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Optimised injection techniques for micro and capillary liquid chromatography

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Abstract

Large volume injections —involving on-column focusing— were evaluated for packed micro and capillary liquid chromatography columns of 300 μm and 1.0 mm inner diameter (I.D.) respectively. It was found that the I.D. of the injection loop plays a critical role in sample dispersion, effecting peak asymmetry and injection reproducibility. The use of injection loops with too large or too small an I.D. resulted in a reduced injection performance. Ideally the I.D. should be in between 100–150 μm. Further it was investigated what influence the loop volume and the use of a low dispersion injection technique had on the column stability. The combination of large volume injections —up to 1 μl and 5 μl for 300 μm and 1.0 mm I.D. columns, respectively— and additional switching of the injection valve did not affect column lifetime. Typical decreases of only 10% in efficiency over 1500 injections were found.

Keywords: Injection methods; Large-volume injections; Capillary liquid chromatography; Micro liquid chromatography

1. Introduction

To maintain the high chromatographic efficiency and resolution obtained on packed capillary and micro liquid chromatography (LC) columns, special attention must be given to extra-column band-broadening processes. These processes are mainly volumetric and originate from dead-volumes in the injection valve, connecting tubing or detection flow cells. Generally, extra-column dispersion must be minimised with packed capillary and micro LC columns. Detailed descriptions and theoretical studies about extra-column band-broadening processes in microcolumn LC are given in several text books [1–3].

The reduction of the injection and detection volume generally decreases the sensitivity of the chromatographic analysis. The decrease in sensitivity as a result of on-column detection in UV absorbance detection has been conquered by the introduction of flow cells with an extended longitudinal path-length [4,5]. Furthermore, to overcome the reduced concentration sensitivity of miniaturised chromatographic LC systems, large volume injections have been applied [6–17].

However, to maintain the chromatographic efficiency the solutes should be dissolved in a non-eluting solvent. This method is based on on-column focusing and has been evaluated thoroughly in theory and practice by other research groups for LC columns with micro [6–10] and capillary dimensions [11–17]. Another approach to introduce large sample

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volumes onto a analytical micro or capillary column is the use of micro precolumns combined with column switching techniques [18–23], but those require an additional pump and injection valve for sample loading and flushing of the precolumn.

In this paper the feasibility of a micro auto sampling system and the optimal use of on-column focusing techniques for 300 μm and 1.0 mm I.D. columns are described. The influence of the loop volume and the loop I.D. was studied in relation to the repeatability of the injection process. Other parameters that were studied included the effect of the loop volume and type of injection technique (standard versus low dispersion injections) on the stability of micro and capillary columns.

2. Experimental

2.1. Instrumentation

All the LC experiments were performed with a 420 conventional HPLC pump (Kontron Instruments SpA, Milan, Italy). A low-pressure in-line filter (0.2-mm disposable filter, Whatman, Clifton, NJ, USA) was placed between the mobile phase reservoir and the pump to prevent contamination or clogging of the chromatographic system. Micro and capillary flow-rates were generated by an IC-400-VAR semi-variable microflow processor (LC Packings, Amsterdam, Netherlands) that was connected to the HPLC pump. The outlet flow of the microflow processor was transferred to a FAMOS micro autosampler (LC Packings). A detailed description about the principle of operation and performance of the micro-auto-sampler is given in an earlier publication [24].

Dispersion studies were conducted with injection loops of different I.D. and length and made of fused-silica capillaries. The effect of the injection loop volume on the stability of the microcolumns was measured with PEEK shielded fused-silica injection loops of 1 μl and 5 μl (LC Packings) volumes. The columns applied in this study had an I.D. of 300 μm (LC Packings) or 1.0 mm (LC Packings) and a length of 15 cm and were packed with C_{18} stationary phase (Shandon Hypersil, Astmoor, UK).

