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Development of system suitability tests for ion-interaction chromatography of colorants on reversed-phase packing materials

S. Angelino[a], A.F. Fell[b], M.C. Gennaro[a,*]

[a]*Dipartimento di Chimica Analitica, Università di Torino, Via P. Giuria 5, 10125 Torino, Italy*
[b]*Pharmaceutical Analysis Research Unit, University of Bradford, Bradford, West Yorkshire BD7-1DP, UK*

Abstract

Ion-interaction reagent (IIR) chromatography has been established as a highly selective and sensitive method for studies on ionic solutes in environmental and food analysis. This technique makes use of a mobile phase containing an ion-interaction reagent (alkylammonium phosphate) which, flowing in isocratic conditions, induces a modification of the stationary phase surface. The formation of an electrical double-layer adsorbed onto the original RP packing material permits the separation of anionic and/or cationic species. As an essential requirement for transferring of IIR methods, it is necessary to devise suitable conditions for validation of the methods. The present work describes the development of system suitability tests (SSTs) based on a group of red dyes as a model system, where the influence of a number of parameters has been examined, including: organic modifier percentage, mobile phase pH, alkylamine chain length, IIR concentration, column temperature, flow-rate, sample composition. Some factors affecting sample stability have also been explored. To extend the results, two yellow dyes were added to the mixture, to provide a group of peaks characterized by critical resolution. Standardized conditions are proposed to assess the suitability of a new column packing material for adequate resolution of the proposed system. © 1998 Elsevier Science B.V.

Keywords: Ion-interaction chromatography; System suitability tests; Mobile phase composition; Dyes

1. Introduction

Ion-interaction reagent chromatography (IIR–HPLC) has been established as a highly selective, sensitive, flexible and low operational cost technique for studies on ionic or ionizable solutes in environmental and food analysis [1,2]. The methods developed generally make use of a RP-C_{18} stationary phase, with a mobile phase consisting of an aqueous or hydro-organic solution of an ion-interaction reagent (IIR) (e.g. an alkylammonium orthophosphate). The principle involves surface modification of the stationary phase, when the lipophilic chain of the IIR is adsorbed onto the ODS, giving rise to a primary positively-charged layer. The anion of the ion-interaction reagent (e.g. orthophosphate) is also bound to the ODS through electrostatic forces. The formation of an electrical double-layer adsorbed onto the original RP packing material permits the retention of anionic and/or cationic species. The anionic species can be organic or inorganic, whereas the cationic species are usually organic.

For transfer of an IIR–HPLC method between laboratories it is an essential requirement to devise suitable conditions for validation of the method, when different column materials, equipments and conditions may be used.

The present work describes the development of a system suitability test (SST) for IIR–HPLC methods

*Corresponding author.

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in general, using a group of red dyes (Table 1) as a model system [3].

The influence of a number of parameters on the IIR–HPLC method has been examined, including: percentage of organic modifier, IIR alkylamine concentration and chain length, mobile phase pH, column temperature, flow-rate and sample solution composition. Some factors affecting sample stability have also been explored for this particular model system. The data have been extended by a limited study on some yellow dyes (Table 1).

2. Experimental

2.1. Apparatus

Chromatographic analysis was carried out with a Hewlett-Packard 1090 Series II liquid chromatograph (Waldbronn Analytical Div., Germany), equipped with a UV–Vis photodiode array detector.

A Corning Delta 240 pH-meter equipped with a combined glass–calomel electrode was employed for pH measurements. A Jones chromatography column Chiller (model 7950) for temperature control was employed for studies on the dependence of the separation on column temperature.

2.2. Chemicals and reagents

Double-distilled water, obtained from a 4 l/min Fistreem Still, after pretreatment with a Fistreem Deioniser (Fisons, UK) was used for the preparation of all solutions.

Gradient-grade acetonitrile and Hypersolve grade orthophosphoric acid were used as received from Merck. Alkylamines (hexyl-, heptyl-, octyl-) were obtained as analytical-grade reagents from Fluka and the dyes (amaranth, new coccine, chromotrope, tartrazine, sunset yellow) were used as received from Aldrich chemicals.

2.3. Chromatographic conditions

An ODS-μBondapack (Waters, Milford, MA, USA) column (30 cm×0.39 cm I.D.), comprising end-capped, irregularly shaped 10-μm particles was used to develop the SST for the analysis of food dyes. The performance of an ODS-2 Spherisorb column (25 cm×0.46 cm I.D.), (Phase Separations, Deeside, UK) packed with end-capped, spherically-shaped, 5-μm material was tested under the SST conditions developed.

