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Gradient Elution Analysis

I. A General Treatment

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In a previous paper [1] the limitations of the two most common procedures for chromatographic analysis, displacement analysis and elution analysis, have been discussed. It is the purpose of this paper to introduce a technique, gradient elution analysis, which has only received scant mention in the literature [2,3] and has not been treated in any way theoretically.

It is usual to point out that elution analysis can not be successfully applied to those substances which have curved isotherms because of the spreading of the zones and the high losses of material on the column ***. To some extent this has been overcome by applying a succession of different eluting solutions [4,5] but in such a case there are a number of grave complications. Firstly many substances are eluted partially by one eluting agent and then appear again in the front of the next eluting solution. It appears that the zones are spread widely and all the material is not elutable from the column in the first eluting agent but is picked up again by the second (Fig. 1). Clearly this can result in poor separation (see later). Again the position in which the substance travels in the eluting agent is dependent upon (a) the column length (b) the amount of the material, (c) the step length. Thus the recognition of an individual compound is not predictable from the position in which it travels in the eluting

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*** For very small concentrations of the substances to be applied to the column, the isotherms are linear, of course. Working in the extremely low concentration regions it is possible to apply elution analysis as in the well-known example of Moore and Stein's analysis of amino-acids. Even in such low concentrations elution analysis may not be always possible *e.g.* with carbon, because of irreversible adsorption.

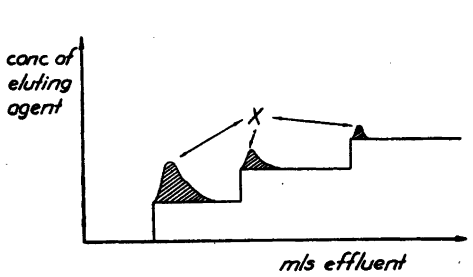


Fig. 1. A schematic representation of a stepwise elution experiment in which one substance occurs three times.

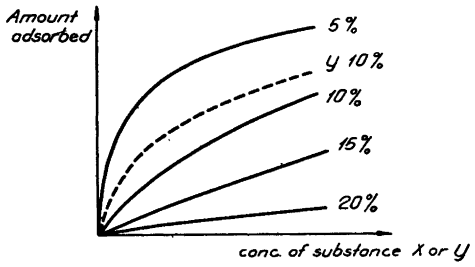


Fig. 2. Adsorption isotherms of two substances, X and Y, in different solutions of ethanol in water. The full lines represent isotherms for X and the percentages are the % ethanol in water in the solvent.

solutions. It was of interest to enquire therefore how substances would travel in a column in which the eluting power was being continuously increased.

The first approach to this problem has been made through the observation of the behaviour of the oligo-saccharides in a gradient of increasing ethanol concentration in water [6].

However, the general effect of eluting agents on the isotherms of a number of different substances [7] is similar and therefore the approach through the isothermal data instead of through column procedure will be outlined as it presents a more widely applicable picture of the method. The data will be treated in a purely qualitative fashion.

THE ISOTHERMS

As was shown by Hagdahl, Williams and Tiselius [1] a saturator depresses the maximum possible adsorption (as shown by the isotherms) of many substances such that the isotherms become linear only at very low adsorption. An eluting agent has a somewhat different effect in that the maximum adsorption remains the same but an adjustment of the isotherm to become more linear occurs already at much higher concentrations of the substance being examined than with a saturator. Fig. 2 shows the way in which the isotherms change for substances such as the sugars and amino-acids adsorbed on carbon from different concentrations of ethanol, or many other eluting agents, in water. Changes of the isotherms of other groups of substances are somewhat different but not sufficiently so to warrant a separate general discussion [6].

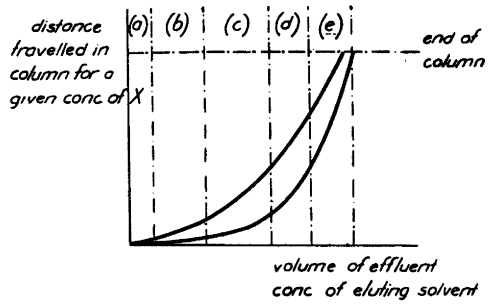


Fig. 3. Diagrammatic representation of the way in which two different concentrations of the same substance would travel in the same gradient. The lower curve is for the lower concentration. The curves can be deduced from a set of isotherms as in Fig. 2. The letters refer to Fig. 4.

