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SIMPLE METHOD OF PACKING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COLUMNS WITH HIGH REPRODUCIBILITY

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SUMMARY

A new method of packing high-performance liquid chromatography columns has been developed. Critical and continuous control of packing operations and optimization of packing parameters result in the production of efficient columns with excellent reproducibility.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) in routine analysis requires that conditions be optimized with respect to time and cost. Columns packed with small particles are the most suitable[1], especially if the column specification for a particular analysis means that the investigator must prepare it.

Many authors have described packing methods for small particles, *e.g.* the equidensity method[2–7], the viscosity method[7,8] and the use of the mobile phase[9,10].

The advantages and disadvantages of these methods have been described[11,12], and very often authors have commented on the probability that badly packed columns will occasionally be produced. Reproducibility of columns is normally variable so that changing a column during routine analysis often means the adaptation of the chromatographic method to the new column, which is time-consuming and needs the attention of an experienced chromatographer.

The method described here, which is similar to that of Linder *et al.*[13], combines (i) constant stirring to maintain the dispersion of the suspension used for packing and (ii) high pressure for rapid packing. The method takes into account the main factors that interfere with the packing operation in order to make them reproducible from one column to another. The very good reproducibility of efficiency and retention that is obtained means that columns can be changed during routine analysis without any other modification to the chromatographic conditions.

MATERIALS AND METHODS

Apparatus

Fig. 1 represents a scheme for the packing procedure. The slurry reservoir

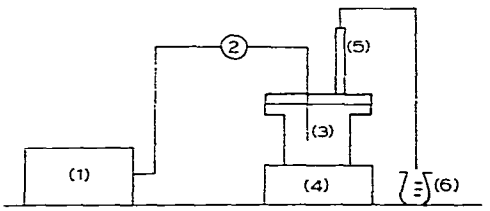

Fig. 1. Diagram of the packing apparatus. 1 = High-pressure pump, Micromeritics 7000 B; 2 = high-pressure valve; 3 = mixing vessel, Micromeritics 705 (slurry reservoir); 4 = magnetic stirrer; 5 = column; 6 = graduated vessel. All tubes are of capillary steel 1/16 in. O.D. and 2/100 in. I.D.

(Stirred-Slurry Column Packer, Micromeritics, Norcross, Ga., U.S.A.) has an internal volume of 32.5 ml. The high pressure (6000 p.s.i.) and flow-rate are provided by the pumping system of a high-performance liquid chromatograph Micromeritics 7000 B (in the constant pressure mode).

Packing procedure

A carefully determined amount of stationary phase is dispersed by mechanical and ultrasonic agitation for 1 min in the dispersing liquid, which is also delivered by the pumping system into the slurry reservoir. The reservoir has a stirrer bar in the bottom and is placed on a magnetic stirrer in order to maintain a homogeneous suspension. When the lid to the reservoir is secured and the outlet valve is opened, the dispersing liquid flows gently into the attached column until the first drop appears at its end. This purges trapped air from the system. The valve is then shut and the pressure increased to 6000 p.s.i. When the pressure is constant the valve is reopened and the suspension introduced into the column, which is vibrated either mechanically or manually. During all these operations and until the packing is complete, the flow-rate of the pumping system is continuously recorded (Fig. 2). When it becomes constant the column is packed. The pumping system and magnetic stirrer are then stopped and when the pressure has fallen to normal, the column is disconnected from the reservoir and sealed. It is now ready for equilibration.

Recording the flow-rate throughout the packing time provides a means for determining when the column is completely packed and also helps to control the quality of the packing because, under similar conditions, the maximum flow-rate, F_M, and flow-rate at the end of packing, F_E, must reach the same values respectively at the same times (see Fig. 2 and Table I).

RESULTS AND DISCUSSION

General considerations for the optimization of a method

In a wet packing method the ideal column can be made only if the initial suspension is entirely dispersed and homogeneous before introduction into the column. It must be introduced into the column without the occurrence of sedimentation, instability of the dispersion or changes in the pressure gradient. This demands the preparation of a stable dispersed solution.