The mobile phase was prepared by mixing the water–organic modifier (ACN) mixture in proportions ranging from 70:30 to 80:20 and then adding the amount of alkylamine required to obtain the desired concentration between 2.5 m*M* and 7.5 m*M*. Finally the appropriate volume of orthophosphoric acid was added to obtain the desired pH. This pH in the aqueous–organic solution is reported as an 'operational pH' value [4].

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline was obtained. About 1 h, at a flow-rate of 1.0 ml/min, was necessary to fully condition the system. This procedure was followed for every new mobile phase used. The column temperature was kept constant at 25°C by the column chiller. The flow-rate was set at 1.0 ml/min, except when studying the effect of the variation of each factor on the separation. Spectrophotometric detection by photodiode array (190–600 nm) was employed at appropriate wavelengths.

3. Results and discussion

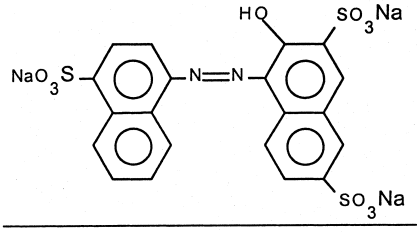
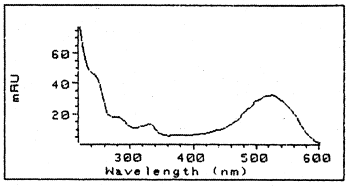
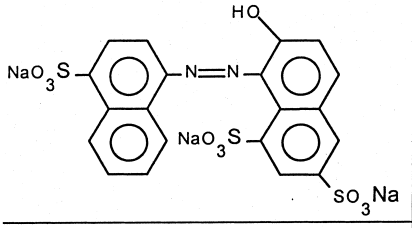
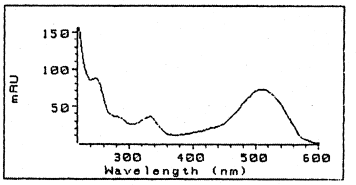
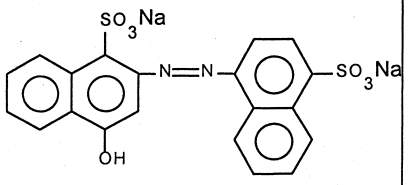
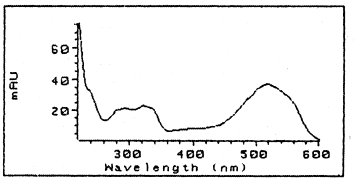
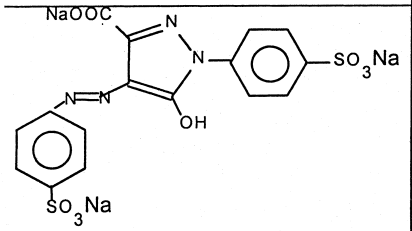
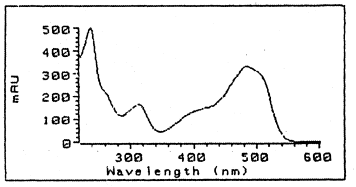
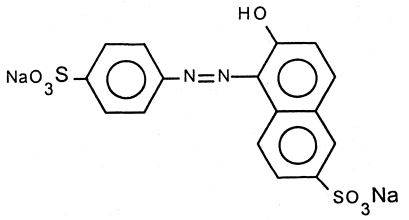
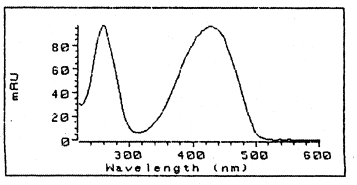
The retention time of analytes in ion-interaction reagent chromatography depends on a variety of parameters and these are usually interdependent. In the present paper a systematic study of the effects of the variation of each factor has led to establishing a set of optimized conditions for a system suitability test (SST) for a typical IIR–HPLC method.

These SST conditions were then used to test the performance of a similar commercial RP stationary phase, the Phase Separation ODS-2 column, in order to assess and validate its suitability for use in this IIR–HPLC method.

