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Automated HPLC optimization — not all systems are the same*

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Abstract: ICOS and DIAMOND are two LC solvent optimization software packages commercially available from Hewlett–Packard and Unicam, respectively. U-83,757 and various related compounds were chosen as the test mixture for the comparison of these systems. Chromatographic data were collected on both systems using the same 10 mobile phase compositions, equally spaced across an iso-eluotropic plane. The comparison focused on determining the performance of both packages with respect to the prediction of the mobile phase composition required to achieve an optimal separation. Both software systems are semi-automatic, with differing amounts of operator involvement required and employing slightly different approaches to interpolating peak movements between the 10 sets of data. The predicted optimal solvent compositions are evaluated in terms of the extent the information collected across the iso-eluotropic plane was used by the various algorithms in the two systems. Our results demonstrate the importance of comprehending the component operations involved in and the limitations of any software package that is used in analytical development. The operator should always remember that both systems are simply tools and design experiments appropriately, since the quality of the final result is highly dependent on a combination of the operator's objective, the capability of the system and the appropriateness of the data input.

Keywords: *Reversed-phase liquid chromatography; solvent optimization; automation; chromatographic peak deconvolution; chromatographic peak tracking; spectral library.*

Introduction

Background

Developing the ideally optimized separation has been the goal of many chromatographers. To that end, numerous practical and philosophical approaches have been developed to help decide on the initial chromatographic components — for example: mobile phase constituents, stationary phase, detectors, pH, temperature — and how to scientifically proceed in order to develop an appropriate separation [1–5]. Historically, though, it has been thought that changing the mobile phase composition was the most powerful method for influencing selectivity [6], and that has been the method of choice for most LC optimization strategies [1–4, 6–20].

LC optimization involves five steps: (i) definition of the criterion of evaluation; (ii) definition of the parameter space; (iii) data collection; (iv) data analysis and interpretation; and (v) prediction and confirmation of the optimum. Optimization methods can, therefore, be divided into two fundamental classes. Univariate methods focus on the effect of changing one discrete variable at a time, such as the particle size in the stationary phase, whereas multivariate methods deal with related variables, such as the proportion of each solvent in the mobile phase. These are related variables since the sum of all the solvent proportions must equal 100% [6].

During the last decade, much work has been invested in developing the theory involved in a number of the multivariate methods, including computerization and automation, with some resources going towards the development of whole or modules within chromatography expert systems. In some cases this has led to the production of commercially available systems and, where appropriate, some of these will be included in the list below. The multivariate methods can be further sub-divided into three groups:

(i) Grid-search methods, in which a large number of experiments are carried out and the best is chosen. This has been referred to as a 'structured trial and error' approach [6], and forms the basis of the commercially available systems known as PRISMA [7, 8] and PESOS [9].

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(ii) Sequential methods, where the results of previous experiments are used to select a subsequent set of conditions. The best known example of this is the Sequential Simplex method [2, 10, 11]. Although these methods are often criticised for determining local rather than global optima, modifications to the basic Simplex methodology have been directed at minimizing this risk [12, 13].

(iii) Interpretive methods, where computer modelling of retention data from a series of chromatographic data sets provides the optimum solvent composition. Commercial systems that belong in this group include DryLab [9], ICOS [9, 14–16] and DIAMOND [9, 17–20].

Of the three groups, the interpretive methods, by definition, are the most efficient at determining the optimum from the least number of chromatographic runs (4–10 sets of data compared with 20–100 sets for the grid search methods) [20]. However, the consequence of this is that they tend to require greater computational capacity and a greater reliance is laid on the algorithms employed. Generally, the chromatographer cannot alter the performance of the algorithms, although the data with which the algorithms interact is operator-dependent.

ICOS and DIAMOND systems

This paper describes the results obtained when the optimal chromatographic separation of a mixture of components was determined using two of the interpretive methods — ICOS and DIAMOND. The same reversed-phase isoeluotropic plane was defined for both systems and the same samples were chromatographed on both systems. Determination of an optimized separation is the result of a combination of factors derived from the system (software + hardware); the sample (including the interaction of the components of the sample with the various chromatographic parameters that are being investigated) and the appropriateness of the data collected, i.e. consider whether the data is of appropriate quality and information content and whether it fits the requirements of the software algorithms used to calculate the optimum separation. In this paper some of the factors that are derived from the system will be considered by comparing the operational and design objective differences of the two systems. In a second paper [21] we will focus on the sample factors, in order to exemplify some of the pitfalls of optimization that await unwary 'black box' users. The appropriateness of the data collected will be considered as an integral part of both papers. The test mixture used was composed of U-83,757 (an amine) and varying numbers of other related compounds, including an aminopyridine (AP) and phenol.

