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INDIRECT UV DETECTION OF NON-ABSORBING SOLUTES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH THE HELP OF UV-ACTIVE, NON-IONIC MOBILE PHASE CONSTITUENTS

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SUMMARY

UV-absorbing, non-ionic benzene derivatives of different polarity, from benzene to benzyl alcohol, were added in concentrations ranging from 5 μmol/l to 5 mmol/l to aqueous methanol eluents in a reversed-phase high-performance liquid chromatography system. Non-ionic solutes without UV chromophores, covering a wide range of polarity, were separated and could be detected by a constant-wavelength UV detector with the help of induced peaks reflecting solute-induced changes in the mobile phase concentration of the detection agent. Relative response factors depend on the relative retention of the solute and the detection agent and the relative polarity of the functional group of the solute and the detection agent.

INTRODUCTION

Pettersson and co-workers[1,2] have shown that hydrophilic UV-active ions dissolved at a constant concentration in moderately polar organic non-aqueous eluents could be used in conjunction with hydrophilic stationary phases to separate and detect non-UV-absorbing ionic solutes indirectly with a UV photometer.

Schill and co-workers[3–5] demonstrated the succesful application of this indirect detection principle in reversed-phase ion-pair chromatography (RP IPC) using both cationic and anionic UV-active ion-pair reagents (and their combinations) as detection agents dissolved at constant concentrations in hydro-organic eluents. Although at first[3] only ionic solutes were separated and indirectly detected in this RP IPC system, it was soon demonstrated[4] that non-ionic solutes also caused induced peaks by which they could be detected and quantified. It was concluded that, depending on the respective charges and relative retentions of the solute and the detection agent, a positive or a negative induced peak of the detection agent coeluted with the solute, followed (or preceded) by an oppositely directed "system" peak of equal area of the characteristic retention volume of the ionic detection agent. The area of the induced peak depended on the amount of solute injected, the structure of both the solute and the detection agent, the stationary phase, the composition of the mobile phase (meth-

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anol concentration) and, above all, the relative retentions of the solute and detection agent. It was found that the ratio of peak area to amount of solute injected followed a maximum curve with its extreme being at $k'_{solute} \approx k'_{reagent}$.

Gnanasambandan and Freiser[6,7] used methylene blue as the ionic detection agent in an RP IPC system for the detection of non-ionic, non-UV-absorbing solutes. They claimed that detection and increased retention of non-ionic alcohols in the presence of the ionic methylene blue detection agent were facilitated by the formation of methylene blue–alcohol complexes.

(In our opinion, the retention of alcohols in the presence of methylene blue ions is not truly larger than in the absence of methylene blue. Rather, the exceedingly high solute concentrations used with the refractive index detector in the absence of methylene blue lead to decreased retention. Thus, solutes injected in low concentrations in the presence of methylene blue appear "more retained". In fact, ion-pairing regents examined so far *decrease* the retention of uncharged alcohols[8].)

Stranahan and Deming[9] developed a computer simulation method to explain the appearance, in an RP IPC system, of these induced peaks. The model does not rely on any particular retention mechanism; it assumes solely that just as the retention of any solute depends on the concentration of the pairing ion (detection agent), the retention of the pairing ion depends on the concentration of the solute. In other words, the injection of any solute will disturb the actual steady-state distribution of the pairing ion in the injection zone and these disturbances travel through the column, leading to an induced peak that coelutes with the solute peak and an oppositely directed peak called the system peak. There are six basic peak patterns possible, depending on the relative retention of the solute and the pairing ion, on the one hand, and on the ability of the solute to increase or decrease, by its presence, the distribution coefficient of the ion-pairing reagent, on the other.

The starting point of the work reported here was the assumption that indirect detection (by any means) is feasible in any chromatographic system if a multi-component eluent is used and if the injection of a solute can disturb the steady-state distribution of the mobile phase constituents to such an extent that it becomes detectable as a local change in the concentration of (at least one of) the eluent constituents.

Recently, a paper by Parkin[10] appeared, demonstrating the use of uncharged *p*-aminobenzoate ester and benzamide in aqueous acetonitrile eluents for the detection of alcohols and esters. No correlation was sought, however, between response and the structures of the solutes and detection agent.

