CHROM. 4741

AUTOMATIC ION-EXCHANGE CHROMATOGRAPHY OF AMINO ACIDS

EXPERIMENTAL STUDIES FOR OPTIMISING RESIN COLUMN DIMENSIONS

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(Received March 27th, 1970)

SUMMARY

Experimental studies are reported concerning the optimisation of two fundamental column parameters in automatic ion-exchange chromatography of amino acids, namely the amount of resin and the cross sectional area of the column. The experiments have been performed employing a crushed Amberlite IR 120 resin, and the amount of resin has been considered disregarding the height of the resin bed. From the results obtained some practical rules have been derived, which permit a better control of the chromatographic working conditions; consequently the solution of the problems of sensitivity and time is facilitated. Regarding the speed of the chromatogram, it is demonstrated how the problem of the overpressure can be avoided without employing spherical resins, which from a theoretical viewpoint are less suitable for the chromatographic process.

INTRODUCTION

Independently of the various theoretical studies on chromatographic separation[3,6,11,12,15], many of which have been followed and investigated by HAMILTON *et al.*[5] in their work dealing with the analysis of the mass transfer mechanisms for amino acids through ion-exchange columns, it has been possible to obtain some practical rules which permit a better control of the ion-exchange chromatography of amino acids starting from the results of the experimental studies reported here.

As a consequence, the operating capabilities and limitations of the resin columns can be more easily anticipated and the problems of resolution, sensitivity, speed of analysis and operating pressure can be solved more easily.

EXPERIMENTAL

The experiments were performed on a new analytical system whose advantages over the traditional amino acid analysers have been reported in a series of earlier papers[8–10] concerning the analysis of protein and biological fluid amino acids and related compounds. It should be mentioned that this new system allows peak eval-

uation simply by measurement of the height in mm above the base-line, with a precision higher than that achieved by measurement of their areas[10]. The representation on the recorder chart is linear with respect to the optical density and for all the experiments reported in this paper, sensitivity was set at an optical density of 1 for a full scale deflection, employing flow-cuvettes having a 10-mm light path and an internal diameter of 2 mm, provided with plane and parallel windows. The chart speed was set at 3 in./h.

The resin employed was a crushed Amberlite IR 120 prepared and conditioned in our laboratory according to the method previously described[8,9], having an average particle size of 35 μ and corresponding to the harvest obtained in HAMILTON's system of classification[4] at a water flow-rate of 50–100 ml/min.

A theoretical interpretation of the experimental observations was not sought intentionally. An experimental solution to the analytical problems was preferred to the development of theories made too complex by the large number of parameters that must be taken into consideration, too many of which are outside the possibility of measurement if not of detection.

THE CHOICE OF THE CORRECT AMOUNT OF RESIN

It is easy to anticipate that in a chromatogram it will not be possible to obtain a good resolution pattern, if, for a given set of conditions (including resin mesh-size, degree of resin cross-linking, buffer flow-rate, buffer pH, temperature, etc.), the quantity of resin is inadequate for the separation of a given mixture of amino acids.

It is likewise easy to foresee, given the same set of conditions as before, that the side effects of diffusion and non-equilibrium, when the quantity of resin is too great, can be undesirable.

Like HAMILTON *et al.*[5] we had observed that peak spreading increased, in a given glass column, when the height of the resin bed (*i.e.* the amount of resin) was increased. We therefore wished to find experimentally a law of variation of the peak height as a function of the apparent volume of resin in the column for our system.

Unlike HAMILTON, who in his experimental work[5] collected fractions emerging from the column and subsequently submitted them to analysis to determine the peaks, automatic colorimetry was applied to the column effluent in order to take into account, also, the effect of the reaction coil and the colorimetric system in deforming peaks, just as happens in every-day analytical conditions. Moreover, while HAMILTON for his purposes was chiefly interested in observing the peak width variation as a function of the column length, we were mainly concerned with studying the peak height behaviour on the recorder chart by varying the amount of resin, since in our system the peaks can be evaluated by measuring their height over the base-line.

For this purpose a jacketed column of pyrex glass, 40 cm high, having an I.D. of 1.2 cm, was set up on the apparatus previously described[8] and thermostated at 40°. According to our method[9] the column was packed four times with four different volumes of crushed Amberlite IR 120 resin. For each amount of resin a chromatogram was performed by loading the column with 100 nmoles of aspartic acid contained in 0.2 ml of 0.1 *N* HCl, which were carefully placed on the drained surface of the column and then forced in under air pressure. The column was then refilled with buffer, pH 3.26, which thereafter was pumped for the elution at a flow-rate of 2 ml/min. At

the same time the ninhydrin colour reagent, prepared as previously referred to[8,9], was pumped at a flow-rate of 1 ml/min. These flow-rates were kept constant for all the chromatograms performed in this experiment. After each chromatogram the resin was taken out of the column and the packing of the subsequent new amount of resin was performed.