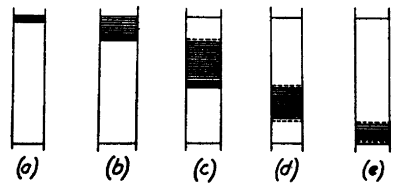


Fig. 4. The development of a zone (see Fig. 3).

This suggests that for a substance which has a curved isotherm in the initial pure solvent, *e.g.* water, the effect of the second eluting agent is generally to decrease the adsorption until the isotherm is linear to the origin — a continuous change of the isotherm being seen [7]. For different substances the isotherm will become linear at different concentrations of the second eluting agent. Now when a step-wise change in the eluting agent, *e.g.* alcohol, concentration is made in eluting a substance X (Fig. 2) from a column of adsorbent taking for example 5 %, 10 % and 20 % ethanol, it may well be that at the first concentration a little of X is eluted, and then at 10 % ethanol most of X will be washed from the column and finally at 20 % all of it will be eluted, see Fig. 1. Only if the steps are made very large can the substance be completely eluted in a concentration of eluting agent less than that of the condition where the isotherm is linear (*i.e.* in this case, in 15 % alcohol). Unfortunately if such a procedure is adopted other compounds may occur in the step. This is illustrated with reference to Fig. 2 where it is clear that Y will be eluted to some extent in 10 % ethanol *i.e.* before all of X is certainly eluted. This explains the painstaking methods which have been found to be necessary in step-wise elution methods. An example is provided by steroid analysis [5].

In order to appreciate the advantages of the gradient procedure consider the movement of the zone of a substance in an increasing concentration of an eluting agent. In the first instance let it be assumed that the eluting agents move without retention volume. This is the case for most eluting agents at the relatively high concentrations used. Then the concentration of the eluting

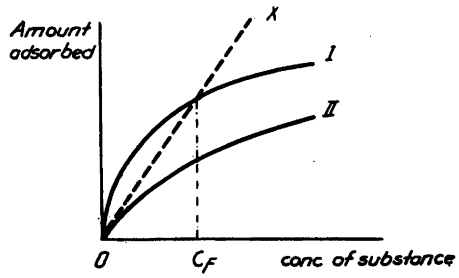


Fig. 5. Two adsorption isotherms taken from Fig. 2 and used to discuss the speed of movement of the front and rear of a zone.

agent, which is assumed for a moment, to increase in a linear manner at the top of the column will also increase linearly with the effluent volume. The movement of the substance X can now be considered in this gradient. There are two aspects of major importance, firstly the zone spreading and secondly the separating power of the gradient.

ZONE SPREADING

On applying the substance X to the adsorbent its spreading during movement down the column in a constant eluting solution depends upon the shape of its isotherm. In the first place if the isotherm is linear there will be the theoretical spreading (discussed in ref. 8 and 9) but if the isotherm is curved the substance will travel as a "tailing" elution peak. In normal elution methods there are no features opposing this zone spreading and tailing. In the gradient two factors adjust these effects. Firstly the gradient itself, which will exist in the column, is such that for all isotherms the "tail" moves in a region of

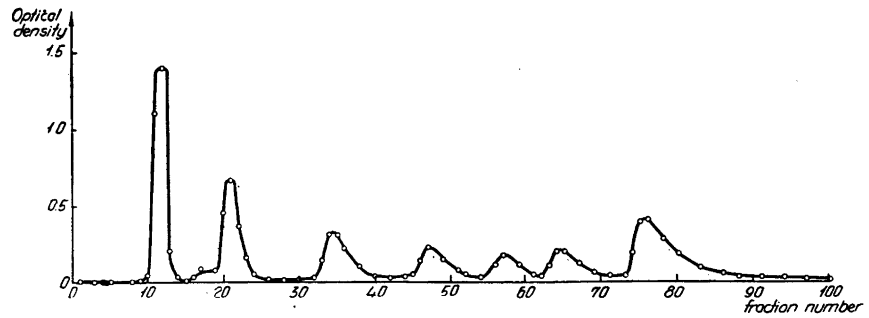


Fig. 6. A plot, optical density (which is proportional to the amount of each sugar) against fraction number. It shows the resolution of an oligosaccharide mixture by a gradient elution analysis. The oligosaccharide mixture, an α-Schardingerdextrine hydrolysate was applied to a pretreated column (carbon Darco G 60) and the elution carried out with 0—20 % ethanol. The experiment will be described in detail in a later publication.