EXPERIMENTAL

Apparatus

A liquid chromatograph was assembled from commercially available components[11] that allowed for the recording of both breakthrough curves (for the determination of adsorption isotherms) and chromatograms (to obtain retention data). For some of the measurements a Type LC 4020 constant-wavelength UV detector (Varian Aerograph, Walnut Creek, CA, U.S.A.) was replaced with a Type HP-1040 diode-array detector (Hewlett-Packard, Avondale, PA, U.S.A.). Separations were carried out on a 250 × 4.6 mm I.D. stainless-steel column (Chrompack, Middelburg,

The Netherlands), packed with 10 μm RP-18 reversed-phase material (E. Merck, Darmstadt, F.R.G.). Aqueous methanol eluents [0–30% (v/v) methanol] containing UV-active detection agents in concentrations ranging from 5 μ*M* to 5 m*M* were used. The eluents were filtered through a GF/D glass-fibre filter (Whatman, Clifton, NJ, U.S.A.) and degassed ultrasonically. The eluent temperature and flow-rate were 25.0 ± 0.2°C and 2.0 ml/min, respectively. The dead volume was determined by injections of ^2H_2O.

First, the columns were equilibrated with detection agent-free eluents. The eluents were then changed in a stepwise manner and the breakthrough curve of the particular detection agent was recorded[11]. Once steady-state conditions had been established, the test solutes were injected and their retention volumes were determined. Once measurements with a certain detection agent were finished, the column was programmed linearly to 100% (v/v) methanol, washed with 100 ml of methanol, reprogrammed linearly to the detection agent-free eluent composition and flushed with 100 ml of this eluent.

Standard solutes were repeatedly injected to check whether the original conditions were re-established.

Chemicals

Benzyl alcohol, benzaldehyde, benzonitrile, nitrobenzene and benzene were reagent-grade chemicals obtained from Reanal (Budapest, Hungary) and were used without further purification. Their purity was analysed by capillary gas chromatography (HP-5880A, Hewlett-Packard) using glass capillaries with gamma-irradiation-immobilized OV-1 stationary phases[12]. Benzyl alcohol was found to contain 0.4% (v/v) of benzaldehyde as an impurity. The solutes were reagent-grade chemicals, obtained from Reanal.

RESULTS AND DISCUSSION

Demonstration of the possibility of indirect UV detection with non-ionic detection agents

In order to demonstrate the feasibility of indirect UV detection in RP systems with non-ionic detection agents, benzyl alcohol was selected as a non-ionic, UV-active detection agent because it was believed to be able to interact with both non-polar and polar solutes, and with the polar and non-polar parts of the RP-18 stationary phase. Benzyl alcohol was added at a concentration of 0.5 m*M* to the 10% (v/v) aqueous methanol eluent. The breakthrough curve was recorded. It contained two steps, as expected, the second being caused by the 0.4% (v/v) benzaldehyde contamination of benzyl alcohol [the absorbances at 254 nm of benzyl alcohol and the 0.4% (v/v) of benzaldehyde were almost equal]. After equilibration, non-UV-active primary alcohols (methanol–*n*-pentanol) were injected (0.3 μl each) and the chromatogram shown in Fig. 1 was recorded. Primary alcohols were selected as solutes for this first test because their interactions with the stationary phase were expected to be similar to those of benzyl alcohol, increasing the chances of a disturbance in the local distribution (in the injection zone) of benzyl alcohol.

It can be seen that the induced peaks that elute at the retention volume of methanol, ethanol, propanol and *n*-butanol are all positive, and although equal

