interface

High-performance liquid chromatographic method-development using computer simulation

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Computer-assisted method-development schemes for high-performance liquid chromatography have taken another step forward with the introduction of 'computer simulation'. Software for the application of this technique is described and illustrated.

High-performance liquid chromatography (HPLC) is today the most heavily used technique for the quantitative analysis of organic mixtures. In many companies, tens and even hundreds of analysts use HPLC on a regular basis. This often requires special groups who have HPLC method development as their main activity. With so much effort spent on creating methods for HPLC analysis, it is unsurprising to see the development of a considerable literature on this subject, with many different approaches to HPLC method development[1-3].

Many workers use a trial-and-error approach to method development that is based on the well known resolution equation

$$R_s = \frac{1}{4}(\alpha-1)N^{\frac{1}{2}}\left(\frac{k'}{1+k'}\right) \quad (1)$$

where α is the separation factor, k' the capacity factor and N is the plate number. Usually they begin by selecting a reversed-phase column (*e.g.*, C_{18}) with a large N. Next they adjust the mobile phase to get the peaks in the middle of the chromatogram (% organic modifier variation to control k'). Often one or more band-pairs will be poorly resolved at this point, so the next step is to vary the selectivity (α) of the system. Some chromatographers prefer to try different columns, *e.g.*, C_8 or C_{18} columns from different sources. Other workers will change the mobile phase in some way: replace methanol with acetonitrile, change the pH, add an ion-pairing reagent, etc. Still another choice is switching to a different HPLC method, usually ion-exchange or normal-phase chromatography.

Whatever method-development procedure is adopted, getting the right conditions often means a large number of HPLC runs. Minimizing the number of experiments and organizing the data for easy interpretation is then a major challenge. An early attempt to solve this problem was the 'window-diagram' approach of Laub and Purnell[4]. Although developed originally for gas chromatography, it can also be applied to HPLC (*e.g.*, ref. 2). This procedure allows resolution (R_s) or separation factors (α) to be mapped as a function of some continuous change in the mobile phase, *e.g.*, variation in pH or the concentration of ion-pairing reagent.

Solvent optimization

A later, more powerful mapping technique for HPLC method development was introduced by Glajch *et al.*[5]. So-called solvent optimization was applied to reversed-phase HPLC for the selection of the best mobile phase (maximum sample resolution) based on mixtures of methanol, acetonitrile, tetrahydrofuran (THF) and water. Solvent mixtures having equivalent strength (giving similar run-times or k' values) were selected to allow computer-mapping of resolution *vs.* mobile phase composition, using only seven runs. This procedure has been extended to ion-pair[6] and normal-phase[7] separations.

Solvent optimization as originated by Glajch *et al.* is a quite powerful approach for HPLC method development. With most samples it is uncommon to find that adequate resolution cannot be achieved in this way. If a satisfactory separation is not obtained based on an initial seven runs with reversed-phase HPLC, then the procedure can be repeated using some other HPLC method[6,7]. Alternatively the column can be changed (*e.g.*, cyano or phenyl instead of C_8), and four-solvent mobile-phase optimization can be repeated with each column[8].