It was necessary to operate with only one amino acid in the feed pulse; if an amino acid mixture were loaded on to the column, separation could not be achieved on the smallest amounts of resin at the speed of elution used and which was kept constant for all the chromatograms.

The height of the resin bed, the apparent volume of the resin and the height of the peaks expressed in millimeters are reported for each chromatogram in Table I.

TABLE I

RESULTS OBTAINED FROM FOUR CHROMATOGRAMS OF ASPARTIC ACID RUN ON FOUR DIFFERENT VOLUMES OF RESIN PACKED IN THE SAME COLUMN

Resin bed height (cm)	34.5	27.0	21.5	16.5
Apparent volume of resin (ml)	40.0	30.5	24.3	18.7
Peak height (mm)	101.0	117.5	133.0	151.7

From the diagram shown in Fig. 1 it can be seen that if the logarithms of the values of the apparent resin volumes employed are placed on the ordinates and the values of the corresponding peak heights expressed in millimeters are on the abscissae,

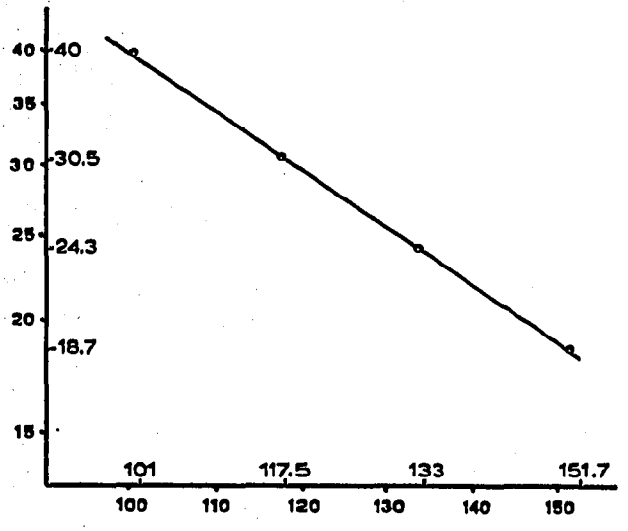

Fig. 1. Variation of the peak height as a function of the amount of resin in the column. On the abscissae are reported the peak heights in mm and on the ordinates the corresponding values of the resin volumes, in ml, on a logarithmic scale, are given.

a straight line is obtained with a negative slope. Thus, it can be deduced that by increasing the volume of resin, at a constant elution flow-rate, the peak height will

decrease according to an exponential function, which can be expressed by the following equation:

$$H_p = A - K \log V_r$$

where

A = constant
H_p = peak height
V_r = resin volume
K = constant

This behaviour may be related to diffusion and/or mass transfer resistance phenomena relating to the amino acids as they travel through the column, and since the diffusion and distribution coefficients are different from one amino acid to another, the value of K (the slope of the line) will be peculiar to each amino acid.

Taking this equation into account some conclusions can be drawn.

Concerning the sensitivity, it must be pointed out that it will vary as an inverse function of the amount of resin employed and specifically according to the above equation. It will then be very convenient to employ the minimum amount of resin to obtain separation of the components of the mixture under examination, avoiding any excess resin.

Furthermore, when a doublet gives an overlap which must be decreased, it will generally be preferable to decrease the elution flow-rate, in order to solve the problem, instead of increasing the amount of resin. As it will be shown later, slowing the flow-rate favours an increase in the peak height with a consequent narrowing of the peak due to a diminution of the band spreading, as a consequence of the diminished diffusional and/or non-equilibrium effects. In this way the overlap will be reduced and, since the separation will be improved, there will be a gain in sensitivity also.

Furthermore, since in our system the peaks can be evaluated by merely measuring their height and consequently the overlapping of peaks has much less influence on the precision and accuracy of the analysis results, it is generally convenient to reduce the amount of resin, augmenting in this way the degree of sensitivity and reducing the total elution volume of the chromatogram, which also involves a reduction of the analysis time.

The following experiment will illustrate these rules more extensively.

A pyrex glass column having an inner diameter of 0.7 cm was set up on the amino acid analyser and thermostated at 58°. An apparent volume of resin of about 7 ml was packed into the column according to the method previously described; then, after equilibration with buffer, pH 5.28, 0.1 ml of a mixture containing 100 nmoles of the following amino acids: tryptophan, lysine, histidine, ammonia and arginine, were loaded on the column. Buffer and colour reagent flow-rates were set at 1.4 ml/min and 0.7 ml/min, respectively, and a chromatogram was performed.