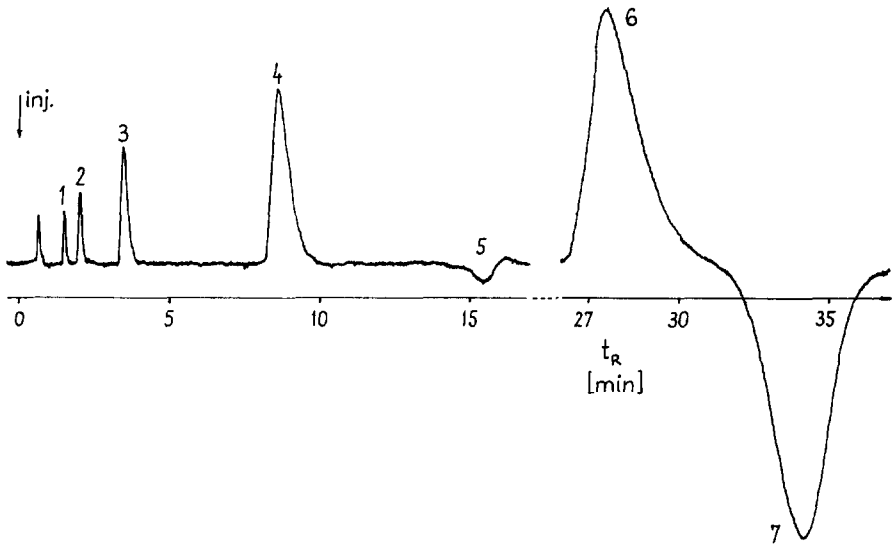

Fig. 1. Chromatogram of *n*-alkanols in 10% (v/v) aqueous methanol eluent containing 0.5 m*M* benzyl alcohol. 1, Methanol; 2, ethanol; 3, propanol; 4, *n*-butanol; 5, benzyl alcohol (system peak); 6, *n*-pentanol; 7, benzaldehyde (system peak).

amounts of alcohols were injected, the peak areas increase with increasing retention. *n*-Butanol is followed by a small negative peak, which elutes at the retention volume of benzyl alcohol, the UV-active detection agent. Benzyl alcohol is followed by a large positive peak at the retention volume of *n*-pentanol, then again at the retention volume of benzaldehyde by a large negative peak. The benzaldehyde system peak is much larger than the benzyl alcohol system peak. Similar chromatograms were obtained with methyl *n*-alkyl ketones and other non-ionic solutes.

These experiments demonstrated that non-ionic, non-UV-active solutes could be detected when the eluent contained a non-ionic, UV-active constituent and also that the solutes disturbed the steady-state distribution in the injection zone of both benzyl alcohol and benzaldehyde. In fact, the eluted solute peaks contained the solutes proper (*n*-alkanols), benzyl alcohol and benzaldehyde, as indicated by the composite UV spectra of benzyl alcohol and benzaldehyde recorded (on-the-fly) at the peak apex with the HP-1040A diode-array detector. Hence it seemed worthwile to examine other UV-active, non-ionic reagents and other solute types to see if there was a correlation between response, solute polarity and reagent polarity.

Various benzene derivatives as non-ionic indirect UV detection agents

Next, benzene, nitrobenzene, benzonitrile and benzaldehyde were used successively as detection agents in eluents of varying methanol concentration. The detection agent concentrations, the methanol concentrations of the eluents and the apparent molar absorptivities of the agents at 254 nm as determined with the HP 1040A diode-array detector are shown in Table I. The detection agents, suitably diluted, were injected successively into the reversed-phase column, in 80% (v/v) methanol eluents, and their full UV spectra were recorded (on-the-fly) at the peak apex, from 230 to 350 nm, at 4 nm resolution. Peak concentrations were calculated by the expression[13]

TABLE I

ELUENT CONCENTRATIONS OF METHANOL AND THE DETECTION AGENTS AND THEIR APPARENT MOLAR ABSORPTIVITIES AT 254 nm (4 nm BAND WIDTH)

Detection agent	*Methanol concentration in eluent (%, v/v)*	*Detection agent concentration in eluent (mM)*	*Apparent molar absorptivity 254 nm (l/mol · cm)*
Benzene	30	5.0	75
Nitrobenzene	20	0.125	760
Benzonitrile	20	1.0	418
Benzaldehyde	20	0.005	7780

$$C_{max} = \frac{q_i}{A\varepsilon v\sigma\sqrt{2\pi}}$$

where

q_i = the amount of material injected;
A = cross-sectional area of the column;
ε = void volume fraction;
v = linear velocity of the eluent;
σ = standard deviation of the eluted peak.

The methanol concentrations of the eluents were selected in such a manner that reasonable retentions were obtained for most of the test solutes ranging from non-polar chloroform to polar *n*-propanol. The detection agent concentrations were selected to obtain eluents with approximately equal absorbances.

The retention data and response factors of the various test solutes obtained with various detection agents are listed in Table II. Response factors normalized to an 0.08 a.u.f.s. detector setting are expressed as peak area per amount injected ($mm^2/\mu mol$).

With benzene as detection agent in a 30% (v/v) methanol eluent, very small induced peaks were obtained. Owing to the low precision of these determinations, these values are not included in Table II.