Detection was performed at 254 nm by using a SPD-10A UV–Vis absorbance detector (Shimadzu, Tokyo, Japan) equipped with a 8 mm, 35 nl Z-shaped capillary flow cell (LC Packings). Data acquisition was performed with MT-2 chromatography software (Kontron Instruments).

2.2. Samples and mobile phase

The dispersion experiments were performed by flow injection analysis with water as the mobile phase and uracil (Fluka, Buchs, Switzerland) as the test component. Typical flow-rates were 5–10 μl/min. The column stability studies, i.e. efficiency experiments, were measured using a standard reversed-phase test mixture containing uracil (4.3 μg/ml), naphthalene (57.5 μg/ml), biphenyl (46.0 μg/ml), fluorene (8.21 μg/ml), anthracene (9.85 μg/ml) and fluoranthene (26.3 μg/ml). All the polyaromatic hydrocarbons were obtained from Fluka. The mobile phase consisted of acetonitrile–water (70:30, v/v) and was delivered to the column through the microflow processor. Acetonitrile and water were both of HPLC grade (LabScan, Dublin, Ireland). The flow-rate was equal to 4 μl/min for the 300 μm I.D. columns and 35 μl/min for the 1.0 mm I.D. columns.

3. Results and discussion

3.1. Injection loop volume

Extra-column band-broadening is mostly regarded as a volumetric dispersion source. Therefore, in the case of extra-column band-broadening originating from the injection valve, the loop volume of the injection valve is usually taken into account [2,7]. The maximum allowed injection volume V_{inj} for an unretained compound that generates a fractional loss θ^2 in column efficiency is given by:

$$V_{inj}^2 = K^2 \sigma_{inj}^2 = \theta^2 (K \pi r^2 \varepsilon_T)^2 HL \quad (1)$$

where K^2 is a constant that characterises the injection profile, σ_{inj}^2 the dispersion introduced by the injection valve, r the column radius, ε_T the total

porosity, H the plate height and L the column length. For an ideal delta function K^2 equals 12. Eq. (1) does not take into account post-injection extra-column band-broadening processes. Those require more complex expressions and will not be addressed in this paper.

From Eq. (1) the maximum allowable injection volume can be calculated when a 5% loss in column efficiency ($\theta^2=0.05$) is permitted. The results for micro and capillary columns are given in Table 1.

Beside the injection volume, the injection time and the I.D. of the injection loop may contribute to sample dispersion. The effect of the injection time is very well described by Colin et al. [25]. Too fast injections were believed to give band broadening because the solute jet hits the chromatographic bed and bounces back. Too slow injections were not recommended because of the effect of injection time on efficiency. Slais et al. [26] have given a detailed description about the effect of the I.D. of the injection loop on extra-column dispersion. Their calculations clearly show that the I.D. of the injection loop is of importance. By applying the peak compression principle Slais et al. [25] calculated that the optimum diameter of the injection loop d_l equals:

$$d_l^2 < \frac{V_0}{V_{inj}} d_p^2 g(k) \quad (2)$$

where V_0 is the dead volume of the microcolumn, d_p the particle diameter and $g(k)$ a function of the retention factor. Eq. (2) fails in calculating exact values in practice, due to the unknown factor $g(k)$, and has therefore not been considered.

Table 1
Maximum injection volumes calculated for micro and capillary LC columns

	Column I.D.	Column length (cm)	V_{inj} (nl)[a]
Micro LC	1.0 mm	15.0	520
		25.0	670
Capillary LC	300 μm	15.0	46
		25.0	61

The total porosity ε_T was taken as 0.70. Ideal injection profiles ($K^2=12$), and optimum mobile phase flow-rate and plate height ($2d_p$) were assumed. The particle diameter was taken as 5 μm and the retention factor k equalled 0.
[a] A 5% loss in column efficiency was allowed ($\theta^2=0.05$).