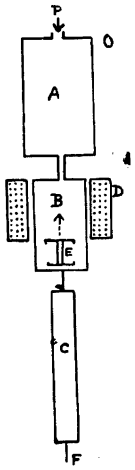
higher eluting power than the front of the elution peak, *i.e.* the peak will be constrained to move in a smaller volume than in a normal elution experiment. The peaks will be somewhat disturbed, of course, from their usual shape. This effect has been mentioned in a theoretical fashion by Strain [2]. Secondly the gradient moves as a whole with no, or little, retention volume so that the zone of the substance is finally moving in an eluting concentration in which it moves rapidly, *i.e.* it does not remain in the column for very large volumes of liquid. The discussion can be clarified by the use of Fig. 3 which illustrates the movement of two concentrations of substance, X, in a column. The isotherms of X in different eluting agent concentrations are assumed to be as in Fig. 2.

When development of such a compound, X, on a column occurs under the influence of a gradient then at first, *i.e.* at low concentrations of the eluting agent, the movement in the column is slight, Fig. 3a, and the zone spreading which takes place is not very different from that to be expected in a normal elution experiment, Fig. 4b. The tail of the peak hardly moves at this stage as the adsorption is very strong at low concentration of the eluting agent. When higher concentrations are reached the zone begins to move as a whole but the tail still moves more slowly than the front. The zone continues to spread. At this time the zone may occupy a considerable region of the column and the concentration in which the tail travels is noticeably higher than that in which the front moves, Fig. 3c and 4c.

A stage will be reached when this difference causes the tail and the front to move at equal rates although the isotherm is still curved. In Fig. 5 this is illustrated with reference to two isotherms from Fig. 2. Isotherm I is that from a solvent of the same composition as that in which the front of the zone is moving. Isotherm II is that from a solvent of the same composition as that in which the tail moves at this stage. Now let the concentration at the front of the zone be C_F, the speed line OX [1], through the point on the isotherm, I, corresponding to this concentration, will not cut isotherm II and is a tangent to it at the origin. The point C_F on isotherm I travels at the same speed as a very low concentration on isotherm II, *i.e.* the tail and the front move at equal speeds. At this stage the spreading of the zone ceases.

The eluting agent concentration is still being linearly increased and as the isotherms are further depressed the tail begins to move faster than the front, Fig. 3(d) and 4(d). Finally the zone travels as an almost symmetrical elution peak Fig. 4(e) as the isotherm becomes linear. The zone spreading is still controlled, however, by the gradient in which the peak travels.

The factor which is clearly important in the above reasoning is the relationship between the column length and the steepness of the gradient. With a



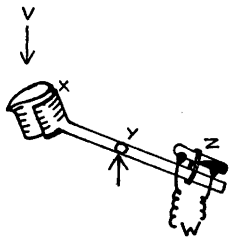


Fig. 8. Simple arrangement for an intermittent current supply. Water runs slowly into the container X until its weight is sufficient to overbalance the mercury switch Z. The swing pivots about Y and empties X. The swing returns to its previous position. The leads W, go to the coil D.

Fig. 7. The arrangement of the column. The effluent from F is collected in fractions.

steep gradient the zone development will occur rapidly and the column length need not be excessive. The less steep the gradient the longer the column must be for a full development of the zone as described above. Again for a slight gradient there is the danger that the compound will leave the column before stage (e), Fig. 4, is reached. Apart from the poor definition of the zone before this stage, the "break through" volume and the "break through" concentration will be ill-defined. A detailed analysis of such points is being undertaken and will be described elsewhere. It is already clear, however, that in many cases the "break through" volume is well defined and that the concentration of the eluting agent at which break through occurs is characteristic of a given compound and independent of the mutual influence of the column length and the gradient steepness. A component eluted from a chromatographic column must be recognisable by the position in which it travels if the technique is to be used analytically. In normal elution experiments this is clearly the case. In gradient elution the "break through" volume of a component will be controlled by factors discussed in later papers and it appears that the normal elution technique is somewhat more convenient as it results in well defined positions of a component relative to such conditions as the eluting medium and the column length. Examples of this are given in ref. 6.