After this run, half of the apparent volume of the resin was withdrawn from the column and a chromatogram was then run under the same conditions as before.

On this new amount of resin and still in the same glass column, another chromatogram was next performed at a buffer flow-rate of 1 ml/min, while the colour reagent was proportionally decreased. The length of the reaction coil was also proportionally decreased.

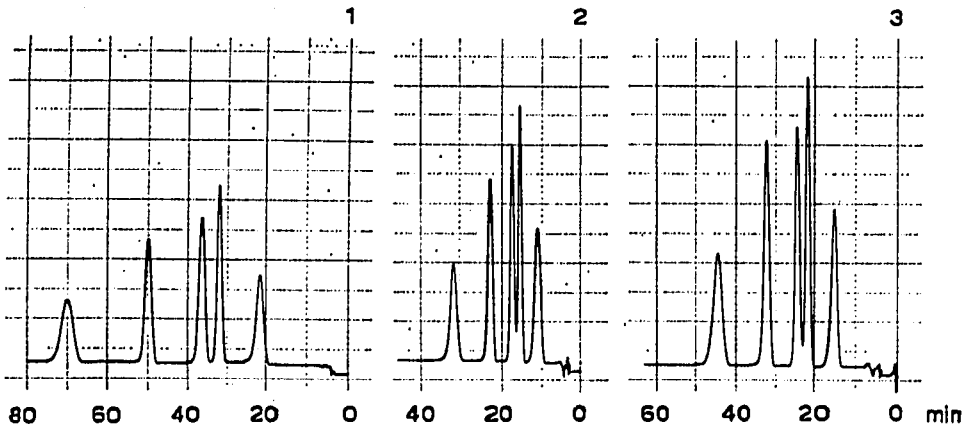

Fig. 2 Each chromatogram shows the separation of the following amino acids (from right to left): tryptophan, lysine, histidine, ammonia and arginine, under the same conditions of colour development and photometry, and employing the same glass column. Chromatograms No. 1 and No. 2 were eluted at 1.4 ml/min, but chromatogram No. 2 was obtained after removing half the apparent volume of resin from the chromatographic column. Chromatogram No. 3 was obtained on this reduced quantity of resin with a buffer flow-rate of 1 ml/min.

TABLE II

PEAK HEIGHTS IN MM AND PEAK HEIGHT-TO-VALLEY RATIOS OF THE CHROMATOGRAMS REPORTED IN FIG. 2

No.	*Tryptophan*	*Lysine*	*Histidine*	*Ammonia*	*Arginine*	*Peak height-to-valley ratio*[a]
1	33.5	67.5	56.0	47.5	23.5	0.5/56.0 = 0.009
2	52.5	99.0	83.5	70.5	37.5	11.5/83.5 = 0.14
3	61.0	112.5	93.0	87.5	43.0	85.0/93.0 = 0.09

[a] These are the ratios of the distance of valley between the peaks of lysine and histidine to the base line to the height of the histidine peak (the lower of the two peaks). The lower the ratio, the higher the resolution.

The three chromatograms are shown in Fig. 2 and the relative data are reported in Table II. Comparing the first and second chromatograms, the increase of sensitivity obtained by reducing the amount of resin for a given flow-rate can be seen. The amount of resin used for the first chromatogram could be considered excessive for the separation of the components in question, particularly at that preselected flow-rate. The resolution of the lysine-histidine doublet, in the second chromatogram, is still fully acceptable, particularly in our system where the peaks are evaluated by simply measuring their heights. Comparing the third and second chromatograms, the effect of reducing the elution flow-rate on sensitivity and separation, for a given amount of resin, can be seen.

From these examples and from the other considerations previously reported, it can be stated that the correct amount of resin can be defined as the least apparent bed volume, which, for a given flow-rate, allows the separation of the components of the mixture with a resolution pattern which does not impair the degree of accuracy and precision required.

THE CHOICE OF THE CORRECT COLUMN DIAMETER

So far an experimental study concerning the effect of the variation of the column cross sectional area on the chromatographic resolution, when the apparent bed volume of the resin and the elution flow-rate (buffer volume/time) are maintained constant, has not yet appeared in the literature.

The results of the experiments reported here checking this variation have led to the solution of many problems in this kind of analysis, mainly regarding the acceleration of the chromatogram running time.

A pyrex glass column having an inner diameter of about 0.7 cm and a height of 100 cm was packed by sections, as reported in a preceding paper[9], with an apparent volume of about 35 ml of the usual crushed Amberlite IR 120 resin.