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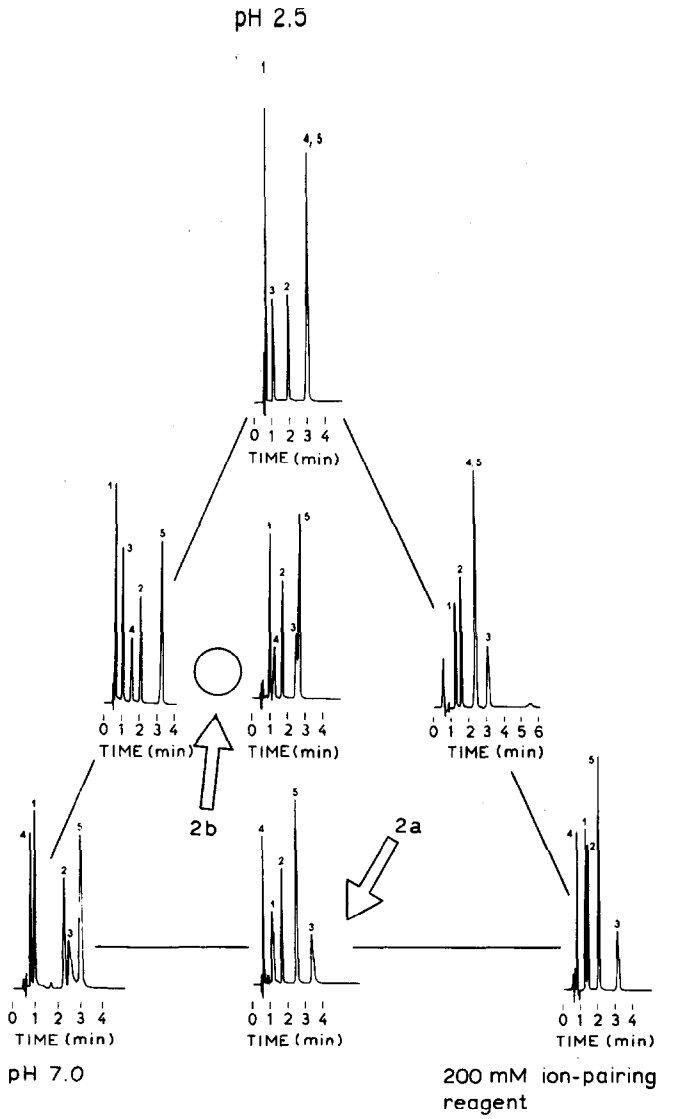


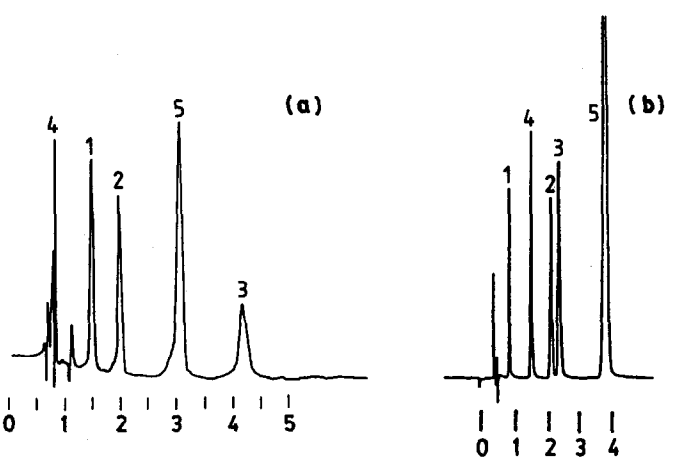
Fig. 1. Retention mapping (solvent optimization) for the ion-pair separation of a five-component cough-medicine sample. C_8 column, methanol–water mobile phase. From ref. 6.

An example of solvent optimization based on the Glajch-Kirkland approach is illustrated in Fig. 1, for the ion-pair separation of a mixture of five compounds in a cough-medicine sample. Here retention is mapped as a function of pH and concentration of the ion-pairing reagent. The three chromatograms shown at the corners of the triangle in Fig. 1 were run with extreme conditions: pH = 2.5 or 7 (no ion-pairing reagent), and pH = 5.5 with a maximum concentration (200 m*M*) of hexane sulfonate as ion-pairing reagent. Intermediate blends of these three mobile phases produced the remaining four chromatograms of Fig. 1. When the retention data for these runs were mapped *vs.* mobile phase composition using a computer, a maximum-resolution composition (arrow, 2a in Fig. 1) was predicted, corresponding to

This adds considerably to the total effort required.

Alternatively, some workers have tried using a diode-array detector to identify sample bands in different runs. This is an expensive and complicated approach that often does not work for samples composed of related compounds, because of similar UV absorption spectra.

Furthermore, many sample mixtures do not require solvent optimization; they are easily separated using a smaller number of separation variables. Finally, previous programs are more or less 'automatic' in nature, and the possible benefits of the chromatographer's experience are generally overlooked. An example is shown in Fig. 2, for the application of solvent optimization to the ion-pair separation of the above cough-medicine sample[6]. Fig. 2a shows the optimum separation predicted by the computer, based on maximum resolution. However this separation would be rejected by an experienced worker, since the first band of interest (peak 4) elutes at t_0. In this case it was possible to use the computer output to select a second optimum mobile phase, but with k' for the first band equal to at least 1. This second attempt (Fig. 2b) gave a more practical separation. However even here the computer can be improved on by a knowledgeable worker. Examination of the original seven chromatograms shows that band 3 can be moved midway toward band 5, with little change in the positions of other bands. Thus the best possible separation exhibits greater resolution than the 'optimized' result of Fig. 2b (the computer erred in this case because resolution changes with pH in a complex fashion that is only roughly approximatable by interpolation).



