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NEW APPROACH TO THE RAPID OPTIMISATION OF CONDITIONS FOR THE ISOCRATIC SEPARATION OF MULTICOMPONENT MIXTURES

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SUMMARY

Despite the rapid advances in chromatographic technology, the development of high-performance liquid chromatographic methods remains expensive with respect to both time and materials. A new approach is proposed here which is suitable for the investigation of eluents and packings for the resolution of multicomponent samples. This is based upon the use of a small column to mimic the retentive characteristics of isocratic analytical systems but on a greatly reduced time scale. The general utility of this approach is illustrated by three applications, using data for purines and pyrimidines on both reversed-phase and ion-exchange systems.

INTRODUCTION

The majority of laboratories involved in quantitative analysis use conventional isocratic high-performance liquid chromatographic (HPLC) systems with columns of 10–30 cm × 4–5 mm I.D. and detectors with *ca.* 8-μl flow cells. Such systems can benefit from the advances made in column technology, but the diversity of bonded phases on particles 3–10 μm in diameter from a large number of manufacturers means that the identification of a packing best suited for a specific application is increasingly difficult. There is a natural conservatism, which tends to inhibit the exploration of potentially better columns or eluents to replace existing methods.

If a new separation system is required, the first steps usually involve injecting standards into familiar columns and using eluents selected with regard to the available information about the chemical nature of the compounds to be separated. When this fails to resolve the standards a series of alternative eluents are tested until acceptable resolution is obtained. To overcome the empirical nature of this approach, the computer-assisted manipulation of limited experimental data for predicting optimal eluent composition has been proposed[1–5]. The application of systematic or simplex searches[1] are complicated, particularly for multicomponent samples or eluents[5]. The use of gradients[2] to assist in the definition of optimal isocratic systems has proved useful for some separations, but for many applications this use of gradients is inappropriate. Problems with the purity of standards and/or peak identification may necessitate lengthy gradient elutions of individual analytes, and this may prove prohibitive if many components are to be resolved. Commercial packages are avail-

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able, but these tend to be expensive and have stringent hardware requirements, together with limited flexibility, *e.g.*, the Perkin Elmer PESOS system[6] requires specific hardware and software costing in excess of £30 000, and any change in the combination of analytes to be resolved necessitates the reinitiation of the optimisation procedure.

A simple, automated system has been developed, which mimics the analytical column in its retentive behaviour but with exceptionally short analysis times. The time saving involved in comparison to the usual semi-empirical approach is considerable and the system is applicable to virtually all isocratic separations. The k' (capacity factor) values generated are plotted against the variable under test, *e.g.*, pH, ionic strength, and organic solvent concentration. Since the retention mechanisms are the same as for the corresponding analytical system, the curves obtained graphically illustrate the separations that are feasible with mobile phases within the range tested. The results can be accumulated to yield a library of data, which may subsequently be used to compare column efficiencies and define optimal eluents for the resolution of many combinations of analytes. The transfer of further data manipulation to spreadsheet or database packages such as Symphony (Lotus), Xchange (Psion) or Smart (Innovative Software) alone or in combination with a graphics package such as Graph-PAD (Academic Press) or Statgraphics (Statistical Graphics) give a manipulative power which is not available in systems dedicated to chromatography. The ease and convenience of using such packages, on a personal computer at a location remote from the laboratory, is a considerable advantage particularly if the hardware and software are already available. When new separation systems are required relevant curves can be superimposed in order to select appropriate columns or eluents. This visual comparison is used in order to accommodate the chromatographer's specific requirements for different applications.