A chromatogram was performed as previously described[8] for each of the following flow-rates: 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 ml/min. The resin was then transferred quantitatively to another column having an inner diameter of about 0.9 cm and a height of 60 cm. A second series of chromatograms was performed at the same flow-rates as before on this new column. The variation of the flow-rates was obtained by changing the gear transmitting motion from the driving synchronous motor to the pump piston, and in order to get the flow-rates of 0.6 and 0.8 ml/min, a motor giving 1 r.p.m. on the buffer feeding pumps and a motor giving 1/2 r.p.m. on the ninhydrin pump were used in place of the usual motors.

The temperature change for the slowest chromatogram was made at 75 min; for the subsequent chromatograms it was made at proportionally shorter times.

The buffer change took place for all the chromatograms after elution of the same buffer volume and after precisely 119 ml had been pumped. In other words the preset volumetric output of the first buffer pump was not varied for all the chromatograms run on the same column.

Regarding the times of colorimetric reaction with the ninhydrin colour reagent, the length of the reaction coil was varied in order to assure an equal residence time of the reaction mixture in the 100° bath for all the chromatograms.

The results of these series of chromatograms are reported in Table III. In order to compare the variation of resolving power, the peak height-to-valley ratio for all the doublets was computed; that is, the ratio of the absorbance measured in millimeters at the minimum of the valley between two juxtaposed peaks to the absorbance measured in millimeters at the maximum of the lower of the two peaks, which is indicative of the resolving power[1].

It can be observed that on increasing the flow-rate the peak heights decrease, since, due to the augmenting diffusion and mass transfer resistance, the peaks spread more and more as the chromatograms are accelerated. This is confirmed by the fact that the resolving power also becomes lower. But even though the spread gets broader and broader on increasing the elution flow-rate, the elution volume of phenylalanine (measured as the number of ml of buffer pumped at the moment when this peak reaches its highest value, *i.e.* the elution volume of the whole chromatogram) remains constant regardless of the flow-rate and the column diameter. This shows that the elution volume of a peak, under these conditions and for a same feed pulse concentration and volume, is only a function of the quantity of resin employed.

Pressures were reaching very high values at 1.6 ml/min flow-rate on the smaller

J. Chromatog., 50 (1970) 260–273

TABLE III

COMPARISON OF THE RESOLVING POWERS OBTAINED ON TWO COLUMNS OF DIFFERENT CROSS SECTIONAL AREA FILLED WITH THE SAME AMOUNT OF RESIN, AT SIX DIFFERENT FLOW-RATES

The following doublets: hydroxyproline–aspartic acid, threonine–serine, glutamic acid–proline, isoleucine–leucine and tyrosine–phenylalanine were considered. In columns 3, 4, 5, 6 and 7 the first figure of the ratio is the height of the valley between the two juxtaposed peaks from the base line, and the second is the height of the lower peak of the doublet, both expressed in millimeters. The lower this ratio (represented by the third figure), the better is the resolving power.

Flow-rate	*Column diameter*	*Hydroxyproline/ aspartic acid*		*Threonine/serine*		*Proline/glutamic acid*		*Isoleucine/leucine*		*Tyrosine/ phenylalanine*	
1.6	0.7	10.0/31.0	0.323	17.0/126.0	0.135	8.0/48.0	0.166	16.5/142.0	0.116	9.0/83.0	0.108
1.6	0.9	9.5/31.5	0.302	18.0/123.0	0.146	7.5/45.0	0.167	17.5/139.0	0.126	9.0/80.0	0.113
1.4	0.7	8.0/31.5	0.254	17.0/132.0	0.129	5.5/53.0	0.104	15.0/155.0	0.097	7.0/88.0	0.079
1.4	0.9	7.5/31.0	0.242	17.0/129.0	0.132	5.5/50.0	0.110	13.0/154.0	0.085	6.5/85.5	0.076
1.2	0.7	7.0/37.0	0.189	16.0/141.5	0.113	3.0/59.0	0.051	12.0/168.0	0.071	6.0/91.0	0.066
1.2	0.9	6.5/37.0	0.176	15.5/140.0	0.111	3.0/53.0	0.057	11.0/158.0	0.070	5.0/90.0	0.056
1.0	0.7	7.0/38.0	0.184	13.5/144.0	0.094	2.5/62.0	0.040	8.0/165.0	0.049	4.0/96.0	0.042
1.0	0.9	6.5/37.0	0.176	12.0/142.0	0.085	2.0/60.0	0.033	8.0/168.0	0.048	4.0/99.0	0.040
0.8	0.7	5.0/38.0	0.131	12.0/153.0	0.079	3.0/67.0	0.045	6.5/182.0	0.036	2.0/107.0	0.019
0.8	0.9	5.0/38.0	0.131	12.0/154.0	0.078	2.5/67.0	0.037	6.0/178.0	0.034	2.5/106.0	0.024
0.6	0.7	6.0/36.5	0.165	12.0/165.0	0.073	1.5/71.5	0.021	5.0/196.0	0.025	1.5/118.0	0.013
0.6	0.9	6.0/37.0	0.162	12.0/163.0	0.073	1.5/75.0	0.020	4.0/196.0	0.020	1.5/120.0	0.013