Dispersion phenomena are very complex but, by using a plot of sedimentation rate against particle diameter, the dispersion can be empirically characterized by the

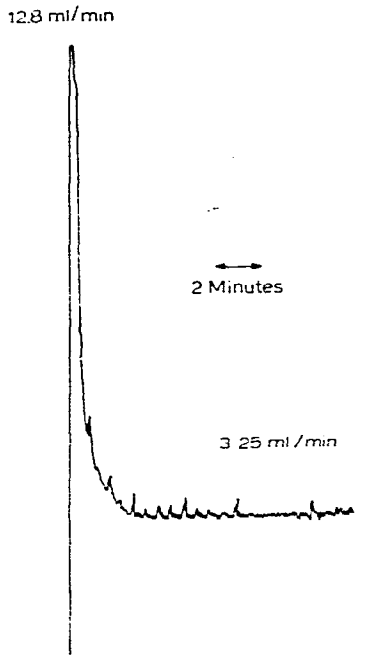

Fig. 2. Flow-rate *versus* time during the packing operation for column E. The amount of packing material was 3.2 g, and the pressure 6000 p.s.i.

TABLE I

EXPERIMENTAL PARAMETERS DURING PACKING OF COLUMNS UNDER THE SAME CONDITIONS

Columns: 15 cm × 4.6 mm I.D.; pressure: 6000 p.s.i.; liquid packing; isopropanol; initial amount of packing: 3.2 g.

Column	*Date (month/year)*	F_M *(ml/min)*	F_F *(ml/min)*
A	10/77	12.8	3.15
B	10/77	13.5	3.20
C	10/77	13.5	3.30
D	12/76	12.6	3.20
E	12/76	12.8	3.25
Mean		13	3.22
S.E. (%)		3.3	1.8

regularity of the plateau corresponding to large diameters, the mean diameter and the proportion of small particles. In Fig. 3 the comparison of curve 1 with curve 2 shows that the former solvent (isopropanol) results in a better dispersion than the latter (0.1 % sodium hexametaphosphate), where reflocculation occurs (cumulative mass % > 100 %).

As packing optimization is a function of the initial state of dispersion of the suspension, two parameters must be considered: the nature of the dispersing liquid and the amount of particles in the initial suspension.

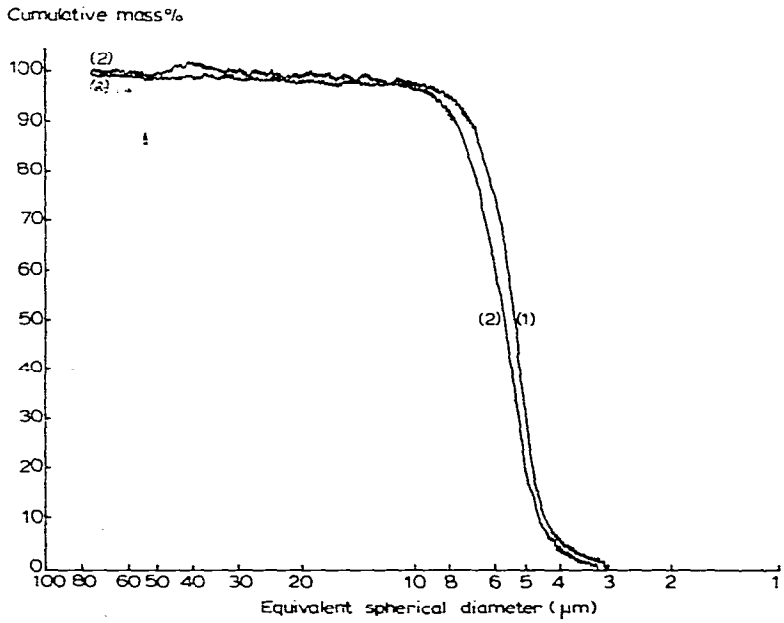

Fig. 3. Particle size analysis by sedimentation of LiChrosorb SI 60 (5 μm) dispersed in two different solvents. Dispersing liquid: 1 = isopropanol; 2 = 0.1% sodium hexametaphosphate in water. Preparation: 2.9 g LiChrosorb SI 60 (5 μm) for 35 ml of suspension. For reproducibility of the analysis, see ref. 18.