EXPERIMENTAL

Chemicals and equipment

All chemicals were of AnalaR grade, with the exception of Aristar orthophosphoric acid (BDH, Poole, U.K.) and far-UV-grade acetonitrile (Romil, Loughborough, U.K.). Buffers were prepared by dilution of acetic, succinic or orthophosphoric acids, with deionised water (Milli-Q; Millipore/Waters, Harrow, U.K.) and titration to the appropriate pH with sodium or ammonium hydroxide. All eluents were filtered before use. The chromatograph (Millipore/Waters) used for these studies consisted of two Model M450 pumps, WISP 710B autosampler, M720 system controller, and a M490 detector, connected to a Trilab 2000 data system (Trivector, Sandy, U.K.). An IBM AT personal computer, running Symphony (Lotus, Windsor, U.K.) and Statgraphics (Cocking & Drury, Reading, U.K.) was used for further data manipulation and presentation.

Procedure

The selected packing material was slurry-packed at 6000 p.s.i. into an Upchurch 20 × 2 mm I.D. precolumn (Anachem, Luton, U.K.). This column has a low internal volume (approx. 60 μl), and when packed with 3 μm Apex ODS (Jones Chromatography, Llanbradach, U.K.) an aqueous flow-rate of 1 ml/min exerts a

backpressure of *ca.* 2000 p.s.i. Equilibration is rapid, and retention times are short, *e.g.*, <200 s for components which would be retained >60 min on a 150 $\times$ 4.6 mm I.D. analytical column with the same eluent.

A series of automatic, isocratic runs (injection volume ≤ 5 μl) of a comprehensive range of compounds of interest and potential contaminants is performed. The composition of the mobile phase for each automatic run is varied, *e.g.*, by 10% increments, from 0 to 100% B, using a gradient controller. There are few constraints imposed upon the pumps or detectors by this method, as an isocratic flow of 1 ml/min is used for each set of automatic sample injections, and the peak shape is relatively unimportant. Some form of data system is essential, due to the very short analysis times (≥ 10 s). However, the analysis of each chromatogram is straightforward, as only one component should be present, and impurities are readily visible. The k' values and plate counts (which may be calculated automatically by the data system, or via a spreadsheet etc.) are stored and plotted, as required.

RESULTS AND DISCUSSION

The basic simplicity of this approach confers a high degree of flexibility as illustrated by the applications outlined below.

Formation of a database for multicomponent separations

Perhaps the most useful long-term application is for the rapid acquisition of data, relating to the retention of a particular group of compounds on a chosen column, in order to obtain a library of information, which can subsequently be extended or used to predict columns or eluents for any combination of analytes within the database. Figs. 1 and 2 are drawn from data obtained when the effects of different

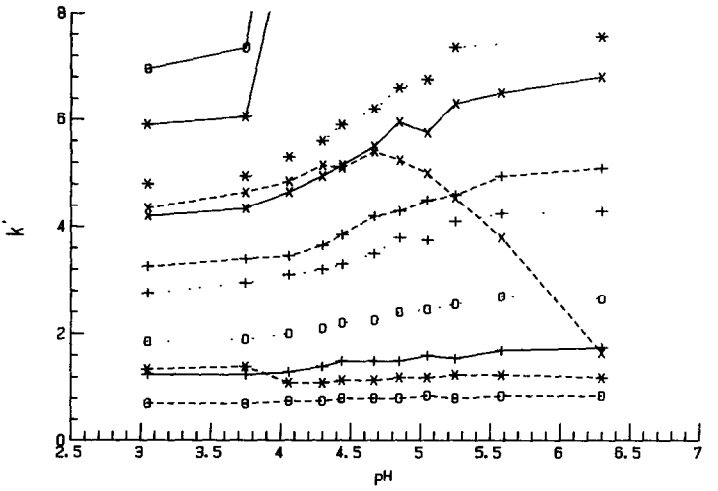

Fig. 1. Dependence of the k' of purines and pyrimidines upon pH. Column: 20 $\times$ 2 mm I.D. Apex 3-μm ODS. Buffer system: 0.1 *M* ammonium acetate. (O—O) 2'-Deoxyadenosine, (*—*) adenosine, (*....*) thymidine, (×---×) xanthosine, (×—×) 2'-deoxyguanosine, (+---+) guanosine, (+....+) inosine, (O....O) adenine, (*---*) cytidine, (+—+) uridine, (O---O) uracil.

buffers on the retention characteristics of 40 standard purines and pyrimidines were investigated (over the pH range 3–6.7). The curves shown in Fig. 1 illustrate 11 of the 40 curves obtained in a single overnight operation when retentions in 0.1 *M* ammonium acetate were investigated.