Alcohols and the chloroalkane solutes do not absorb light at 254 nm, so the response factors could be readily calculated from the area of the induced coeluting peaks. However, the other test solutes do absorb some light at 254 nm. Therefore, their response factors were calculated from the area of the system peak generated by individual injections of each solute. Some of the chromatograms are shown in Figs. 2–4.

It can be seen from Table II that the response factors depend both on the relative retentions of the solute and detection agent and on the structures (relative polarities) of the solute and detection agent. For any combination of detection agent and solute class the response factor increases as the k' of solute approaches the k' of the detection agent. For example, for the benzaldehyde detection agent, $k'_{benzaldehyde} = 11.9$ and the response factor of the alcohols increases from 6 to 522 while their k' increases from 1.0 to 10.7.

TABLE II

RESPONSE FACTORS AND RETENTION FACTORS FOR VARIOUS TEST SOLUTES AND DETECTION AGENTS

Retention factors of the detection agents in the actual eluents are also shown

Solute	*Non-ionic detection agent*					
	Benzaldehyde (5 μM; k' = 11.9)		*Benzonitrile (1 mM; k' = 16.3)*		*Nitrobenzene (0.125 mM, k' = 18.6)*	
	k'_{solute}	*Response factor*	k'_{solute}	*Response factor*	k'_{solute}	*Response factor*
Alcohols						
n-Propanol	1.0	6	1.2	23	1.0	19
n-Butanol	3.3	21	3.9	114	3.2	72
n-Pentanol	10.7	522	13.4	1300	10.8	530
Nitrile						
Butyronitrile	1.7	4	2.0	27	1.8	28
Aldehyde						
Butyraldehyde	3.4	13	3.9	54	3.4	50
Ketones						
Methylethylketone	1.8	11	2.1	38	1.8	18
Methylpropylketone	5.4	66	6.2	119	5.2	83
Nitro compound						
Nitrobutane	9.0	25	10.5	33	9.2	23
Chloroalkanes						
Dichloromethane	3.4	2	3.9	2	3.3	10
Chloroform	11.0	59	12.2	19	11.2	111

For a given detection agent and an approximately identical solute retention factor, the response factor increases with increasing solute polarity. For the benzaldehyde detection agent ($k'_{benzaldehyde}$ = 11.9) the retention factors of dichloromethane (k' = 3.4), butyraldehyde (k' = 3.35) and *n*-butanol (k' = 3.3) are approximately equal, yet the response factors (*RF*) increase in the order $RF_{CH_2Cl_2} \ll$

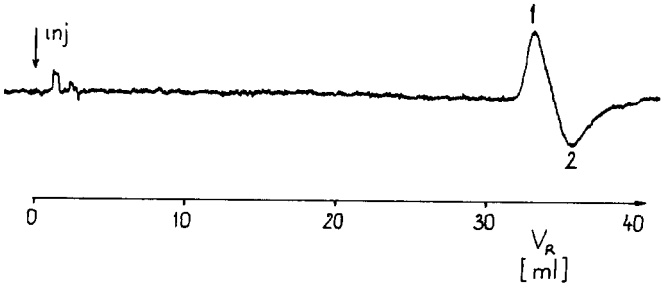

Fig. 2. Chromatogram of chloroform in 20% (v/v) aqueous methanol eluent containing 5 μM benzaldehyde. 1, Chloroform; 2, system peak of benzaldehyde.

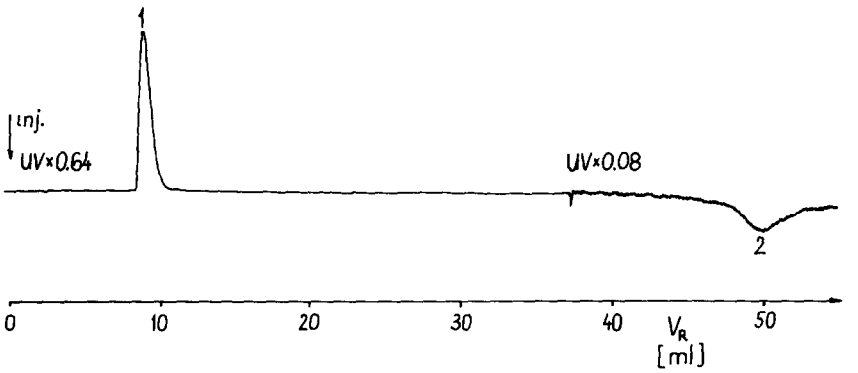

Fig. 3. Chromatogram of methyl ethyl ketone in 20% (v/v) aqueous methanol eluent containing 1 m*M* benzonitrile. 1, Methyl ethyl ketone (UV sensitivity, 0.64 a.u.f.s.); 2, system peak of benzonitrile (UV sensitivity, 0.08 a.u.f.s.).