Choice of dispersion liquid

For columns packed with very small particles, which can be easily regenerated[14,15], polarity is not an important consideration in the choice of packing liquid. Thus liquids with a strong dispersing power and with a large range of polarity can be used. The liquids used in this study were chosen by reference to their physical characteristics (Table II) according to Stokes sedimentation law and literature data.

TABLE II

PHYSICAL PROPERTIES OF DISPERSING LIQUIDS

Liquid	*Density at 20°*	*Viscosity at 20° (cP)*
Isopropanol	0.785	2.3
Water + 0.1% hexametaphosphate	0.998	1.002
Carbon tetrachloride	1.594	0.969
Sedisperse	0.755 (24°)	1.496 (24°)
Heptane	0.684	0.409
Benzene–dibromoethane (5:95)	2.114	—

The dispersion state of silica gel was determined in two steps. First, a particle size analysis was carried out on a Coulter Counter TA_2 (Coultronics, Margency, France) to determine the particle mass distribution relative to the particle diameter. Second, a particle size analysis by sedimentation was carried out on a Sedigraph 5000 (Micromeritics) at a concentration near that used for packing to determine the dispersion state, by comparison with the first analysis.

In carbon tetrachloride and Sedisperse®* the silica gel agglomerated and stuck to the sides of the vessel immediately following wetting. In mixtures of benzene and 1,2-dibromoethane, the suspension was very stablebut flocculated strongly. In heptane, the suspension was very unstable. In 0.1 % sodium hexametaphosphate in water, flocculation was not completely suppressed (see Fig. 3). Isopropanol (IPA) provided a good dispersion.

To check if the dispersion in IPA depended on the characteristics of the silica gel, we carried out particle size analyses on three different commercial batches with a mean diameter of *ca.* 5 μm. The dispersion was satisfactory: no agglomeration or flocculation occurred. The size distribution data (see Fig. 4) indicate a very large (and

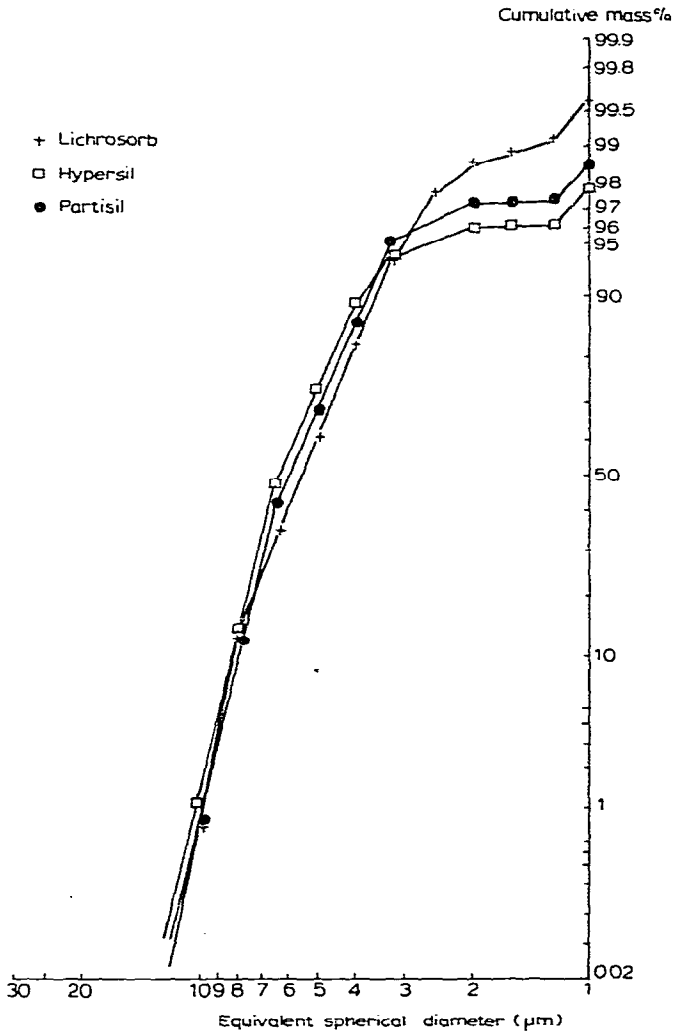

Fig. 4. Cumulative mass *versus* size distribution for different 5 μm silica gels.