TABLE IV

SEPARATION OF THE LYSINE–HISTIDINE DOUBLET PERFORMED ON TWO COLUMNS HAVING A DIFFERENT CROSS SECTIONAL AREA, WITH THE SAME VOLUME OF RESIN

$H_{lys.}$ = height in mm of the lysine peak; $H_{his.}$ = height in mm of the histidine peak; V = height in mm of the valley between the peaks; V/H = peak height-to-valley ratio; P = operating pressure; $H_{0.292}$ = height in cm of the apparent resin bed in the 0.292 cm^2 column; $H_{0.608}$ = height in cm of the apparent resin bed in the 0.608 cm^2 column.

Chromatogram No.	*Column cross sectional area 0.292 cm^2*						*Column cross sectional area 0.608 cm^2*					
	$H_{lys.}$	$H_{his.}$	V	V/H	P	$H_{0.292}$	$H_{lys.}$	$H_{his.}$	V	V/H	P	$H_{0.608}$
1	177.0	162.5	12.0	0.074	5	21.6	176.5	161.0	11.9	0.074	0.35	11
2	175.0	160.0	12.0	0.076	5	21.6	178.0	163.0	11.8	0.072	0.35	11
3	179.0	161.0	11.9	0.074	5	21.5	175.0	160.0	11.8	0.074	0.35	11
4	175.0	159.5	12.1	0.075	5	21.5	177.0	163.0	11.9	0.073	0.35	11
5	176.0	160.0	12.0	0.075	5	21.6	175.0	161.0	11.8	0.073	0.35	11
6	178.0	163.0	11.9	0.073	5	21.6	175.5	162.0	11.9	0.073	0.35	11
Mean	176.6	161.0	12.0	0.074	5	21.6	176.1	161.6	11.85	0.073	0.35	11
Coefficient of variation	±0.92	±0.90	±0.74	±1.4	—	—	±0.80	±0.75	±0.50	±1.0	—	—
	Apparent resin volume 6.2072 ml						Apparent resin volume 6.688 ml					

column and it must be stressed that this experiment would not be possible if traditional reciprocating pumps were used, since in this case the pressure values would be higher. But the most important observation which can be made by examining the reported data is that, even on different column cross sectional areas, provided that the quantity of resin is kept constant, equal flow-rates (buffer volume/time) correspond to equal resolving powers.

As it will be shown later, this observation has been of valuable help in solving the problem of ultrarapid chromatograms, because the overpressure resulting from the acceleration of the elution feed can be diminished by employing a column having a larger cross sectional area but the same volume of resin.

These results have been confirmed by means of a simpler experiment, easily repeated many times for statistical purposes, which is described below.

Two columns were chosen so that the cross sectional area of the first was half the cross sectional area of the second. To be more precise, one column had an I.D. of 0.61 cm, corresponding to a cross sectional area of 0.292 cm^2, and a height of 30 cm; the other column had an inner diameter of 0.88 cm, corresponding to a cross sectional area of 0.608 cm^2 and a height of 15 cm. Both columns were jacketed. They were set up on our amino acid analyser and thermostated at 50°, then an apparent volume of about 6.5 ml of the usual crushed Amberlite IR 120 resin suspended in 0.2 *N* NaOH was packed in one of them by sections. After equilibration with pH 5.30 buffer, 0.1 ml of a solution containing 1 μmole per ml of lysine and histidine was loaded on the column which was then chromatographed at a flow-rate of 1 ml/min. The ninhydrin colour reagent was pumped at 0.5 ml/min. When the chromatogram was completed, the resin was withdrawn from the column, converted to Na form and then, after repacking the column, a subsequent chromatogram was performed. A total of 6 chromatograms were run in this way and extreme care was taken in order not to loose any of the resin in all the operations. After that, the same resin was transferred to the second column, where a further 6 chromatograms were run operating in the same way as before.