Naturally the zone spreading depends upon the amounts of the substance applied to the column. It has been found that the gradient elution technique is particularly suitable for the analysis of small quantities of material.

Before discussing factors controlling separation a further point must be made. If the eluting agent employed has a curved isotherm itself then instead of a gradient of eluting concentration emerging from the column a step will occur covering the low concentration range. After this initial step a gradient will still be obtained. It is important that the substances to be examined are not eluted in concentrations of the eluting agent lower than that of the initial step. Thus if the eluting agent has not a linear isotherm it must be chosen so that its adsorption is considerably less than that of the examined substances.

SEPARATION OF SUBSTANCES

The only effect which has been mentioned so far as effecting a separation of two substances is that of the different rate of travelling in the gradient of eluting concentration. However, as the substances are applied to the column a displacement of the more weakly adsorbed by the more strongly adsorbed compounds will occur especially for those cases in which the substances to be separated belong to the same homologous series or to the same group of compounds [1]. It must be clear that displacement occurs first and then the zones of the different compounds are separated by the eluting agent. The displacement will be very strong as the isotherms are initially very curved.

The main limitation to separation of two substances which have a given difference in adsorption is the zone spreading.

An example of the experimental recordings is given in Fig. 6 which shows a typical analysis of a mixture of oligo-saccharides. It should be observed that the compounds emerge as almost symmetrical elution peaks. This probably means that the length of the column has been correctly adjusted to the given gradient so that complete development of the zones has occurred.

The application of a gradient elution can not increase the separation over that of the simpler elution techniques already devised in those cases where the substances have linear isotherms.

Apart from the adjustment of the concentration of the eluting agent the choice of this agent is important. It has been found for adsorption on silica gel and alumina that a series of eluting agents of increasing power can be utilised. This series, the Trappe series, can be used here too. Experiments on carbon have been made which show the effect of different eluting agents. A discussion of this problem will be given in a later paper [6]. However, other data [10] already exists in the literature which shows that the choice of the eluting agent can have a great influence on the rate of movement, the separation and the zone spreading in a chromatogram of the usual type. Similar somewhat specific factors are to be expected here.

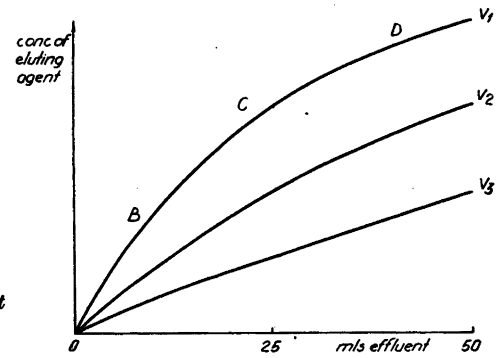


Fig. 9. Three gradient curves for different volumes of the mixing chamber.

The mechanism of the adjustment of the adsorption of compounds by eluting agents can be described under two headings. In the first instance the eluting agent may alter the solubility of the compounds to be examined in the moving phase. Secondly the adsorption of the eluting agent may well produce a change in the adsorbent surface. At present an investigation is being carried out into the relative power of eluting agents on carbon in order to elucidate this aspect of the problem.

PRETREATMENT OF THE ADSORBENT

The effect of pretreatment of the adsorbent has been discussed in several previous papers [1,11,12]. Its first successful application by the gradient elution procedure will be described in connection with the separation of sugars [5]. It has been found in this work and more recently in the separation of peptides [7] that saturation or pretreatment improves the shape of the zones, increases the recovery to a value very close to 100 % and lastly permits the extension of the technique to very strongly adsorbed compounds such as the higher oligosaccharides [6] and the peptides [7]. The combination of a suitable saturation and a gradient elution seems at present to be of extraordinary power in its range of application.

APPLICATION OF THE GRADIENT TO A COLUMN

The above has been concerned in demonstrating the method of gradient elution from a theoretical stand-point. The application of the gradient to a column is not simple as the column must be operated under pressure. The diagram Fig. 7 shows the special construction which has been utilised.