Aside from the problems illustrated in Fig. 2, numerous other separation goals must be addressed in method development beyond maximizing resolution. Run-time should be minimized, pressure held below some practical maximum, peak detection enhanced, etc. Each of these and other objectives must generally be compromised in any final method. Chromatographers, rather than computers, are best able to make these choices among different separation requirements. In the remainder of this article we will describe a new approach that makes fuller use of the chromatographer's abilities, and is less wasteful of experimental effort.

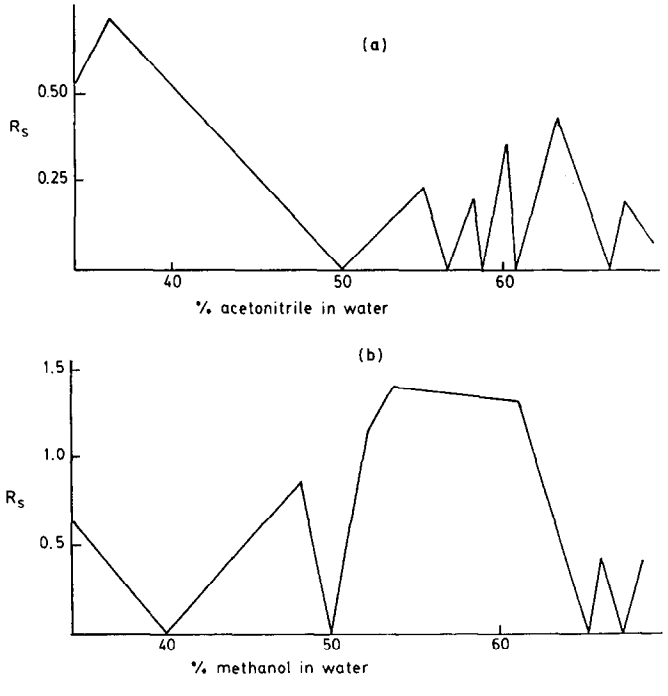
Computer simulation

Previous examples of method development by computer rely mainly on interpolative procedures, so as to increase the information obtainable from a small number of experimental runs. An alternative use of the computer is to utilize chromatographic theory in combination with a few initial experiments. One or two experimental runs can be used to measure certain separation parameters that are a function of the system: sample, mobile phase, column and temperature. Once these critical parameters are defined, theory can be used to predict separation as a function of some change in experimental conditions: column dimensions, particle size, flow-rate, mobile phase composition (% organic modifier), etc. The results for any choice of separation conditions are then presented either as performance characteristics (resolution, run-time, pressure, etc.) or as actual (simulated) chromatograms.

Computer simulation proceeds in the same way as experimental method development. For example, trial-and-error selection of different conditions can be tried, but each 'run' now requires less than a minute. Many more possible separation conditions can be explored under these conditions. Alternatively, the experience gained with computer simulation can be used to design new approaches to HPLC method development. In either case, the chromatographer is able to use computer simulation in the most effective way for a given sample and any special requirements.

Initial examples of computer simulation have included the prediction of (a) the plate number N as a function of the column and flow-rate[9], (b) retention as a function of the mobile phase[10], and (c) retention as a function of computer simulation for HPLC method development.

It was required to separate a ten-component mixture of aromatic compounds (benzene, toluene, xylene and certain of their nitrated derivatives). The first system tried was reversed-phase HPLC with acetonitrile–water as mobile phase. Two gradient elution runs (different gradient times) were carried out initially, and the experimental retention times were entered into the computer (DryLab 4 software). Now we can use the computer to predict isocratic retention as a function of the percentage of acetonitrile in the mobile phase. More convenient at this stage, however, is to ask for a relative resolution map (RRM) as shown in Fig. 3a. An RRM is a plot of minimum resolution (R_s for poorest-resolved band-pair in the sample) *vs.* % organic modifier in the mobile phase for a 10 000-plate column; it is similar to a window diagram. For example, Fig. 3a shows that (at least) two bands in this sample overlap completely ($R_s = 0$) with a mobile phase consisting of acetonitrile–water (50:50). With acetonitrile–water (38:62) as mobile phase, on the other hand, all bands are separated with a resolution of at least 0.7 — if a column of 10 000 plates is used. The RRM of Fig. 3a indicates that no acetonitrile–water mobile phase can provide acceptable resolution (*e.g.*, $R_s > 1.5$) for this sample, without an impractical value of N, *e.g.*, $N = 40\,000$.