3.2. Injection loop inner diameter

In the present study flow injections analyses were conducted to examine the effect of the loop I.D. on band-broadening. The criteria for judging the injection profiles was the peak asymmetry at 10% of the peak height. Also studied was the effect of the I.D. of the injection loop on injection reproducibility. Typical injection volumes in capillary LC range from 50 nl to 5 μl. To cover the high end side —large volume injections— a 5 μl loop was used to study the effect of the injection loop I.D. on the peak asymmetry. The effect of the loop I.D. on injection dispersion for volumes at the low end side —i.e. injection volumes ranging from ~50–200 nl— were conducted with a 1 μl loop.

The results of the effect of the injection loop I.D. on the peak asymmetry for the 5 μl loop are given in Table 2, as well as the obtained R.S.D.-values on peak asymmetry obtained for 10 consecutive injections. The injected sample volume equalled the loop volume. The linear speed of sample aspiration was kept constant for all loop I.D.s. Up to a loop I.D. of 100 μm no significant increase in peak asymmetry was observed. For the 200 μm I.D. injection loops however the peak asymmetry increased almost by a factor of 2. This is most likely caused by the fact that the 200 μm I.D. loop behaves as a mixing coil which subsequently results in more dispersion, which is indicated by the reproducibility of the injection was (with R.S.D. values of 0.3% on peak height and peak area) comparable for the injection loops of 75, 100 and 150 μm I.D. The reproducibility of injections with the 200 μm I.D. injection loop was (for the same reason as the peak asymmetry) worse than for the other sized loops.

Table 2
Effect of the loop I.D. on peak asymmetry

I.D. (μm)	Peak asymmetry[a]	R.S.D. (%)[b]
75	1.14	3.7
100	1.08	3.1
150	1.42	3.7
200	1.82	9.5

Flow injection analysis of full loop injections performed with a 5 μl sample loop.
[a] Peak asymmetry measured at 10% of the peak height.
[b] R.S.D.-values based on 10 consecutive runs.

Similar observations were made for 1 μl injection loops with I.D.s of 50, 75 and 150 μm. Peak asymmetries and reproducibility of injection were comparable for the 75 and 100 μm I.D. loops applying 200 nl injections. Peak asymmetries ranged from 1.10 to 1.25 and the R.S.D.-value on injection reproducibility equalled 0.6% for partial loop fill injections. In these experiments less good numbers on the reproducibility were found for the 50 μm I.D. loop which was caused by the restrictive nature of the small 50 μm I.D. tubing. Hence, all further experiments were conducted with 150 μm I.D. injection loops as the best compromise between good injection reproducibility and tolerable increase in peak asymmetry for this type of micro autosampling systems.

3.3. On-column focusing

The effect of on-column focusing on column efficiency was studied by injecting different amounts, ranging from 50 nl to 1 μl, of a polyaromatic hydrocarbon (PAH) test mixture onto the 300 μm I.D. capillary LC column. Fluoranthene, the last eluting component of the mixture, was used for evaluation. Three different type of experiments were performed. In the first type of experiments the test sample was not diluted. This means that the amount of injected sample increases proportionally with the injected sample volume. In the second type of experiments the test sample was diluted in mobile phase to give equal amounts of test sample per injection volume. Note that in this case the concentration of the samples is different but the total amount of injected sample remains identical. In the third type of experiments the test sample was diluted in a weaker solvent [acetonitrile–water (20:80, v/v)] to give equal amounts of test sample per injection volume. Also in this case the concentration per sample differs but the injected mass of sample remains constant.

The results —in terms of relative efficiency— for each type of experiment are shown in Fig. 1. As observed, only high volumes of sample can be injected without considerable band broadening effects when on-column focusing is applied, that is in the third type of experiments. In the other situations mass or volume overload of the column, or exceeding the linear range of the detector are responsible for the decrease in efficiency. From the results in Fig. 1, it can also be deduced that the maximum injection volume in capillary LC —when no on-column focusing is applied— is equal to about 100–200 nl. As was shown earlier, these values compare favourably with the theoretical calculations in Table 1. It should be mentioned that the maximum injection volume was calculated for an unretained compound and the practical experiments were carried out with a compound that had a retention factor of approximately 4, resulting in two to three fold higher injection volumes.