Table IV reports the results obtained in both series of chromatograms. Even in this case it appears that, on keeping the volume of resin constant, *i.e.* it was exactly the same for both columns, the resolving power remained constant, in spite of the fact that the cross sectional area from column to column as well as the height of the resin bed varied by a factor of two.

Regarding the operating pressure, some interesting comments can be made. We know that the pressure is a direct function of the resin bed height (H) and of the flow-rate (cm^3t^{-1}) and is an inverse function of the cross sectional area (A) of the column. This relationship can be written according to the equation

$$P = K\frac{H \cdot cm^3t^{-1}}{A}$$

Consequently, if $P_{0.292}$ is the pressure of the column having a cross sectional area of 0.292 cm^2, and $P_{0.608}$ that of the column having a cross section of 0.608 cm^2, when they are eluted at the same flow-rate and contain the same volume of resin, we can say:

$$\frac{P_{0.608}}{P_{0.292}} = \frac{H_{0.608} \cdot 0.292}{0.608 \cdot H_{0.292}}$$

which is equivalent to

$$P_{0.608} = \frac{H_{0.608} \cdot 0.292 \cdot P_{0.292}}{0.608 \cdot H_{0.292}}$$

By inserting in this equation the experimental values of $H_{0.608}$, $H_{0.292}$ and of $P_{0.292}$, which are reported in Table IV and the values of the cross sectional areas of both columns, the theoretical value of $P_{0.608}$ should give 1.22 kg/cm^2. In practice the observed value of this pressure was equal to 0.35 kg/cm^2. Consequently it can be stated that the diminution of pressure which can be achieved by enlarging the column cross sectional area, for a constant quantity of resin, is much greater than that expected.

On the other hand, if a same height of resin, 10 cm, is packed on both columns, thermostated at 50°, and the buffer is pumped through the one having 0.608 cm^2 cross sectional area at 2 ml/min, whereas the buffer is pumped at 1 ml/min through the other of 0.292 cm^2 (that means the same linear flow-rate for both columns), the pressures observed, instead of being equal, were 3.6 kg/cm^2 for the larger column and 2.5 kg/cm^2 for the smaller one. This would seem to be in apparent contradiction with what has been observed before.

As a matter of fact, the behaviour of both can be explained by taking into account the variations of the apparent density of the resin, which increases with the resin bed diameter, with the height of the resin bed, with the flow-rate and with the resin particle size, as has already been observed by KUNIN on spherical resins[7].

In the first case, as can be seen from the data reported in Table IV, the apparent density of the resin, that is the ratio between the resin weight and the apparent bed volume, was higher in the smaller diameter column.

In the second case, considering the pressure values observed on the two columns, it can be deduced that the apparent density was higher in the wider column. In other words, in this column the amount of resin was greater than that expected. Consequently it can be stated that, if two columns of different cross sectional area are poured and packed with resin to the same height, the actual amounts of resin are not geometrically proportional.

However, it is not within the scope of this work to discuss the theoretical interpretation of this behaviour or the fact that the resolving power does remain constant for a given quantity of resin when this is transferred to columns of different diameters. We will merely state that this is governed by numerous factors and is the result of many concomitant causes.

However, from these observations practical guidance can follow concerning the choice of the correct column diameter. *E.g.*, the quantity of resin in a given column was chosen in order to obtain, in a given time of analysis, and at a given flow-rate, a satisfactory degree of resolution of the components which are to be separated (and under these operating conditions the requirements of sensitivity as well as of precision and accuracy are satisfied). The operating pressure can, under these conditions, reach excessive values almost at once or packing down of the resin from run to run can take place with a consequent progressive pressure rise[2]. In order to avoid this, instead of reducing the flow-rate of the eluting buffers and consequently increasing the time of analysis, the resin can be transferred quantitatively into a column of larger diameter, on which all the other operating parameters are kept as before. The chromatogram obtained on the new column, as shown from data reported in Table

III, is just the same from every point of view as that obtained on the smaller column without any sacrifice of analysis speed.

It can be asked to what extent this can be done. It has already been shown that a diminution of the resin bed height, for a constant weight of resin and for a constant flow-rate, is accompanied by a reduction of the operating pressure to values which are lower than those expected. If the cross sectional area of the column is enlarged excessively (with a consequent excessive height reduction), the operating pressure will decrease to values which are too low to permit a good packing of the resin in the column; the void fraction will become too large and uneven, so that the chromatographic resolution will be consequently affected.

Concluding we can say that, for a given quantity of resin, by varying the elution flow-rate and the column cross sectional area, optimum conditions of working pressure can be found at which the progressive packing of the resin and the consequent pressure rise from chromatogram to chromatogram does not take place.