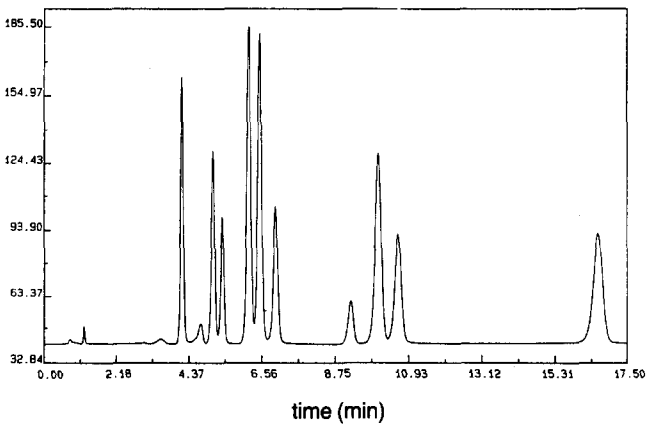


Fig. 4. Separation of aromatic compound sample of Fig. 2 with methanol–water (55:45). Column, 25-cm, 5-μm Zorbax C_8; flow-rate, 2 ml/min.

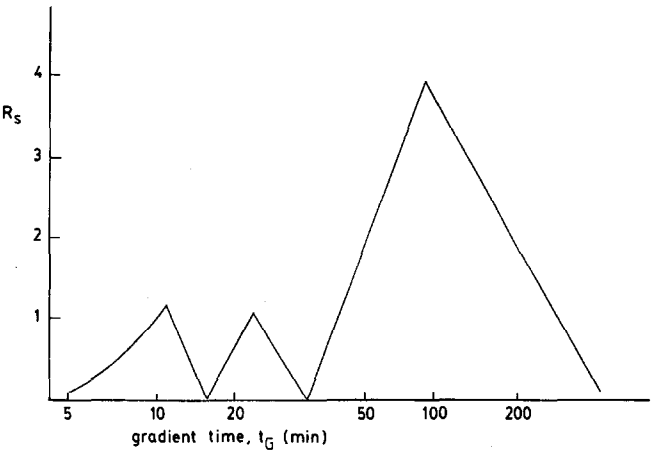


Fig. 5. Relative resolution map for separation of process waste stream by gradient elution[13]. Sample: benzene, toluene, benzyl alcohol, benzaldehyde, acetophenone, anisole, diphenyl ether, benzophenone; column, 30 cm, 10 μm C_{18}; flow-rate, 1 ml/min. DryLab G simulation.

At this point it is worthwhile to investigate another solvent: either methanol or THF. Two more experimental gradient runs were made, with methanol–water as mobile phase. The data were again entered into the DryLab 4 program, with the resulting RRM shown in Fig. 3b. Sample-resolution is now much improved; it appears that a good separation can be achieved with a mobile phase of 55–60% methanol in water. Fig. 4 confirms this prediction, for a mobile phase of 55% methanol. In this case a total of five experimental runs have resulted in a final method for this fairly complex sample. It should also be noted that changes in the percentage of organic modifier can be quite effective for controlling band spacing; Fig. 3b shows four changes in band position between 40 and 70% methanol in water.

Once relative retention has been optimized in this fashion, computer simulation can be used to improve the separation by varying column length, particle size and flow-rate ('column conditions'). Table I summarizes some results from the DryLab 1 program for this same sample, where we see that the separation of Fig. 4 can be further improved by the right choice of column conditions. The particular conditions chosen will depend on what resolution, pressure and run-time we require.

Routine gradient elution

Computer simulation can be extended to the prediction of separation for gradient elution runs, as well as isocratic runs[15]. This is fortunate, because many workers are not presently exploiting the full potential of gradient elution for routine analysis. One problem faced by practical chromatographers is the complex interrelationship of resolution and gradient conditions (gradient time, gradient range, column length, flow-rate). Actually this is less a 'problem' than an opportunity. Once we have the means to optimize gradient conditions, the full use of this option can mean a considerable increase in our ability to separate complex samples.