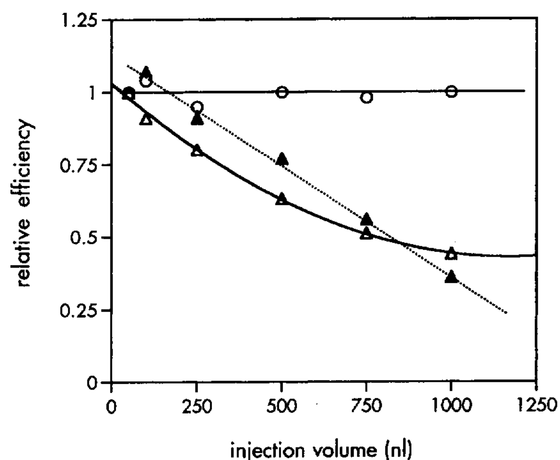

Fig. 1. Effect of sample solvent on relative column efficiency. (△) Test sample dissolved in mobile phase (injected sample mass increases linearly with the injection volume); (▲) Test sample diluted in mobile phase to give equal amounts of sample mass per injection volume; (○) Test sample diluted in a weaker solvent to give equal amounts of sample mass per injection volume applying peak compression. For chromatographic conditions see Section 2.

Peak asymmetry was not affected by on-column focusing, whereas in the two other experiments the peak asymmetry factor rose very quickly from about 1.08 to 1.25. To illustrate the effect of on-column focusing, the chromatogram of two different type of large volume injections are given in Fig. 2. Fig. 2A represents the chromatogram of a 5 μl injection of the PAH test sample dissolved in mobile phase [acetonitrile–water (70:30, v/v)]. Fig. 2B represents the chromatogram of a 5 μl injection of the same sample but this time diluted in a weaker solvent [acetonitrile–water (20:80, v/v)]. Dramatic sample dispersion and breakthrough occurs when the sample

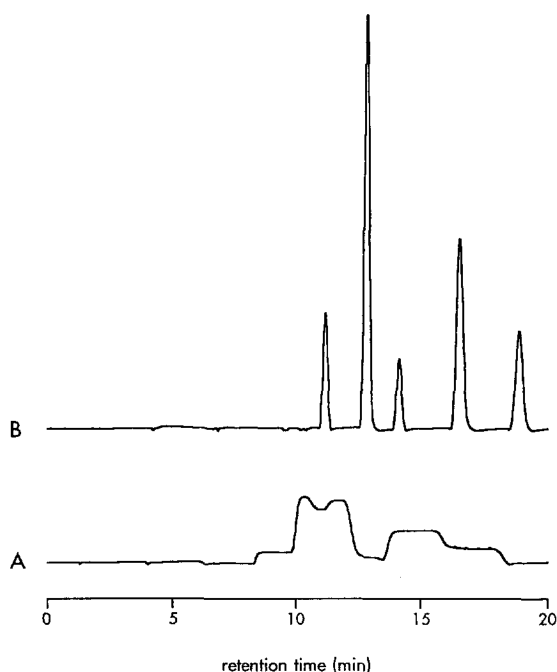

Fig. 2. Effect of peak compression for large volume injections of 5 μl on a 300 μm I.D. capillary LC column on the chromatographic separation of a PAH mixture. (A) represents the chromatogram of an injection of a PAH test sample dissolved in mobile phase. (B) corresponds to the chromatogram of the same PAH sample dissolved in a weaker solvent [CH_3CN-H_2O (20:80, v/v)]. Chromatographic conditions as in Fig. 1.

is dissolved in mobile phase, meanwhile on-column focusing results in efficient separation.

3.4. Column stability

Although the on-column focusing technique looks very promising at first sight, maximum injection volumes must be considered with respect to column stability. In a previous paper we already showed that the lifetime of 300 μm I.D. columns is comparable with that of conventional sized columns when 60 nl injection volumes are applied [27]. So far, the effect of large volume injections on the lifetime of packed micro and capillary LC columns has not been investigated comprehensively.