It should be mentioned that the volume of the feed pulse should be proportional to the cross sectional area. For example, we can load a feed pulse having a 0.1 ml volume on a 0.9 cm diameter column; on a 1.2 cm diameter column, the volume of the feed pulse must be at least 0.2 ml. Otherwise uneven distribution of the solute on the resin surface may occur with the consequent alteration of the shape of the chromatogram peaks and thus of the resolving power.

PRACTICAL APPLICATIONS AND CONCLUSIONS

Taking into account the practical rules emerging from the experimental observations previously reported, the problem of obtaining operating conditions which will give a rise in sensitivity as well as acceleration of the chromatographic procedure can be more easily resolved, since the operating difficulties occurring in both cases can be more easily overcome.

The solution to the sensitivity problems will be dealt with in a forthcoming paper, where a high sensitivity methodology suitable for 10 nmole loads will be fully discussed. Here it will be only mentioned that high sensitivity conditions are obtained by a simple dramatic reduction of the amount of the resin employed, which is followed by lowering the eluting buffer flow-rate until the separation and resolution of the chromatographed components is achieved. The choice of the column cross sectional area only takes into account the value of the operating pressure which must be appropriate to the instrument used and must remain constant from chromatogram to chromatogram. Since the operating column pressure can be very easily controlled in this way, for ultrasensitive chromatograms, the employment of a resin having a smaller particle size should also be taken into consideration.

Concerning the problem of accelerating the chromatographic separation, we have found that by applying the rules previously reported, it becomes a matter of routine to employ a procedure which gives, on our analytical system, a full chromatogram in 90 min with a crushed Amberlite IR 120 resin. With a possible sacrifice of accuracy, but still with a high degree of precision, it is also very easy to obtain the same chromatogram in 60 min. Figs. 3 and 4 show chromatograms run in 90 and 75 min, respectively. The acidic and neutral amino acids were separated on a column having an inner diameter of about 1.2 cm with an apparent resin bed height of 31 cm,