3.1. Organic modifier concentration

The concentration of organic modifier, in this case ACN, greatly affects the retention times of the three solutes and therefore the resolution R_s. In the range

Table 1
Molecular structures, common names, Colour Index (CI) and European Community (EC) numbers and diode array spectrum of the three red and two yellow food dyes considered

Molecular structure	*Common Name CI & EC numbers*	*Diode Array Spectrum (λ: 190-600 nm)*
	**Amaranth** **CI 16185** **E 123**	
	**New Coccine** **CI 16255** **E 124**	
	**Chromotrope** **CI 14720** **E 122**	
	**Sunset Yellow** **CI 15985** **E 110**	
	**Tartrazine** **CI 19140** **E 102**	

of ACN concentrations studied, (from 20% to 30%, v/v), the dependence of log k' on ACN content was linear and inversely proportional in the usual way.

With the IIR (octylamine) concentration at 5.0 m*M*, and the mobile phase at pH 6, an optimum resolution R_s was observed at ACN 27% (v/v), with a total analysis time of 8 min for the three red dyes. In order to resolve two typical yellow dyes, such as tartrazine and sunset yellow, the optimum ACN percentage with the same IIR concentration was 25% (v/v), giving a total analysis time of 28 min.

3.2. Ion-interaction reagent concentration

The IIR concentration, which is regarded as correlating with the extent of surface modification, was examined over the range 2.5–7.5 m*M*. Within this range, the larger the amount of octylamine, the higher the number of active sites available to ion-interact with the analytes. This resulted in longer retention times, as shown in Fig. 1. From the chromatograms reported it is possible to note that the IIR concentration also somehow influences the sensitivity, which is much lower at the highest IIR concentration examined. As noted above, with 27% of ACN, a concentration of 5.0 m*M* was found to be the optimal combination for resolution of the red dyes, together with adequate sensitivity.

To confirm that IIR retention mechanisms are really taking place, an experiment was performed with the same mobile phase composition, viz. ACN–water, (27:63, v/v, pH 6.0), but in the absence of octylamine. Under these conditions the dyes could not be separated and coeluted at the solvent front, as confirmed by diode array spectra.

3.3. IIR alkyl chain length

In principle, the longer the alkyl-chain of the amine, the higher is its adsorption capability onto the RP-C_{18}, resulting in a stronger retention of analytes. Three alkylamines, at the same 5.0 m*M* concentration (the others conditions being the same as in Fig. 1), were tested, with chain lengths C_6, C_7, C_8; the C_8 is the most widely used since, as shown in previous work, it is the most versatile as concerns R_s and total analysis time. The analyses performed by using heptylamine and octylamine led to similar t_R, R_s and N values, so that in this case their use can be considered equivalent. The use of hexylamine, on the contrary, led to much shorter t_R values and the separation of the dyes could not be achieved, so octylamine was selected for further work.

3.4. Mobile phase pH

The pH value strongly influences k' and R_s. Thus, at pH 4.0 the total analysis time was >80 min, whereas at pH 8.0 it was <4 min. Fig. 2 reports the chromatograms obtained at three different pH values and the plots of t_R vs pH. Over the pH range allowed by silica-based stationary phases (pH 3.0–8.0), neither the extent of ionisation of IIR protonated octylamine (pK_a 10.6), nor that of anionic sulphonate dyes would be expected to vary greatly. The t_R variations may be attributable to different concentrations of the phosphate anions able to compete with the dyes for the positively charged sites on the stationary phase to form octylammonium ion-pairs of different stoichiometry and different stability, or may be attributable to different electrical double layer potentials. Both these factors were studied and reported in earlier work [5].

Moreover, the relationship between pH and column efficiency (expressed as the number of theoretical plates, N) illustrated by the plot of N vs. pH (Fig. 3), shows the lowest value of N at a pH of 6.7–7.0 for two of the dyes (amaranth and new coccine). However it doesn't show any effect on chromotrope. The chromatograms reported in Fig. 2 show that the detection sensitivity of all three dyes is affected over the same range of pH.

For the present work pH 6.0 was chosen, giving both good resolution and efficiency, corresponding to small plateau and reasonable total analysis time.

3.5. Column temperature

Temperature can influence both the surface modification equilibria and the retention equilibria of the analytes. Thus to study the role of temperature on the IIR–HPLC separation, experiments were carried out in the range between 5°C and 45°C.