Hence, lifetime experiments were conducted with a 1 μl injection loop for the 300 μm I.D. capillary LC columns and with a 5 μl injection loop for the 1.0 mm I.D. micro LC columns. Injection volumes were equal to 100 and 500 nl respectively using partial loop fill injections. Note that the total injected volume of liquid onto the columns is equal to 1 and 5 μl. Each 50th injection the column efficiency was determined. Additionally, low dispersion injections were performed to study whether additional valve switching could affect column lifetime. In the low dispersion injection routine the injection is terminated by switching the valve, whereof the undefined tailing part of the injection plug can be neglected. The system was run continuously for 6 days. After 6 days of operation and 1500 analysis the experiment was stopped.

The results of the life time experiments for the 300 μm and 1.0 mm I.D. columns are given in Fig. 3a and b respectively. The loss in column efficiency is about 10% over a total number of 1500 injections for both type of columns. These results are similar to those reported earlier using small injection volumes of 60 nl [27]. For optimal column lifetime injection volumes should not exceed 1 μl for 300 μm I.D. columns and 5 μl for 1.0 mm I.D. columns.

3.5. The effect of low dispersion injections on column stability

In the previous section low dispersion injections were mentioned as a tool to improve peak shape and column efficiency. A very detailed description about the low dispersion injection routine is given elsewhere [24,28]. To demonstrate the enhancement in efficiency and peak shape, standard and low dispersion injections were carried out on the same column. The results of these experiments are shown in Fig. 4 for 200 nl injections of the PAH test sample. The upper trace in Fig. 4 shows the standard injection and the lower trace the low dispersion injection routine.

The efficiencies for three of the PAH test compounds are summarised in Fig. 5. The investigated components, biphenyl, fluorene and fluoranthene, had a retention factor k of 1.4, 1.7 and 3.6 respectively. As can been seen from the results in Fig. 4 the low dispersion injection routine is more effective for the earlier eluting compounds. The column efficiency does not seem to be affected in column for compounds with a relative high k value. The average enhancement in column efficiency is about 7–10%,