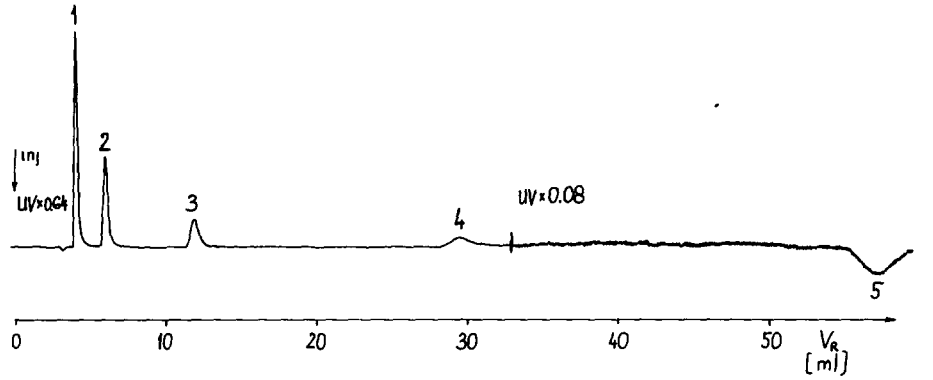

Fig. 4. Chromatogram of nitroalkanes in 20% (v/v) aqueous methanol eluent containing 0.125 m*M* nitrobenzene. 1, Nitromethane; 2, nitroethane; 3, nitropropane; 4, nitrobutane (UV sensitivity, 0.64 a.u.f.s.); 5, system peak of nitrobenzene (UV sensitivity, 0.08 a.u.f.s.).

$RF_{\text{butyraldehyde}} < RF_{\text{butanol}}$. Also, for chloroform ($k' = 11.0$) and *n*-pentanol ($k' = 10.7$) $RF_{CHCl_3} \ll RF_{\text{pentanol}}$. The same trends occur for the other detection agents.

As for a given combination of solute family and detection agent there is an increase in the relative response factor as $k'_{\text{solute}}/k'_{\text{agent}}$ approaches unity. The response factors obtained with various detection agents for the same solute can also be inferred, although indirectly, from Table II. For example, $k'_{\text{pentanol}}/k'_{\text{benzonitrile}} = 0.9$ and $RF_{\text{pentanol}} = 522$, and $k'_{\text{pentanol}}/k'_{\text{benzonitrile}} = 0.82$ and $RF_{\text{pentanol}} = 1300$, *i.e.*, a higher response is obtained even though $k'_{\text{pentanol}}/k'_{\text{agent}}$ is lower (less favourable) for benzonitrile. The same applies to the benzaldehyde–nitrobenzene pair: $k'_{\text{pentanol}}/k'_{\text{agent}}$ are 0.90 and 0.58, while RF_{pentanol} are 522 and 530, respectively. If less polar solutes are considered, *e.g.*, methyl *n*-propyl ketone (MPK), the same trend is found: $k'_{\text{MPK}}/k'_{\text{agent}}$ are 0.45 and 0.38 for benzaldehyde and benzonitrile, while RF_{MPK} are 66 and 119, respectively. For benzaldehyde and nitrobenzene $k'_{\text{MPK}}/k'_{\text{agent}}$ are 0.45 and 0.28, while RF_{MPK} are 66 and 83, respectively. This means that in the systems

examined, the less polar the functional group of the detection agent with respect to the polar functional group of the solute, the higher is the response factor (at identical k'_{solute}/k'_{agent}).

Relationship between solute retention and detection agent concentration

As the first step in the quantitative study of solute retentions, response factors and detection agent concentrations, a model was sought that describes the dependence of solute retention on the detection agent concentration, and a computer model was developed to simulate the generation of induced peaks.