The dependence of retention on temperature is given by:

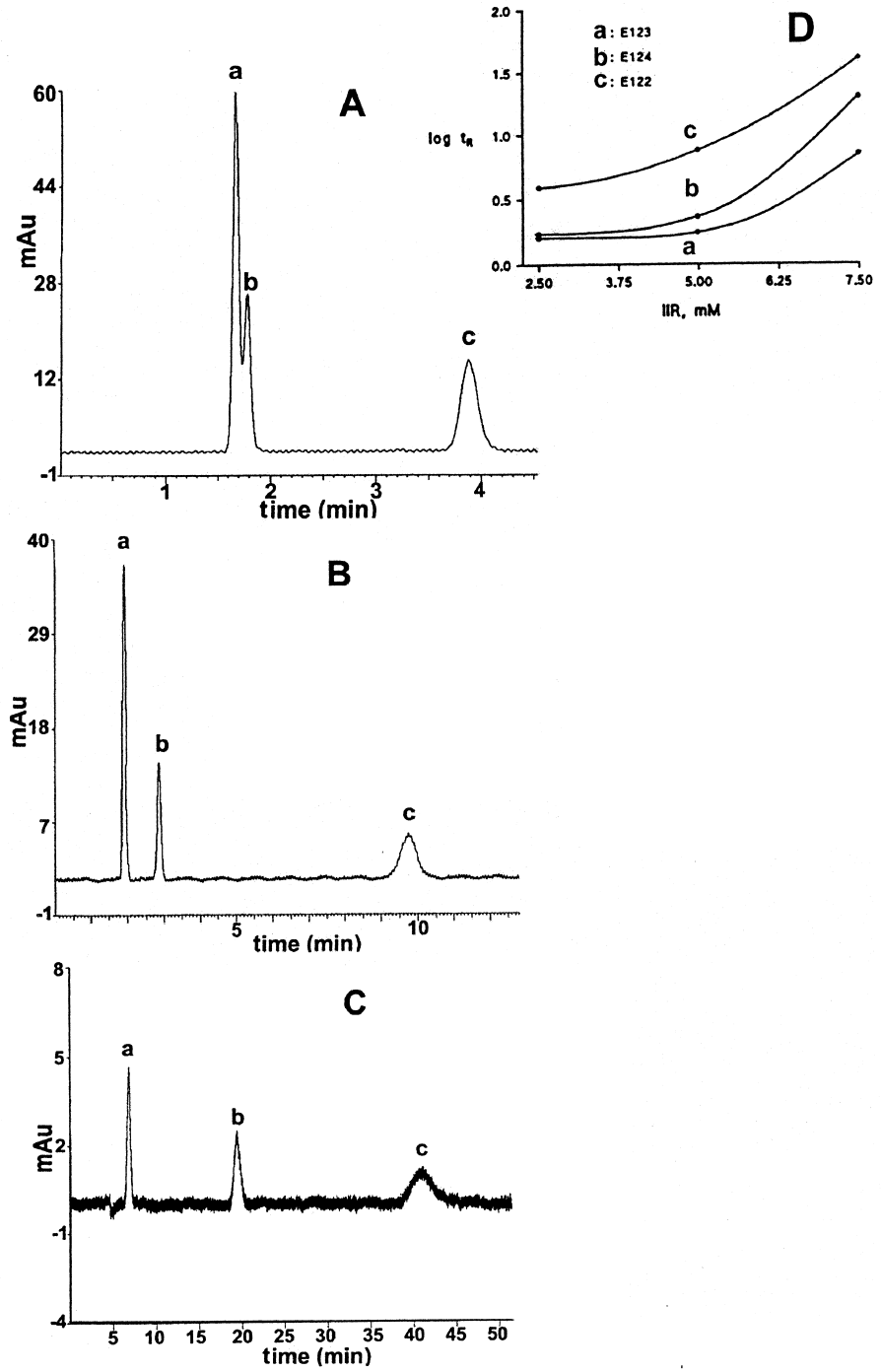


Fig. 1. Elution of the red dye mixture performed with three different concentrations of IIR. Stationary phase: Waters ODS μBondapack, 30×0.39 cm I.D., 10 μm, end capped. Detector: photodiode array, λ range: 190–600 nm. Mobile phase: (A) 2.5 m*M*, (B) 5.0 m*M*, (C) 7.5 m*M* octylammonium phosphate in water–ACN (63:27, v/v), pH=6.0, flow-rate: 1.0 ml/min. Peak identification: *a*=E123, *b*=E124, *c*=E122, 100 μg/l each. (D) Plots correlating log t_R of the three red dyes to the ion-interaction reagent concentration.