* Sedisperse® (Micron Data Laboratory, Grimsby, Canada), a mixture of condensed light alkane, is a good dispersing agent.

very badly controlled for the finest particles) granulometric range. In the three batches studied more than 1% of silica gel was found to be less than 2 μm.

Choice of the amount of stationary phase

Rosset *et al.*[9] have shown that in the classical packing method there exists an optimal composition of slurry suspension. Linder *et al.*[13] have noted that, in their system, the best results were reached with double the amount required to pack a column.

These results correspond with the observation, made during particle size analysis by sedimentation[16], that too weak a concentration can increase the discrimination between large and small particles, whereas too strong a concentration often results in an unstable suspension.

Columns (15 cm × 4.6 mm I.D.) were packed by the method described with various amounts of LiChrosorb Si 60 (5 μm) in the slurry reservoir. Table III and Fig. 5 show the HETP evolution of different condensed aromatic compounds (eluted with hexane at 1 ml/min) *versus* the initial amount of stationary phase in the slurry reservoir. A sharp maximum occurs near 3.2 g. On the other hand, F_E seems to be independent of the initial amount of stationary phase in the reservoir. The amount introduced into the column, therefore, seems to be independent of the initial amount, and the difference in efficiency appears to be related to the properties of the initial suspension.

TABLE III

INFLUENCE OF THE INITIAL AMOUNT OF PACKING MATERIAL ON THE REDUCED HETP OF DIFFERENT SOLUTES

Analytical conditions: column, 15 cm × 4.6 mm I.D.; mobile phase, hexane; flow-rate, 1 ml/min. A commercial chromatograph was used.

Solute	*Weight 2.4 g*	*Weight 2.8 g*	*Weight 3.2 g*	*Weight 3.6 g*
Benzene	7.2	5.5	5.0	6.1
Naphthalene	7	4.9	3.9	6.4
Anthracene	–	5.5	4.4	–
Fluorene	–	4.4	3.5	–
Chrysene	8	4.8	3.7	7.7

Reproducibility of packing

Table I lists the experimental data obtained when five identical columns were packed by this method. Three (A, B, C) were packed the same day, the other two (D and E) on different days. F_M and F_E have respectively a mean value of 13 and 3.22 ml/min with a relative standard deviation of 3.3 and 1.8%.

Efficiency and reproducibility of columns

In order to check the quality of the packing method, column D was tested on laboratory equipment in which dead volumes were kept as small as possible (septum injector; connection, < 5 cm; I.D., 0.5 mm; volume of detector cell, 8 μl). Two tests were carried out:

(1) Separation of condensed aromatic compounds (Fig. 6) under the conditions previously described;