At first, the adsorption isotherms of benzyl alcohol as detection agent and *n*-butanol as solute were determined in pure water as the eluent, using the method of breakthrough curves[11]. The isotherms are shown as a Langmuir representation in Figs. 5 and 6, respectively. It can be seen that in the concentration range encountered in actual chromatographic practice, both the detection agent and the solute closely follow the Langmuir isotherm.

Next, the retention data of *n*-butanol were determined in pure water–benzyl alcohol eluents, then the retention data of benzyl alcohol were determined in pure water–*n*-butanol eluents.

The three-parameter thermodynamic retention model of Tang and Deming[14] was fitted to the retention data. This model assumes that retention in reversed-phase high-performance liquid chromatographic (RP HPLC) systems in the presence of non-ionic surfactants is described by the difference of a constant term representing retention in the absence of surfactant, and a term reflecting the effect of surfactant concentration on the surface pressure. If adsorption of the non-ionic surfactant follows the Langmuir isotherm, the second term can be described by the Szyszkovsky equation[15]. Hence, the distribution coefficient, K_i, of a solute can be expressed as

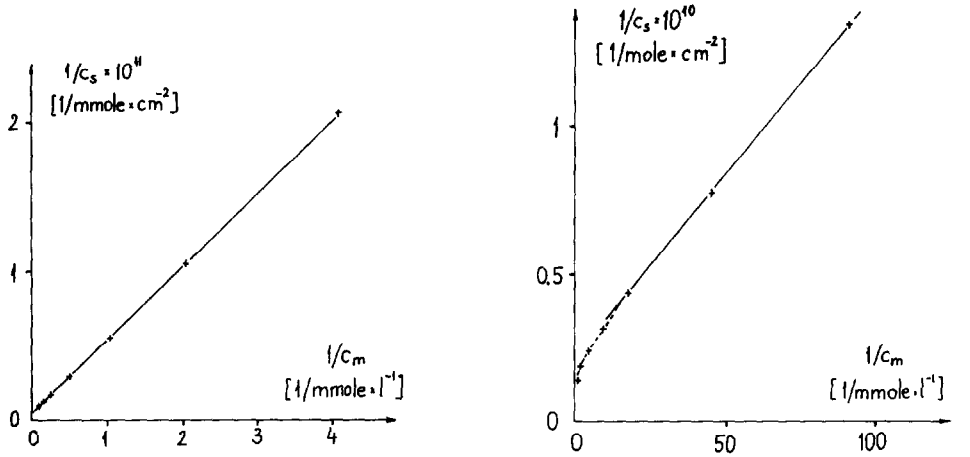

Fig. 5. Adsorption isotherm of benzyl alcohol in a water–RP-18 system (Langmuir representation). $C_{benzyl\ alcohol}$, 0–8 m*M*.

Fig 6 Adsorption isotherm of *n*-butanol in a water–RP-18 system (Langmuir representation). $C_{butanol}$, 0–900 m*M*.

$$K_i = \exp\left[B_{i0} + B_{i2} \ln\left(1 + \frac{C_s}{B_s}\right)\right] \quad (1)$$

where

K_i = distribution coefficient of solute i;
C_s = concentration of the non-ionic surfactant;
B_s = parameter related to the adsorption of the surfactant;
B_{i2} = parameter related to the adsorption of solute i;
B_{i0} = surface area of solute i in the system;
B_{i0} = $\ln K_i$ of solute i in the absence of non-ionic surfactants.

Application of this model seems jusitified because the adsorption of both the solute and the detection agent follows the Langmuir isotherm (Figs. 5 and 6). Consequently, the Szyszkowski equation can be used to describe surface pressure as a function of the bulk concentration of the non-ionic surfactant. Although this model neglects even the first-order interactions of solute and surfactant (a term proportional to the stationary phase concentration of the surfactant), they are implicitly accounted for by the apparent B_{i2} values (it has been shown[16] that the Langmuir terms and the Szyszkowski terms have very similar shapes in the low concentration range).

In order to determine the formal distribution coefficients to be used in further calculations, K_{butanol} and $K_{\text{benzyl alcohol}}$ had to be assessed from measured retention volumes.

The mass of the dry packing in the column is 2.46 g and its specific surface area 175 m^2/g[11], so the total surface area of the stationary phase is 430.5 m^2. The volume of the mobile phase, as determined by the injection of ^2H_2O, is 2.90 cm^3.