The retention curves obtained for eight of the analytes with acetate, succinate and phosphate buffers over the same pH range, are illustrated by Fig. 2a–c. The considerable variations seen in the retentive characteristics of these compounds is representative of the differences observed with this group of analytes.

This approach differs from available methods in that no attempt is made to predict optimal conditions for the separation of a particular mixture of components. The aim is instead to allow the chromatographer to graphically visualise potential separation systems by the superimposition of families of curves. The individual k' determinations are not precise but this does not affect the relationship between curves

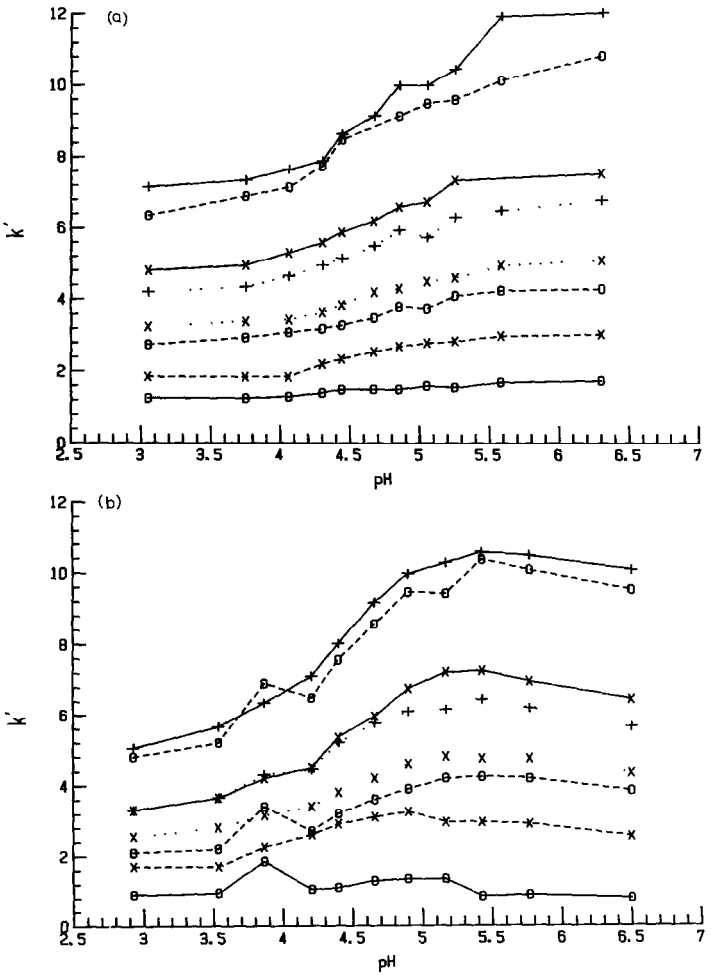

Fig. 2.