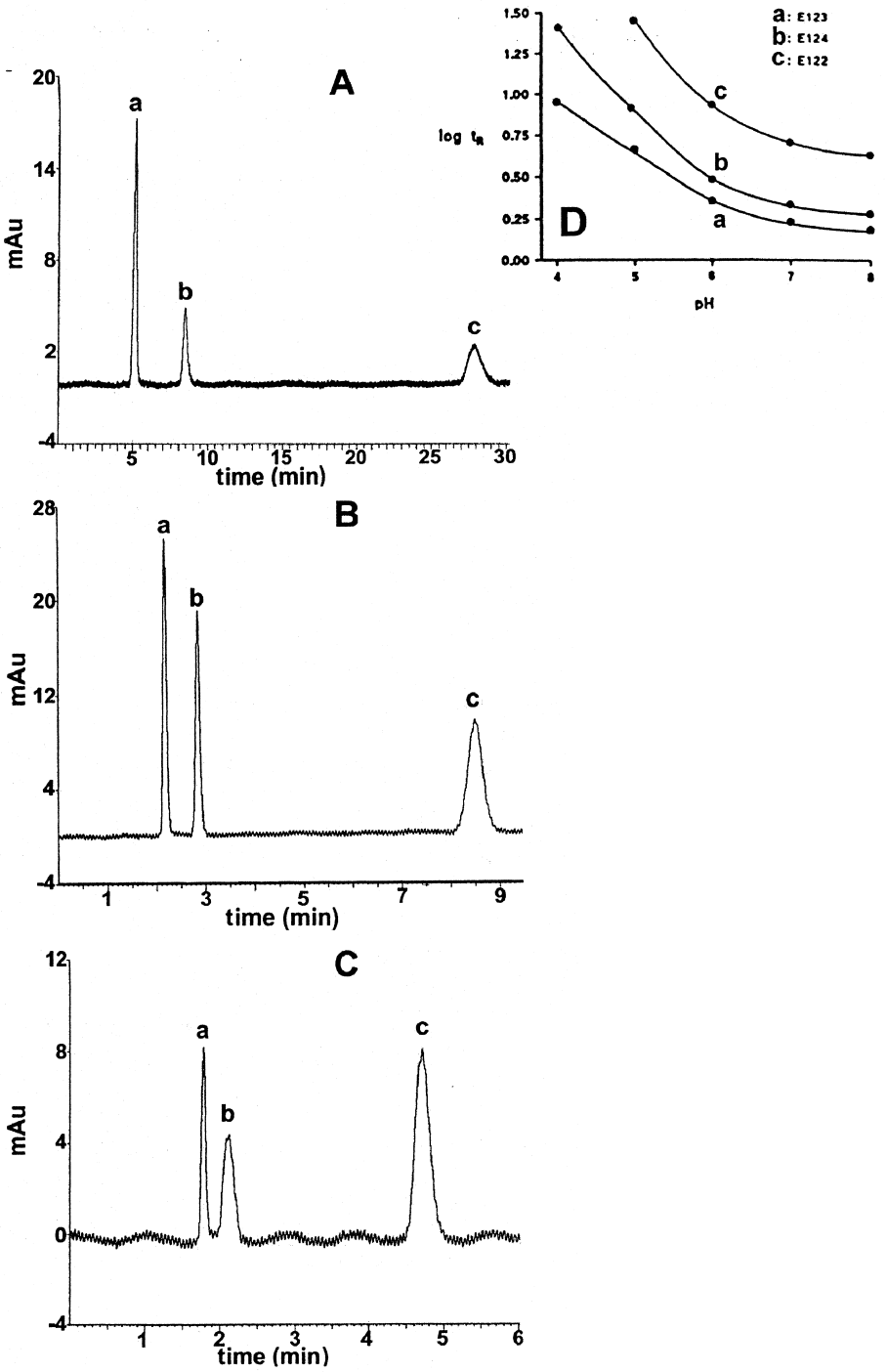


Fig. 2. Elution of the red dye mixture performed at three different pH values. Stationary phase: Waters ODS μBondapack, 30×0.39 cm I.D., 10 μm, end capped. Detector: photodiode array, λ range: 190–600 nm. Mobile phase: 5.0 m*M* octylammonium phosphate in water–ACN (63:27 v/v), flow-rate: 1.0 ml/min. Mobile phase pH: (A) 5.0, (B) 6.0, (C) 7.0. Peak identification: a=E123, b=E124, c=E122, 100 μg/l each. (D) Plots correlating log t_R of the three red dyes to the mobile phase pH.