A least-squares non-linear curve-fitting program, written in Basic[17], was used to determine the parameters in eqn. 1, yielding

$$K_{\text{butanol}} = \exp\left[-2.4540 - 0.2971 \ln\left(1 + \frac{C_{\text{benzyl alcohol}}}{0.002378}\right)\right] \quad (2)$$

and

$$K_{\text{benzyl alcohol}} = \exp\left[-1.6392 - 0.6519 \ln\left(1 + \frac{C_{\text{butanol}}}{0.01511}\right)\right] \quad (3)$$

A simulation program similar to that described in ref. 9 was constructed using a Craig extraction model (with 100 transfer units), the mass balance equations and the equilibrium relationships for both *n*-butanol and benzyl alcohol, eqns. 2 and 3.

An actual and a simulated chromatogram were obtained with $C_{\text{benzyl alcohol}}$ = 2 mM and a 20-μl injection of an *n*-butanol solution of concentration 10 μl/ml. The simulation program allows us to follow the development of the chromatogram inside the column, as shown in Fig. 7. Here, *n*-butanol (lowest curve) moved through about half of the column (its peak maximum is at a relative column length of 0.54).

Coeluting with the *n*-butanol peak is an induced benzyl alcohol peak (second curve from the bottom), followed by a deficiency peak at a relative column length of

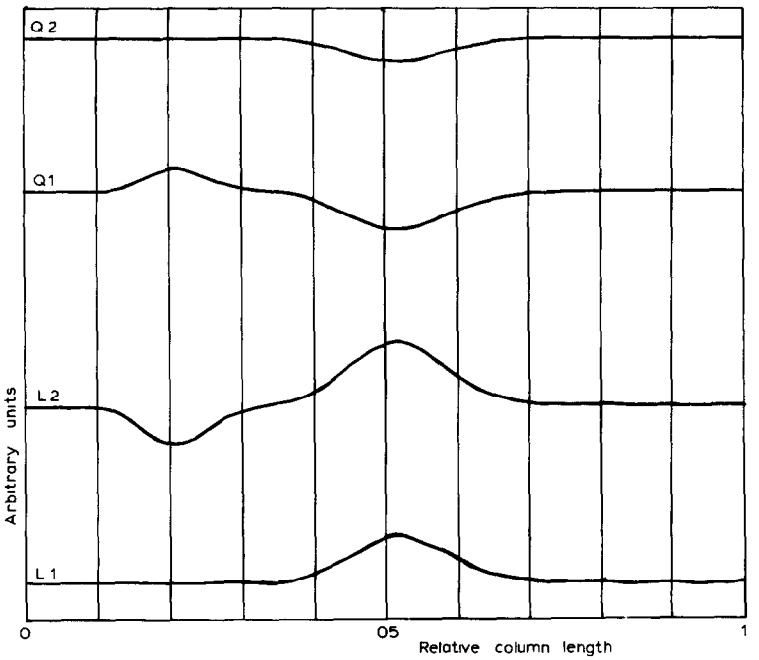

Fig 7. Concentration distribution along the column of *n*-butanol and benzyl alcohol (calculated by the simulation program). L1, *n*-butanol concentration in the mobile phase; L2, benzyl alcohol concentration in the mobile phase; Q1, distribution coefficient of *n*-butanol; Q2, distribution coefficient of benzyl alcohol

0.22. At this point the areas of the two induced peaks are not yet equal. Also shown are the actual distribution coefficients of *n*-butanol (third curve) and benzyl alcohol (fourth curve) along the column.

The actual and simulated chromatograms are shown in Figs. 8 and 9. The areas of the positive and negative induced peaks are now equal, in both the actual and the simulated chromatograms. The actual and simulated capacity factors of the two components in the two chromatograms are as follows: *n*-butanol, 10.7 and 10.5, respectively; and benzylalcohol, 21.7 and 28.6, respectively. The capacity factors of the solute *n*-butanol in the actual and simulated chromatograms are in close agree-

