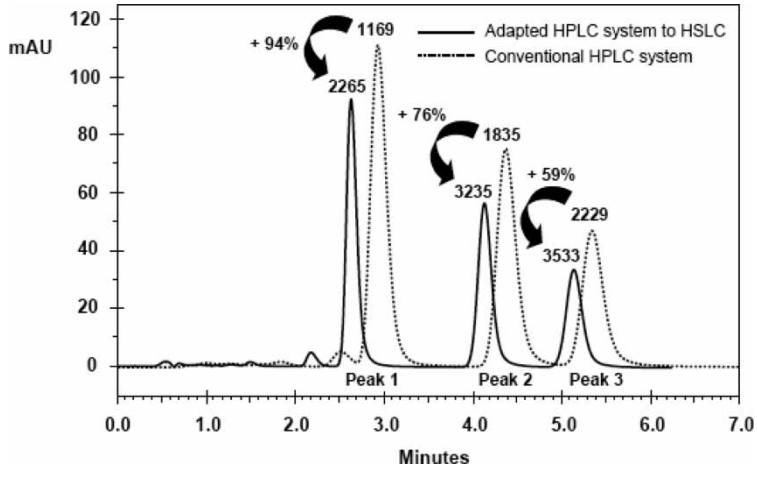

Figure 7. Separation of a test mixture to compare the provided effectiveness (as theoretical plate number) by conventional HPLC equipment and by the modified HSLC setup with injector bypass A. Column: 50 mm × 2.1 mm I.D., 3.5 μm, Kromasil 100 C_{18}. Mobile phase: water and acetonitrile (35 : 65) at 0.2 mL min^{-1}. Temperature: 30°C. Detection UV-Vis at 254 nm. Injected volume: 2 μL. Response time: 80 ms. Identification: naphthalene, 100 mg L^{-1} (Peak 1); acenaphthene, 200 mg L^{-1} (Peak 2); anthracene, 4 mg L^{-1} (Peak 3).

bypass A, in keeping with high speed HPLC and mass spectrometry requirements for short and narrow bore columns.[24,25] Likewise, 1 mL was obtained for the conventional HPLC system.

CONCLUSIONS

The instrumental setup, according to HSLC requirements, is relatively simple and economical, about 1500 $ as regards the detector micro flow cell, the bypass union tees, and connecting tubes, plus the price of substituting the classical injection valve by an internal loop micro valve, if this should be necessary. The main drawback is the higher pressure drop imposed by the adapted system.

The construction of the injector bypass is critical as regards tubing bore and length, in order to eliminate pressure shocks and to minimize instrumental bandwidth. Its use is only recommended when the columns are susceptible to pressure damage.

Extracolumn effects are one of the main reasons for the low performance of 2 mm I.D. short columns on normal HPLC instruments. To achieve roughly the same performance level as that obtained with the large diameter column, it is necessary to reduce extracolumn band broadening by the same factor as the change in column volume. In the typical case, from a 4.6 mm I.D. column to a 2.1 mm I.D. column, extracolumn effects should be reduced by a factor of approximately 5. This means that equipment should be downscaled by the same factor, which includes the extracolumn, injection and dwell volumes.

Our results illustrate that careful optimization of instrumental parameters and hardware for HSLC may lead to better results for the desired applications. Although, the modifications were applied to a specific system, the basic approach should also be generically applicable to different apparatuses.

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