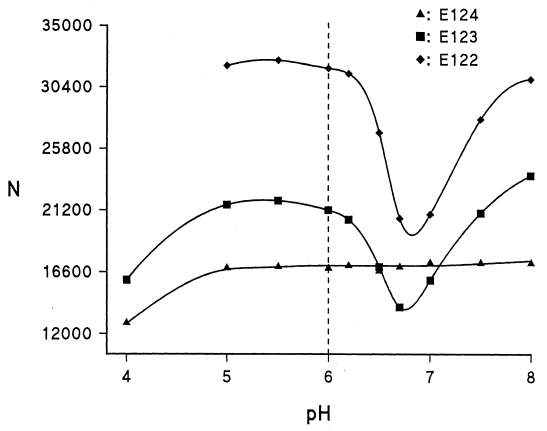


Fig. 3. Plots of N (number of theoretical plates) for Microbond column vs pH of the mobile phase.

$$\ln k' = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \Phi$$

where k' is the capacity factor of the analyte, ΔH° and ΔS° are respectively, the enthalpy and entropy of transfer of the analyte from the mobile phase to the stationary phase, R is the gas constant, T is the absolute temperature and Φ is the phase ratio of the chromatographic column (i.e. the volume of the stationary phase divided by that of the mobile phase); in this case, Φ was 0.625 [6]. This equation leads to the Van't Hoff plots reported in Fig. 4, showing as expected an increase of log k' against reciprocal T. The linearity of the Van't Hoff plots indicates that over this range of temperature the retention mechanism does not change [7]. The calculated ΔH° and ΔS° values are reported in Fig. 4.

The column efficiency was also slightly affected by temperature, the number of theoretical plates being higher as T increased. This result may be ascribed to a faster exchange of analytes between stationary and mobile phases taking place as the temperature increases, resulting in a higher N. The best temperature range for the resolution of the dyes lies between 25 and 30°C.

3.6. Mobile phase flow-rate

A series of experiments were performed for flow-rates (f_v) between 0.2 and 2.0 ml/min, keeping the other factors (IIR concentration 5.0 mM, ACN percentage 25%, pH=6.0, T=25°C) constant. In order to achieve more information on the influence of f_v on the resolution of analytes characterized by very close retention times, yellow tartrazine and sunset yellow were also added to the model system. It was shown that f_v significantly influenced the

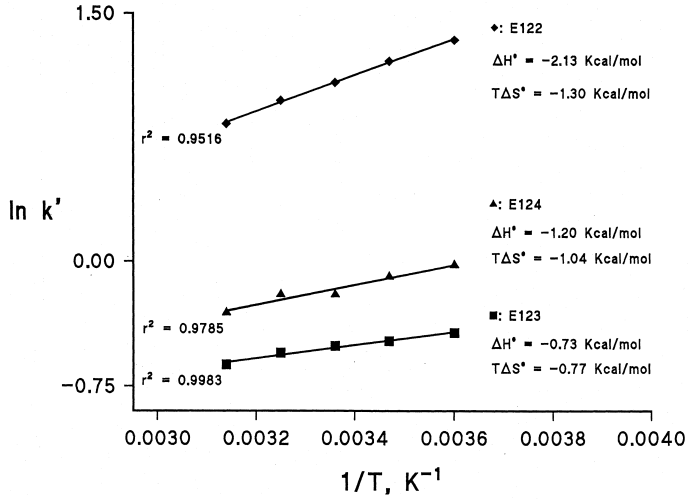


Fig. 4. Van't Hoff plots of log k' vs $1/T$, obtained for the three red dyes on the μBondapack column: r^2=correlation coefficient, ΔH°=enthalpy, ΔS°=entropy, T=absolute temperature.

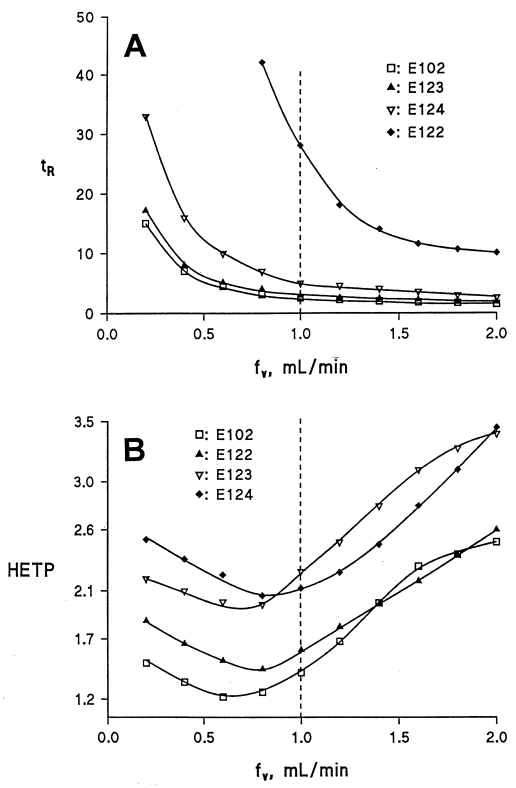


Fig. 5. Plots correlating the mobile phase flow-rate (f_v) to: (A) retention times (t_R, min) of the analytes; and to (B) HETP.

retention times of the analytes (Fig. 5A), although R_s was always satisfactory (≥ 1).