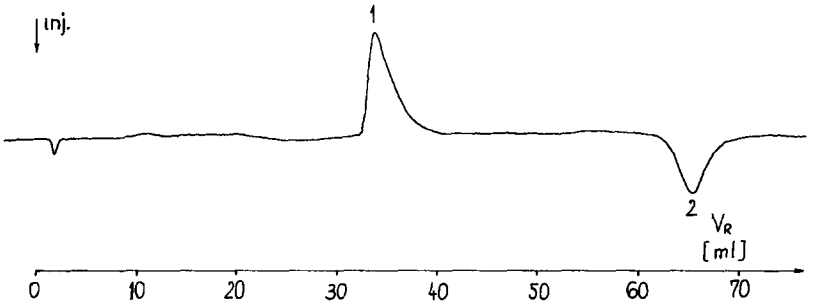

Fig. 8. Actual chromatogram of a 20-μl injection of a 10 μl/ml *n*-butanol sample into 2 m*M* benzyl alcohol–water eluent. 1, Elution position of *n*-butanol; 2, elution position of benzyl alcohol. UV detector at 254 nm, × 8 a.u.f.s

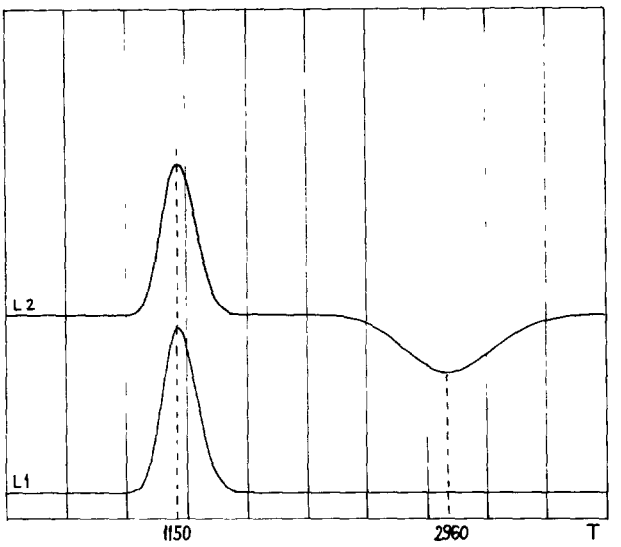

Fig. 9. Simulated chromatogram of a 20-μl injection of a 20 μl/ml *n*-butanol sample into 2 m*M* benzyl alcohol–water eluent. L1, concentration of *n*-butanol in the eluent; L2, concentration of benzyl alcohol in the eluent. T = number of transfers.

ment. However, the sytem peak (benzyl alcohol) is eluted much faster in the actual chromatogram than in the simulated chromatogram. This discrepancy is believed to be caused by the fact that the simple expression used (eqn. 1) does not describe the situation fully; one should add at least another term which shows that the distribution of a component depends on its concentration, too (that is, the system peak of benzyl alcohol elutes faster as the benzyl alcohol concentration in the eluent is increased). It seems that this dependence is almost an order of magnitude stronger for benzyl alcohol than for *n*-butanol (B_s in eqn. 1 is 0.002378 for benzyl alcohol and 0.01511 for *n*-butanol). Further work is in progress to account for this effect.

CONCLUSIONS

Benzene, nitrobenzene, benzonitrile, benzaldehyde and benzyl alcohol were added, in concentrations ranging from 5 μM to 5 m*M*, to 0–30% (v/v) aqueous methanol eluents in an RP HPLC system. When solutes of varying polarity with no indigeneous UV absorption were injected, induced peaks were observed that could be detected by a UV detector. One of the induced peaks coeluted with the solute, while the other induced peak, of equal area but opposite polarity, eluted at the characteristic retention volume of the non-ionic detection agent (system peak). This indirect detection scheme seems fairly universal as solutes with a non-polar to highly polar character all caused induced peaks in the systems studied.

For a given UV-active, non-ionic detection agent the response factor increases (at constant k'_{solute}/k'_{agent}) with the polarity of the functional group of the solute. For a given detection agent and solute family (identical polar functional group), the response factor increases as k'_{solute}/k'_{agent} approaches unity. For a given solute and

k'_{solute}/k'_{agent}, the response factor increases as the polarity of the functional group of the detection agent decreases.

It has been shown that in the chromatographically meaningful concentration range both the detection agent and the solute follow Langmuir-type adsorption isotherms. The simple three-parameter thermodynamic retention model in ref. 14 fits the experimental data fairly well, but when incorporated into a simulation model developed to generate induced peaks, it proves only a first approximation. Further work is in progress to refine both the retention model and the simulation program to allow for an examination of the effects of the various model parameters on the magnitude of the induced peaks (detection sensitivity), and to relate the response factor quantitatively with solute and detection agent structure and other constituents of the chromatographic system.

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