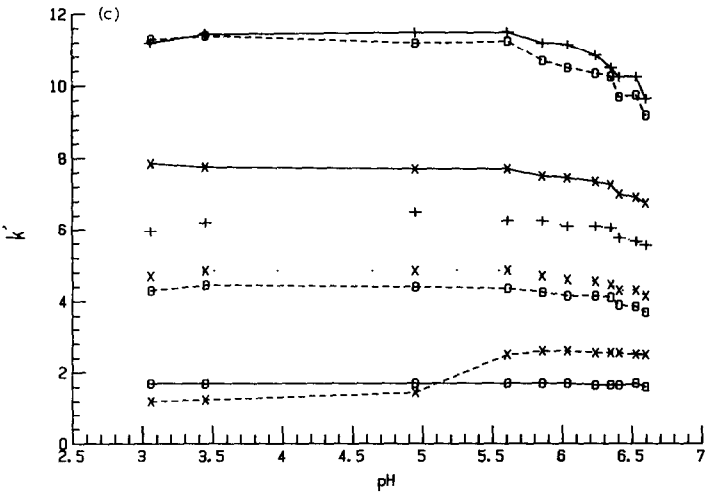

Fig. 2. k' *vs.* pH curves for purines and pyrimidines. Column: 20 × 2 mm I.D. Apex 3-μm ODS. Buffer systems: (a) 0.1 *M* ammonium acetate, (b) 0.1 *M* sodium succinate and (c) 0.1 *M* sodium phosphate. (+—+) 1-Methylguanosine, (O---O) 1-methylinosine, (×—×) thymidine, (+....+) 2'--deoxyguanosine, (× ...×) guanosine, (O---O) inosine, (×---×) adenine, O—O) uridine

as the errors incurred are consistent. An example of a chromatogram obtained with a 100 × 4.6 mm I.D. 3-μm Apex ODS column (Jones Chromatography) is shown in Fig. 3 together with a comparison of the k' values (relative to thymidine) for this separation and those obtained on a 20 × 2 mm I.D. column eighteen months earlier. The elution pattern produced on the small columns has been found to be predictive

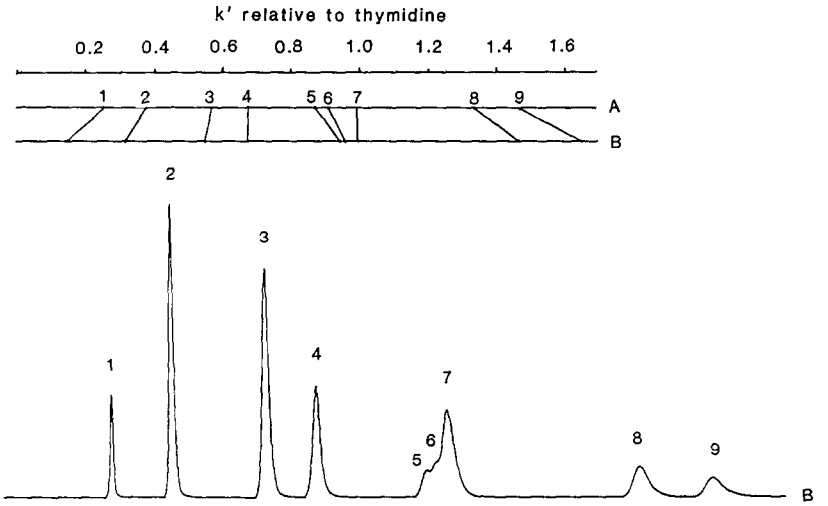

Fig. 3. Separation of standards on Apex 3 μm ODS columns. (1) Uridine, (2) 2'-deoxyuridine, (3) xanthosine, (4) inosine, (5) guanosine, (6) 2'-deoxyguanosine, (7) thymidine, (8) 1-methylinosine, (9) 1-methylguanosine. The lines represent the k' of the standards relative to thymidine run on (A) 20 × 2 mm I.D. and (B) 100 × 4.6 mm I.D. Apex 3-μm ODS columns running in 0.1 *M* ammonium acetate pH 3.3, thymidine having a k_1 of 10.93 on column A and 4.88 on column B.