As regards HETP the minimum value for each analyte, on the Van Deemter curves was found to occur at slightly different values of the flow-rate. Fig. 5B reports the plot of HETP vs f_v. On average the best value of f_v is between 1.0 and 1.2 ml/min for balance between adequate t_R and sufficient N.

3.7. Sample solution composition

This factor was shown to be of primary importance for the satisfactory separations of dyes.

A series of experiments showed that it was essential for the dyes to be dissolved in the same mobile phase used for their elution, since any difference in the water–ACN ratio and in the pH value led to badly shaped peaks, variable t_R values and to the appearance of artefact peaks.

Moreover, the sample solution had to be stored in a tightly closed container, to avoid any contact with atmosphere. If this was not observed, after a few hours, some of the dyes gave rise to peaks characterized by the same spectrum of the pure analyte, but at different t_R values, probably due to the formation of oxidation products.

4. System suitability test conditions

4.1. Microbondapack column

The following conditions represent the preliminary conditions for a system suitability test for these model solutes in IIR–HPLC, namely: 5.0 m*M* octylamine aqueous solution–ACN (73:27, v/v), adjusted to pH 6.0 by orthophosphoric acid, $f_v = 1$ ml/min, $T = 25$°C. In order to extend this work further, two yellow dyes (Sunset Yellow and Tartrazine) were added, to provide a group of peaks with critical resolution. Since factors like ACN and IIR concentrations have opposing effects on the t_R (t_R increases when ACN decreases and when IIR concentration increases), it would be certainly possible to find other conditions leading to equivalent results. For the sample containing the two yellow colorants, in order to separate these dyes from the other three, the required ACN percentage was 25% (v/v) (Fig. 6a). The optimum flow-rate was 1.0 ml/min, and the column temperature was maintained constant at 25°C.

As noted above, in order to equilibrate the stationary phase surface modification, the mobile phase must be allowed to flow for at least 1 h before injection and the sample must be prepared in the same mobile phase and stored in closed vials.

4.2. Phase separation column

A Phase Separation column (25 cm×0.46 cm I.D., with ODS end-capped material packing, 5 μm of size and spherical shape) has also been tested under the conditions reported above, in order to assess its suitability in resolving the red and yellow dyes by IIR–HPLC. The results obtained show that this