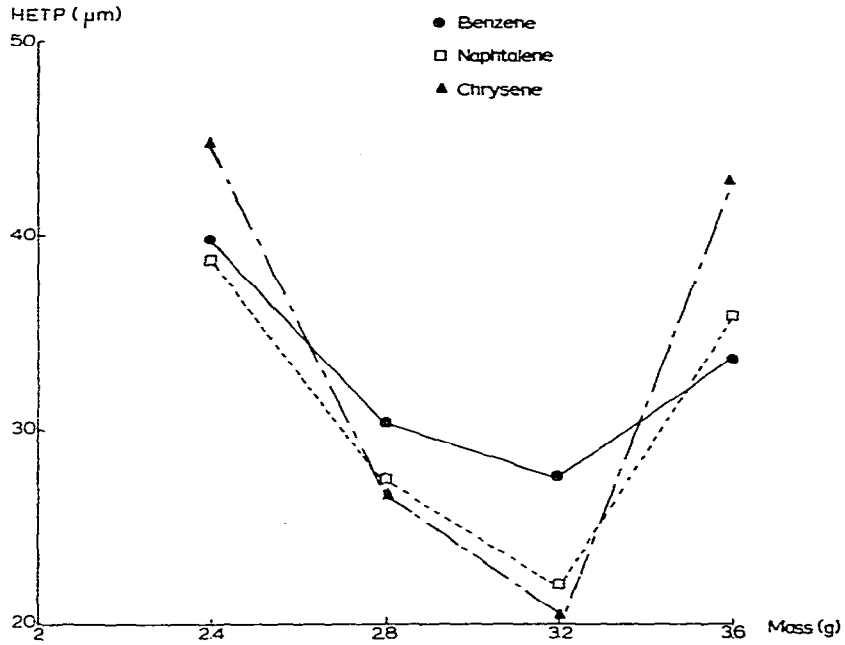

Fig. 5. Variation of HETP with the initial amount of packing material.

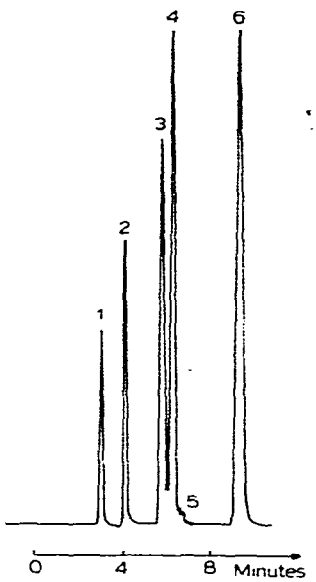

Fig. 6. Separation of condensed aromatics on column D. Peaks: 1 = benzene; 2 = naphthalene; 3 = anthracene; 4 = fluorene; 5 = impurity; 6 = chrysene. Column: 15 cm × 4.6 mm I.D. LiChrosorb SI 60 (5 μm). Mobile phase: hexane. Flow-rate: 1 ml/min.

(2) Separation of the three isomers of nitroaniline, eluted by hexane–IPA (80:20) at a flow-rate of 1 ml/min (Fig. 7).

The reduced HETP (mean particle diameter 5.6 μm) were: benzene, 3.0; naphthalene, 3.7; fluorene, 3.2; chrysene, 3.1; 2-nitroaniline, 3.4; 3-nitroaniline, 3.2; and 4-nitroaniline, 3.6.

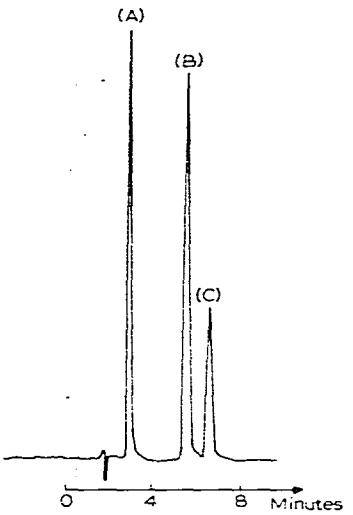

Fig. 7. Separation of the isomers of nitroaniline on column D. Peaks: A = 2-nitroaniline; B = 3-nitroaniline; C = 4-nitroaniline. Column: 15 cm × 4.6 mm I.D. LiChrosorb SI 60 (5 μm). Mobile phase: hexane–isopropanol (80:20). Flow-rate: 1 ml/min.

The values of reduced HETP are equal to those obtained by other packing methods described in the literature[2–13].

In order to check their reproducibility the five columns (column D included) were tested with a mixture of the three nitroaniline isomers under the conditions previously described, with commercially available equipment that has a lower performance than the one described above (injector valve; total connecting tubing, ≈ 100 cm; I.D., 0.5 mm; volume of detector cell, 16 μl; chromatograph Micromeritics 7000 B, detector DuPont 836). Table IV lists the data obtained. The relative standard deviation of efficiency and capacity factor are, respectively, 5.2 and 1.8% for 2-nitroaniline ($k' = 1.45$), 7.0 and 3.4% for 3-nitroaniline ($k' = 3.57$) and 5.3 and 2.2% for 4-nitroaniline ($k' = 4.31$); the retention time of a non-retained compound had a relative standard deviation of 1.2%.