for that seen on analytical columns, for a wide range of packings and eluents. The 1-methylguanosine peak (9, Fig. 3) shows considerable broadening as would be expected with a retention time of 1623 s; on the equivalent 20 × 2 mm I.D. column a retention of 66 s was recorded. As peaks with retentions of at least 200 s can be evaluated on the small columns the investigation of analytes whose retentive characteristics would prohibit similar studies using analytical columns, can readily be undertaken. This allows the maximum degree of flexibility, with respect to the analytical requirements, with the minimum of prior knowledge of the chromatographic behaviour of the analytes. Having accrued data for a large number of components it is possible to visually assess the suitability of the columns and eluents for a particular application by superimposition of families of curves on the computer screen. After selecting what appears to be an appropriate system, for the resolution of components of interest, it is then possible to interrogate the database to find any other analytes which, if present in the sample, would interfere if these conditions are used. The resolution of standards, diluted in the chosen eluent and sample matrix, may then be checked on an analytical column in order to identify any problems associated with interactions between components or the presence of interfering peaks. Should the resolution prove to be inadequate alternative conditions may be selected and additional information, relating to unknown peaks etc., entered into the database for future reference. This can be particularly useful for biological samples where the complexity of the sample matrix is a major problem which is not addressed by many of the alternative approaches.

The rapid optimisation of eluents for a specific application

The lifetime for an application may vary from days to years and this may be a significant parameter in the feasibility of developing a method for a particular estimation. The rapidity with which results can be obtained and interpreted is important for short term application and also for the rapid optimisation of new systems where several parameters may need to be varied. This is illustrated with the example below.

It was necessary to determine whether a nucleotide analogue (with unknown chromatographic properties) was metabolised to its natural congener during incubation in a crude enzyme assay. This required the resolution of the analogue, and

TABLE I

RAPID OPTIMISATION OF AN ION-EXCHANGE SEPARATION OF NUCLEOTIDES FOR A SHORT TERM APPLICATION

Operation	*Buffer A*	*Buffer B*	*Runs*	*Objective*
1	0.1 *M* sodium phosphate (pH 4.5)	2.0 *M* sodium phosphate (pH 4.5)	55	To define suitable buffer concentration *x* for operation 2
2	*x M* sodium phosphate (pH 3.0)	*x M* sodium phosphate (pH 6.5)	55	To define the optimal pH *y* for the separation
3	*x M* sodium phosphate (pH *y*) 0% acetonitrile	*x M* sodium phosphate (pH *y*) 10% acetonitrile	55	To improve the resolution of the analogue and product from the large ATP peak

potential product at micromolar concentrations in the presence of three other nucleotides, including a millimolar concentration of adenosine triphosphate (ATP).

A 3-μm Apex Amino packing (Jones Chromatography) was chosen and the three operations in Table I were carried out as described in the Experimental section. Where the five standards were repeatedly injected as the mobile phase was varied in 10% increments from 0 to 100% B.

The first two operations were successful, but the resolution was not adequate in the presence of the large amounts of ATP. By the selection of an appropriate concentration of acetonitrile from operation 3, it was possible to overcome this problem. An analytical separation fulfilling the objective was operational within two days of initiation of the study which was itself completed within five days.

The comparison of the performance of different columns

Data accumulated over an extended period to form a database can be used for the comparison of the retentive characteristics of column packing materials. This may be either for the comparison of different batches of packing material or comparison of different types of manufacturers' packings. If less efficient packings are to be investigated a larger column may be used, as illustrated in the example where a number of Machery-Nagel reversed-phase packings (Camlab, Cambridge, U.K.) were compared to μBondapak C_{18} (Millipore/Waters). A series of 50 × 4.6 mm I.D. columns were packed, and a number of analytes with a wide range of physico-chemical properties were individually injected under conditions which varied from 0–35% methanol. As each column was treated in the same manner, it was possible to compare the plate counts and capacity factors for all the analytes tested. The relative