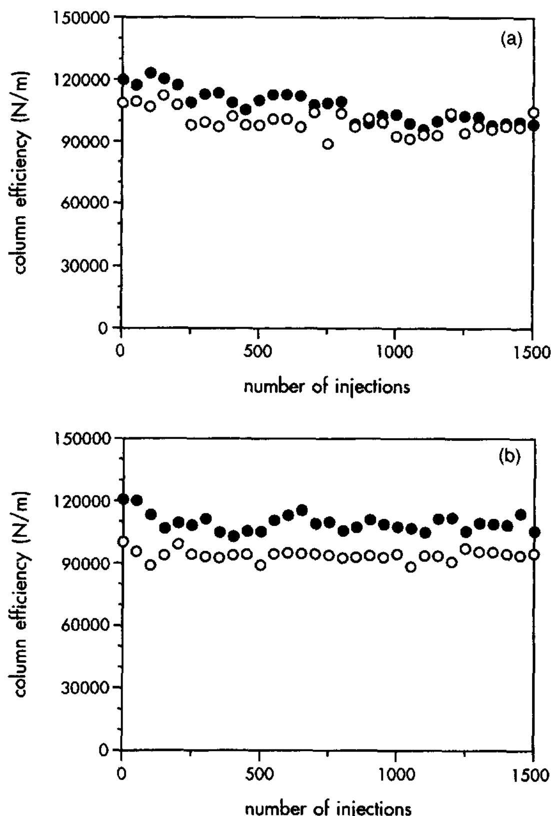

Fig. 3. (a) Number of plates per meter (N/m) as a function of the number of injections using a 300 μm I.D. capillary LC column. Test component fluoranthene (k~3.6); Mobile phase flow-rate 4 μl/min. (○) Standard injection; (●) Low dispersion injection. Other chromatographic conditions as described in Section 2. The total volume of injected sample liquid was equal to 1 μl and the sample volume equal to 100 nl. (b) Number of plates per meter (N/m) as a function of the number of injections using a 1.0 mm I.D. micro LC column. Test component fluoranthene (k~3.6); Mobile phase flow-rate 35 μl/min. (○) Standard injection; (●) Low dispersion injection. Other chromatographic conditions as described in Section 2. The total volume of injected sample liquid was equal to 5 μl and the sample volume was equal to 500 nl.

meaning a 3–5% gain in peak resolution. Meanwhile the peak shape using the low dispersion injection routine is significantly enhanced.

4. Conclusions

Using on-column focusing large volume injections can be applied easily on packed capillary and micro LC columns of 300 μm and 1.0 mm I.D. respectively with virtually no loss in column efficiency. The chromatographic separation of a large volume injection using on-column focusing is almost identical to that of a small volume injection. Furthermore, on-column focusing allows to improve the limits of detection by several orders of magnitude.

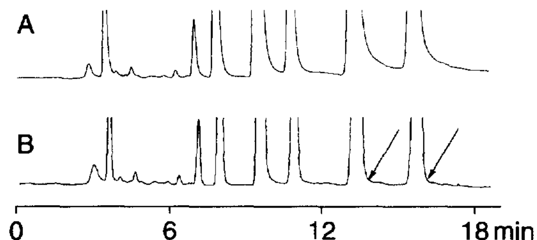

Fig. 4. Effect of low dispersion injections on peak asymmetry and resolution for a 200 nl injection. Upper trace (A) standard injections. Lower trace (B) low dispersion injection.

For optimal injection in micro and capillary LC special attention must be given to the I.D. and the volume of the sample loop. Too small I.D. injection loops can result in a decrease in injection reproducibility because of restriction during sample aspiration. Too large I.D. also results in lowered injection reproducibility. The reason therefore is the fact that the loop acts as a mixing device. The optimal injection loop I.D. —in terms of dispersion

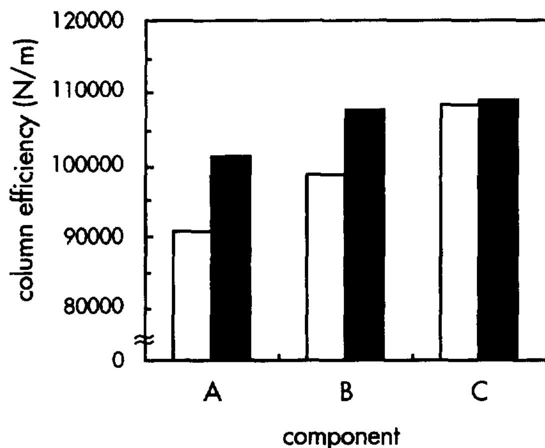

Fig. 5. Enhancement in plate number using low dispersion injections as compared to standard injections. (□) standard injection; (■) low dispersion injections. Test compounds: A = biphenyl (k~1.4), B = fluorene (k~1.7) and C = fluoranthene (k~3.6). Experimental conditions as in Fig. 1.

and injection reproducibility— was found to be equal to 150 μm. The volume of the loop is not a critical parameter as long as on-column focusing techniques are applied, but is of critical importance in column lifetime and column stability. Extensive stability experiments have shown that column lifetime is preserved when large volume injections do not exceed 1 and 5 μl for 300 μm and 1.0 mm I.D. columns respectively. It should be noted though that not only the injection volume but also continuous operation i.e. continuous flow and mobile phase delivery is know to be critical and may affect column lifetime too. In our experiments this has been realised by using conventional HPLC pumps and flow splitting. Additional valve switching, as required when low dispersion injection techniques are applied, was not found to influence column lifetime. With the low dispersion injection routine significant better peak shape and resolution can be obtained as compared to standard injections.

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