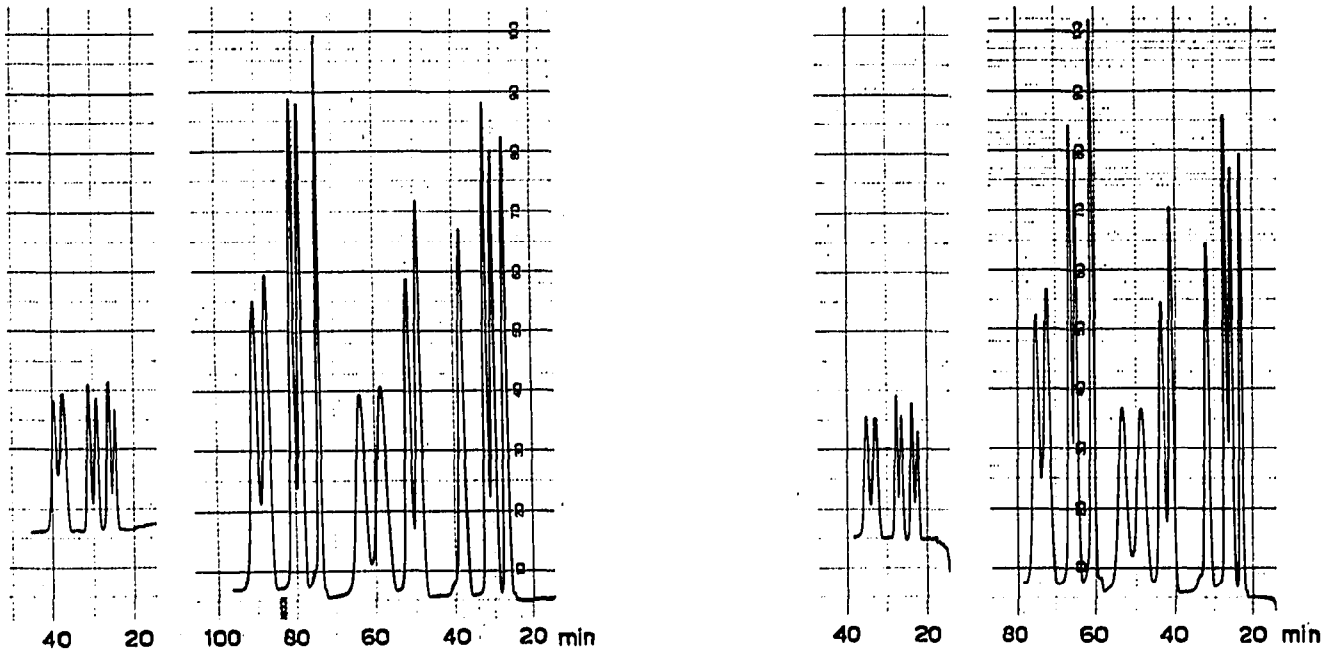

Fig. 3. Chromatogram run in 90 min on a 1.2-cm diameter column at a flow-rate of 2 ml/min. The feed pulse of 0.2 ml contained 200 nmoles of each amino acid. On the right hand side of the figure, the 570 mμ reading is reported and shows the separation, from right, to left of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine. On the left hand side of the figure, the 440 mμ reading shows the separation of hydroxyproline, aspartic acid, threonine, serine, glutamic acid and proline. The temperature was kept at 40° for the first 19 min and then raised to 58°.

Fig. 4. Chromatogram run in 75 min on a 1.2-cm diameter column at a flow-rate of 2 ml/min. The feed pulse of 0.2 ml contained 200 nmoles of each amino acid. On the right hand side of the figure, the 570 mμ reading is reported and shows the separation, from right to left, of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine. On the left hand side of the figure, the 440 mμ reading shows the separation of hydroxyproline, aspartic acid, threonine, serine, glutamic acid and proline. The temperature was kept at 40° for the first 15.8 min and then raised to 58°.

that is an apparent resin bed volume of about 35 ml. The 90-min chromatogram was obtained at a flow-rate of 2 ml/min against a total pressure (column plus coil) of 13 kg/cm² at the lower temperature, while the 75-min chromatogram was run at 2.4 ml/min with a total pressure of 17 kg/cm² at the lower temperature. The basic amino acids were separated on a 0.7 cm inner diameter column with an apparent resin volume of about 3.5 ml at a flow-rate of 1 ml/min; since this chromatogram is completed in 30 min (see Fig. 2/2), if recording is started on the second channel just after the breakthrough of the proline from the long column, both recordings will be finished at the time at which the phenylalanine is over on the long column chromatogram. Consequently the times of 90 min and of 75 min for the acidic and neutral amino acids which are chromatographed on the first channel, become the total chromatogram times[8].

It must be stressed that these results can be obtained with crushed Amberlite IR 120 resin, of the same type and mesh as that employed by SPACKMAN *et al.*[13]. At the same time we would like to mention that this type of resin has also been generally abandoned because it was thought unsuitable for obtaining a protein amino acid chromatogram in less than 4 h (ref. 14). The crushed resin has been generally sub-

stituted by much more sophisticated specially prepared spherical resins; in this way it was hoped to overcome the problems of overpressure when high flow-rates of buffer are pumped through the column. As a matter of fact, this expectation was not realised[2], because, in order to get resolution when spherical resin is employed, a smaller particle size has to be adopted. We agree with HAMILTON, who reported[4] that irregular 30-μ particles gave resolution comparable to that obtained with spherical 25-μ particles. A possible explanation of this fact may be that equilibrium can take place more rapidly in nonspherical particles than in spheres of the same mass, as the time necessary for diffusion to their centres is governed by the smallest of the three dimensions.

Consequently, since the ion exchange kinetics are strictly related to the extent of available surface presented by a given amount of solid phase, it is to be recommended that the irregular crushed resin should be retained as much more suitable for chromatographic purposes than the spherical resins, considering that the sphere is characterised by the smallest surface area per mass unit.

The experimental results reported here demonstrate that in order to avoid the overpressure, which occurs when the chromatographic elution is accelerated, it is not necessary to change the geometrical form of the resin, since this problem can be solved perfectly by a suitable choice of column parameters (that is the amount of resin and cross sectional area), and by abandoning the single reciprocating pumps usually employed to feed the columns. During the positive stroke, these pumps supply a pumped volume which is the double of that of the preset output, and consequently the pulsing pressure at the top of the resin bed reaches values which are higher than those obtained with our syringe-type pumps for the same flow-rate.

It has only to be stressed that when working with a crushed resin, particular care must be taken concerning the packing operation.

As a matter of fact, the dispersion of the particle classification which occurs in a pulverised resin is, of course, always much greater than that which can be achieved by regrading of spherical shapes.

Consequently, the column packing must be accomplished according to the method described in a preceding paper[9] in order to get an evenly dispersed column.

ACKNOWLEDGEMENTS

The author wishes to express his thanks to Professor G. SEGRE for his valuable criticism, to Dr. G. BONGIOVANNI, Mr. I. RAFFAELE and Mr. C. VILLA for their skilful assistance during the experimental stage, to Dr. M. FERRO-MILONE and Dr. G. BONGIOVANNI for reading the manuscript and to Miss E. TAYLOR for correcting the English style.

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