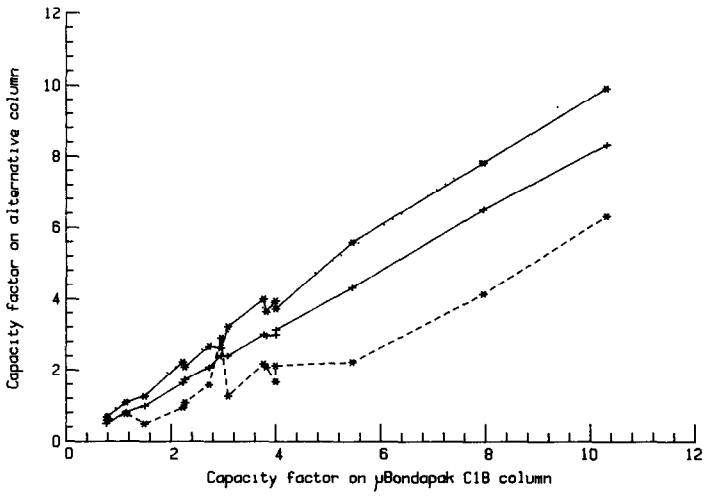

Fig. 4. Retention of purines and pyrimidines on Machery-Nagel columns relative to μBondapak C_{18}. (.....) k' μBondapak C_{18} *vs* k' μBondapak C_{18}, (*—*) k' Nucleosil 5 μm C_{18} *vs.* k' μBondapak C_{18}, (+—+) k' Polygosil 10 μm C_{18} *vs.* k' μBondapak C_{18}, (*---*) k' Polygosil 10 μm C_8 *vs.* k' μBondapak C_{18}. The analytes plotted sequentially are cytosine, uracil, 2'-deoxyuridine 5'-monophosphate, cytidine, inosine*, hypoxanthine, adenine*, uridine, thymine, 2'-deoxyguanosine*, 2'-deoxycytidine, thymidine*, 2'-deoxyuridine, adenosine*, 2'-deoxyadenosine*. The buffer used for all analytes contained 0.1 *M* sodium acetate pH 4.75. For those marked with an asterisk the buffer also contained 7% methanol.

TABLE II

COMPARISON OF A RANGE OF MACHERY-NAGEL PACKINGS TO μBONDAPAK C_{18}

Packing name	*Particle size*	*Shape*	*Carbon load (%)*	*Pore size (Å)*	*Plates/metre*
μBondapak C_{18}	10 μm	Irregular	10	125	11 745
Polygosil C_{18}	10 μm	Irregular	12	60	2 123
Polygosil C_{18}	10 μm	Irregular	–	100	3 355
Polygosil C_{18}	5 μm	Irregular	12	60	26 265
Polygosil C_{18}	5 μm	Iregular	–	100	17 970
Nucleosil C_{18}	4 μm	Spherical	14	100	28 064
Polygosil C_8	5 μm	Irregular	7	60	12 686

retentions of fifteen purines and pyrimidines on three Machery-Nagel columns, compared to a μBondapak column, are presented in Fig. 4. The use of 5-cm columns gave retentions in the range 32–240 s, thereby allowing the rapid evaluation of the column packings. The average plates per metre for the fifteen analytes in Fig. 4 (under these conditions, see legend) for some of the packings tested are shown in Table II.

CONCLUSIONS

This approach permits the rapid evaluation of a range of mobile phases or column packings for a particular separation ten to twenty times faster than the standard semi-empirical approach.

The basic simplicity of this approach confers a high degree of flexibility with the following advantages:

(i) The results may be accumulated into an ever-expanding library of information, suitable for predicting conditions for the resolution of many combinations of analytes.

(ii) It is not restricted to reversed-phase columns, and has been particularly useful for ion-exchange columns where methods development is exceptionally time-consuming, due to long equilibration times and lower efficiencies compared to reversed-phase columns.

(iii) Virtually any HPLC system with an autosampler and data system can be used.

(iv) The level of automation may range from minimal to complete.

(v) The range of each variable tested may be narrow or broad.

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