One illustration is the optimization of the separation of a process waste-stream sample[16], using the recently developed DryLab G software. This eight-component mixture was first separated by gradient elution, using a C_{18} column with a gradient of 5 to 100% methanol in water, and gradient times of 20 and 60 min. A relative resolution map as a function of gradient time was next requested, as shown in Fig. 5. This shows several changes in band spacing as a function of gradient time, with an optimum gradient time of about 90 min; R_s is predicted to be about 4 for these conditons. The simulated chromatogram from DryLab G is shown in Fig. 6a.

It is possible to further improve resolution, or minimize run-time, by varying the gradient range (initial and final percentage organic modifier), flow-rate or column dimensions. The linear gradient of Fig. 5 can also be replaced with non-linear or segmented gradients of various shapes. All of these separation conditions can be studied using computer simulation rather than actual experiments. Because gradient runs tend to be longer than corresponding isocratic runs, this enhances the advantage of computer simulations (each simulation requires less than a minute to carry out).

The choice of a poor gradient time (35 min from Fig. 5) is illustrated in the chromatogram of Fig. 6b; bands 4 and 5 (arrow) are now completely unresolved. The separation of Fig. 6a can be further improved by computer simulation. Usually after retention has been optimized as in this case, the gradient range should be narrowed to save wasted time at the