There are two distinct chambers: the first of which A, contains the solution of the eluting agent which is to be used for development, and the second, B,

of volume V, the mixing chamber, contains the solvent in which the column C was packed. The mixing chamber is equipped with a magnetic stirrer which consists of an iron rod enclosed in plastic material which is not attacked by the eluting solvent. The chamber itself can be made of brass or plastic. An intermittent magnetic field produced in a coil, D, surrounding the mixer, is sufficient to lift the iron rod, D, in the mixer. The up and down movements of the rod mixes the liquid in the chamber. The intermittent current is produced by the simple arrangement shown in Fig. 8.

The operational procedure is simple. The column is set up and the substances to be examined are applied to the top of the column in the first solvent which has little if any eluting action on the compounds. The column is then connected to the mixing chamber, which should be made in pieces so that its volume can be adjusted, and then to the chamber A. Naturally the chamber B must be completely filled. When pressure is applied to A the first solvent starts to move down the column and the second solvent moves into the mixing chamber. The gradient of the second solvent applied to the column in this way is expressed by the formula:

$$2.303 \log_{10} \frac{X}{X-x} = \frac{v}{V}$$

x is the concentration of the second solvent in the mixing chamber when a volume, v, has flowed into this chamber, and X is the concentration of the second solvent in the chamber A. V is the volume of the mixing chamber. A plot can be made of $\frac{x}{X}$ in the mixing chamber against v. The shape of this plot is independent of X but is dependent upon V. A set of such curves can be obtained for different V values. Thus the steepness of the gradient is adjustable by an alteration of the value of V. This is shown in Fig. 9 where $V_3 > V_2 > V_1$.

The second adjustment of the gradient that is possible lies in the choice of the initial solutions in A and B. Thus it is possible to choose a gradient of any desired slope in any required concentration range.

In Fig. 9, and the above equation, v can be replaced by $v_1 - v_2$ where v_1 is the volume of the effluent and v_2 the dead volume of the column, providing the eluting agent travels without retention volume.

Now in Fig. 9 if the curve for the volume of the mixing chamber is V_2 and the curve for this volume is divided into three parts OB, BC and CD then it is seen that the gradient is steep over the region OB, moderate from B to C and slight from C to D. Substances eluted in the region OB will be somewhat crowded together and substances eluted in the region CD will be well separated but the peaks will be more spread than those eluted in the earlier parts of the

gradient. Clearly a small gradient should be applied to similarly adsorbed substances and a steep gradient to compounds adsorbed in very different strengths. However, as a saturator can be used to adjust the strength of the adsorption the further question arises as to whether a small gradient should be employed together with high saturation or a steep gradient together with low saturation. These features will be discussed in detail for specific cases in later papers.

It is obvious too that an improvement in the existing technique can be immediately made by the production of a linear gradient in the mixer. Methods for this are under consideration. It is of interest to note devices suggested by Desreux [15] in a somewhat parallel apparatus for the production of gradient changes in solvent composition.

The gradient elution technique has a value apart from its immediate application to separation problems. It can be utilised in initial experiments in order to obtain a survey of the chromatographic behaviour of an unknown mixture of compounds.

The introduction of gradient elution procedures has already met with considerable success which will be described elsewhere. However, the application of the method is very wide and its full value has not been appreciated so far. Thus a gradient can be applied in many ways, a continuous change of pH, of salt concentration [13], of displacing strength [1,7], are all feasible and a similar variation is possible in partition extraction procedures [14]. All these methods must be thoroughly examined before their usefulness can be accessed. It is known however, that very good separations can readily be achieved in the oligosaccharides, fatty acids, sterols, peptides, and amino acids. The method is being tried also for proteins and alkaloids. Full reports of these experiments will appear shortly.

SUMMARY

A new general procedure for the chromatographic analysis of mixtures of compounds is outlined. The method consists of the use of a continuously changing eluting medium which is produced external to the column in a mixing chamber. The mode of operation of this "gradient elution method" (as we propose it should be named) is outlined and factors such as the spreading of zones, the separation of components and the pretreatment of the adsorbent are discussed. From the experiments which will be published elsewhere it is clear that the method has widespread application.

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