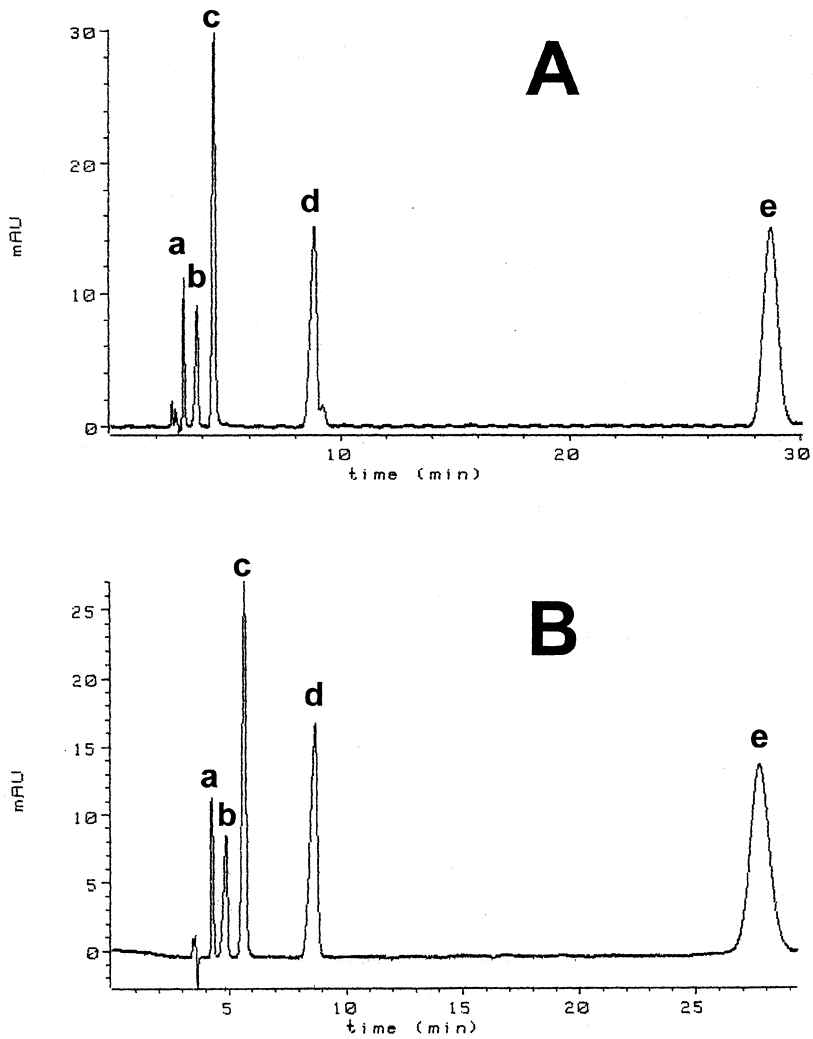


Fig. 6. Separation of five food dyes under the optimized SST conditions. (A) Stationary phase: μBondapack ODS, 30 cm×0.39 cm, end-capped, irregular 10-μm particles. Mobile phase: 5.0 m*M* octylammonium phosphate in water–ACN (65:25 v/v), pH=6.0, flow-rate: 1.0 ml/min; column temperature 25°C. Detector: photodiode array, 190–600 nm. (B) Stationary phase: ODS-2 Spherisorb, 25 cm×0.46 cm, end-capped, spherical 5-μm particles. Mobile phase: 5.0 m*M* octylammonium phosphate in water–ACN (66:24, v/v), pH=6.0, flow-rate: 1.0 ml/min; column temperature 25°C. Peak identification: (a) Amaranth, (b) Tartrazine, (c) Sunset yellow, (d) New Coccine, (e) Chromotrope, 100 μg/l each.

stationary phase is less retentive than the μBondapack, leading to lower k' values. Nevertheless the separation obtained within a total analysis time of 5 min was still satisfactory.

On the other hand it was not possible to resolve the mixture comprising the additional two yellow dyes, even with a reduced ACN percentage of 25%. The separation would appear to need further optimization. In this case, it was found that reducing the ACN percentage to 24% was sufficient to obtain the separation of all five analytes (Fig. 6B). In Fig. 7, the relationship between log t_R and ACN (%) illustrates the method for identifying the concentration of ACN required for comparable t_R on the new column.

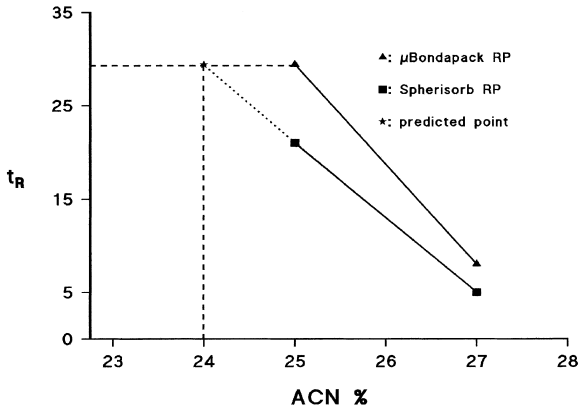


Fig. 7. Method used to find the ACN% for obtaining comparable retention times when transferring the method from a μBondapack RP to a Spherisorb RP column.

5. Conclusions

The IIR–HPLC method, which can offer very good performance in separation, clearly requires careful control of the delicate equilibria for its successful application. Thus it is good practice to accurately measure the volume of organic modifier and of ion-interaction reagent when preparing the mobile phase. It is also necessary to stabilize the operating temperature and the flow-rate as well as to carefully control the mobile phase pH, which exerts a dominant effect on the overall separation process. It is essential to equilibrate the column as noted above.

With the SST standardized conditions proposed above, it should be possible to assess the suitability of a new column packing material for adequate resolution of the proposed model system. However, if a different packing material were substituted, then it is helpful to fine-tune the local optimum conditions before the method can be satisfactorily transferred to this new column. Thus it is essential, for successful applications of the IIR–HPLC method on an international basis, to establish satisfactory SSTs for each particular application.

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