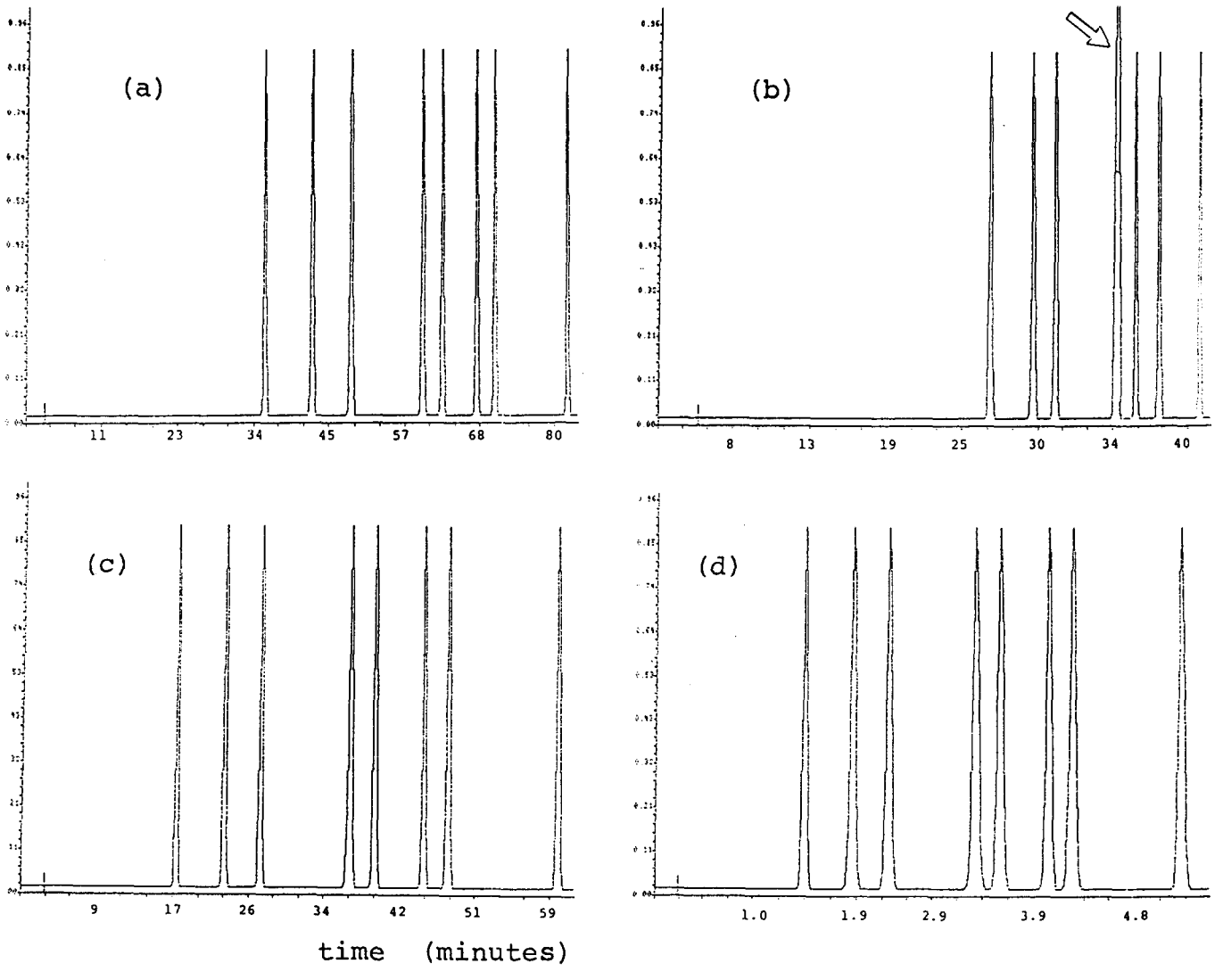


Fig. 6. Computer-simulated chromatograms for the process waste sample and HPLC system of Fig. 5. Same conditions unless noted otherwise. Gradients: (a) 5 to 100% methanol in water in 90 min, (b) same in 35 min, (c) 29 to 87% methanol in water in 70 min, (d) 29 to 87% methanol in water in 4.8 min using a 5-cm column with 5-μm particles.

beginning and end of the chromatogram. DryLab G will recommend an optimum gradient range for separations such as this: 29 to 87% methanol in water for the sample of Fig. 6. After optimizing gradient time again (as in Fig. 5 for the initial 5 to 100% methanol gradient, the separation of Fig. 6c results (gradient time 70 min). Resolution is a little better in the chromatogram of Fig. 6c than in 6a, and the separation time is 20 min less. Further improvement in the separation will require optimizing column conditions as in Table I for the preceding nitro-compound sample. A change from a 30-cm column with 10-μm particles in Fig. 6c (flow-rate, 1 ml/min) to a 5-cm column of 5-μm particles in Fig. 6d (2 ml/min) is seen to drastically shorten run time (5 min), while maintaining adequate resolution.

TABLE I. Computer simulation (DryLab 1) of aromatic compound separation for changing column conditions

Column	Flow-rate (ml/min)	R_s	Run-time (min)	Pressure (p.s.i.)
25 cm, 5 μm particles	1.0	1.9	36	1800
	2.0*	1.7	18	3600
10 cm, 3 μm particles	2.0	1.7	13	2200

* Original conditions (first two experimental runs).

The preceding examples of computer simulation for HPLC method development are only a start. Software packages that are now under development in various laboratories should greatly expand our ability to minimize experimental effort while achieving much better HPLC methods.

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Indirect detection in high-performance liquid chromatography

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Indirect detection is a technically simple method to follow and quantify compounds without inherent detector response in high-performance liquid chromatography. The underlying principle can be expressed in simple equations. These equations indicate clearly how the detection sensitivity can be optimized and how disturbance effects can be avoided when the mobile phase gives detector response.

Compounds that give low response on UV, fluorescence and electrochemical detectors are difficult to follow and quantify in low amounts in normal high-performance liquid chromatographic (HPLC) systems. The detection problems can be solved by pre- or post-column derivatization, but a third, technically much simpler alternative is the so-called indirect detection method, which is based on the use of a mobile phase with detector response in the chromatographic system.

An indirect detection technique has for a long time been used for the detection of ionic compounds in liquid–liquid chromatography. An ultraviolet absorbing ion is applied in the aqueous stationary phase and it gives ion pairs with solutes of the opposite charge that migrate with the organic mobile phase. The ion pairs are easily detected owing to the absorbance of the counter ion[1].

Reversed-phase chromatography is, however, the main field of application of the indirect detection methods, and they can in such systems be used for uncharged as well as charged solutes. The technique is very simple. A detectable component is included in the mobile phase and is then distributed to the stationary phase. Solutes that are injected in the system give rise to detector response as a result of their influence on the distribution of the detectable component (the probe). The response pattern depends on the properties of the solute and the system.

The detection of a series of alcohols in a system with nicotinamide as probe is shown in Fig. 1[2]. Two kinds of retained peaks appear, one for each of the injected solutes and one additional peak that is characteristic for this chromatographic system (the system peak). This peak appears with the same retention whenever injections are made but its direction depends on the properties of the sample. The so-

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