The good spread of these data allows the chromatographer to be free from the step-equilibration test in routine work. If the values of F_M and F_E correspond to those obtained for previous packings (made under the same conditions), column performance will be reproducible and the column will be usable for routine analysis directly after mobile phase equilibration without the need for determining a new set of chromatographic conditions. If the flow-rates differ, the packing is not correctly spread out, *i.e.* if F_E is too large a badly packed and insufficiently compact column is produced, and if F_E is too small a heterogeneity of the column results, probably owing

TABLE IV

REPRODUCIBILITY AND ACCURACY OF COLUMNS PACKED UNDER THE SAME CONDITIONS

Abbreviations: t_0, retention time of unretained peak; t_R, retention time of the compound; N, number of theoretical plates; k', capacity factor; H and h, height and height reduced equivalent to one theoretical plate.

Compound	*Column*	*N*	*H (μm)*	*h*	t_R *(min)*	*k'*	t_0 *(min)*
2-Nitroaniline	A	2338	64	11.5	2.85	1.45	1.15
	B	2481	60.5	10.8	2.76	1.46	1.15
	C	2296	65.3	11.7	2.78	1.41	1.15
	D	2604	57.6	10.3	2.88	1.45	1.175
	E	2510	59.8	10.7	2.91	1.48	1.175
	Mean	2446			2.84	1.45	1.16
	S.E. (%)	5.2			2.3	1.8	1.2
3-Nitroaniline	A	3831	39.2	7.0	5.24	3.56	1.15
	B	3557	42.2	7.5	5.22	3.54	1.15
	C	4016	37.3	6.7	5.20	3.52	1.15
	D	4306	34.8	6.2	5.23	3.45	1.175
	E	4009	37.4	6.7	5.41	3.77	1.175
	Mean	3944			5.30	3.57	1.16
	S.E. (%)	7			3.3	3.4	1.2
4-Nitroaniline	A	4226	35.5	6.3	6.2	4.39	1.15
	B	4015	37.4	6.7	6.05	4.26	1.15
	C	4344	34.5	6.2	6.05	4.26	1.15
	D	4619	32.5	5.8	6.13	4.22	1.175
	E	4190	35.8	6.4	6.38	4.43	1.175
	Mean	4279			6.16	4.31	1.16
	S.E. (%)	5.3			2.3	2.2	1.2

to poor homogeneity of the initial suspension. This fact was apparent during assay without stirring of the suspension after dispersion.

A comparison of the results obtained with the two different chromatographs shows that the more consistent (reduced HETP between 3 and 3.7 for different solutes under very different conditions of mobile phase) and the more efficient (Table V) performance was obtained from the laboratory equipment. Such variations between two chromatographs modify considerably the performance that can be hoped for with a given column.

In order to eliminate these variations as much as possible, the different contributions to peak broadening, which decrease the efficiency of the commercial chroma-

TABLE V

RELATIVE EFFICIENCY LOSS (%)

Solute	*Calculated for connecting tubing*	*Calculated for volume of detector cell*	*Experimental*
2-Nitroaniline	166	25	203
3-Nitroaniline	57	8.5	110
4-Nitroaniline	35.5	5	78

tograph, were calculated from equations established by Martin[17]. The misreading of time constants and the great difference between the two injection systems made it impossible to determine all the contributions. However, the influence of volumes of the connecting tubing and the detector cell were calculated (Table V). About 50% of the loss of efficiency is due to the connecting tubing being too long, which is made necessary by poor design of the equipment. According to Martin, the contributions of the connecting tubing and the detector cell decrease when k' increases. The other contributions, which could not be calculated, seem to increase with k', possibly owing to differences between the injection techniques. These findings have resulted in the modification of the commercial equipment used in this study.

CONCLUSIONS

The efficiency of columns packed by the method described here (reduced HETP ≈ 3 for different solutes under very different conditions of mobile phase) is at least equal to the efficiency of columns packed by other methods. The ease of use, the low cost, the short packing time (≈ 20 min), the constant control of packing quality and especially the very good reproducibility of performance are features of the method. It is particularly suitable for laboratories in which a large number of routine analyses are performed. A column can be changed by a relatively inexperienced operator in a short time, and the chromatographic conditions need not be changed for the new column.

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