Both ICOS and DIAMOND, ostensibly, have the capacity to interpret chromatographic data derived from various points within a solvent space. The solvent space for reversed-phase chromatography is usually represented as a tetrahedron with water (or buffer), methanol, acetonitrile and THF at the vertices. With respect to the work discussed in this paper, all the data points were derived from a triangular plane, designed with methanol, acetonitrile and THF binary aqueous mixtures at the corners. However, other than providing reference values for calculations, the data interpretation algorithms employed in both systems are not limited to these solvents. In theory, any set of binary solvent mixtures can be used, providing one of the binary components is common to all the corners, as water was in the previous example.

Since the operation of the ICOS [9, 14–16] and the DIAMOND [9, 17–20, 22] software have been extensively described elsewhere, only the sections of the software that are involved in the delineation of the iso-eluotropic plane will be discussed here.

Table 1 provides an overview of the major similarities and differences between both systems. In both cases, the optimization software is a stand-alone package that needs to be purchased separately from the main chromatographic operating software, with which it operates in a symbiotic-type relationship. With ICOS, the necessary chromatographic and data collection conditions can be set up from within the software, samples run and the data manipulated and interpreted, with respect to the optimization, without necessarily leaving the ICOS shell. On the other hand, while chromatographic pump methods can be created from within DIAMOND, data collection methods cannot be created from within the shell. Instead, samples are run, data collected and stored using the UICS operating software. The data can then be accessed from the DIAMOND environment, from where it can be displayed and interpreted, following any appropriate manipulation. The various

Table 1
A comparison of the major components in the ICOS and DIAMOND software packages

Attribute	DIAMOND	ICOS
HPLC method: set-up run	Semi-automatic Outside DIAMOND shell	Semi-automatic Within ICOS shell
Solvent plane determination	PLANE software Default range: $1 < k' < 10$ Methanolic gradient + solvent transfer rules	ESS software User defined limits
Chromatographic data collection	Variable sequence Default: fixed steps Semi-automated Other data can be added subsequently	Fixed sequence User-defined steps Fully automated Other data can be added subsequently
Optimization process	Interactive Determines global optimum Semi-automated	Interactive Determines local or linear optimum Semi-automated
Procedure exploits:		
Reference spectra	Yes — spectral reference set created within DIAMOND shell Reference set specific for that optimization	Yes — spectral library set up outside ICOS shell Spectral library independent of optimization
Peak tracing	Manual or automatic Integral part of optimization database generation Tracking weighted (user-defined values) by spectral, peak volume or retention time matches Results automatically entered into database, but user can modify results of tracking	Manual Optional — spectral library search can be activated manually from within ICOS shell Tracking by spectral and retention time matches (user-defined acceptance criteria) Results need to be manually entered into ICOS
Peak homogeneity investigation	Integral part of data investigation Uses — PCA information ITTFA	Optional during data collection Uses spectral comparison (Upslope/Apex/Downslope)
Peak retention time input into database	Integral part of data investigation Results can be modified by user Final input into database requires user confirmation	Chromatographic peak retention data generated during data collection User-defined retention times for individual components Manual user input of times into database
Information from	Overlapping resolution maps for each peak of interest Non-linear interpolation between data points	Linear interpolation between retention times or k' values of peaks of interest

manipulations will be described below, for both ICOS and DIAMOND systems.

Both systems have a sub-part that can be used to determine the corners of the iso-eluotropic plane. In ICOS, this is called the Elution Strength Selection (ESS) program, while in DIAMOND it is referred to as the PLANE program. The ESS program utilizes an iterative process wherein the components of the binary mixture are variously combined until the retention time of the last eluting peak falls within a user-defined time-window. Solvent transfer rules can be applied to establish either the other two corners of the plane, or the starting points for the ESS operation at those corners.

The approach employed in the PLANE software evolved more directly from the solvent transfer rules and isocratic vs gradient elution transfer rules that have been generated (see refs 3, 4, 17–20, 22 for further references and information). Initially, the sample mixture is chromatographed using a methanolic gradient. Using the retention times of the first and last eluting peaks; the t_0 of the system; the methanolic profile information and a user estimation of the potential number of components in the mixture, the software calculates an appropriate isocratic aqueous methanolic solvent composition such that the final peak will elute with a k' of approximately 10. Built into the algorithm at this point, is a consideration of the situation exemplified in Fig. 1, where A is the binary solvent composition calculated such that the first peak will elute with a $k' = 1$, and Z is the composition required to elute the last peak with a $k' = 10$. If $A > Z$, the system proceeds. However, if $A < Z$, the system will suggest a value of Z such that $A > Z$. This may require the chromatographic time to be extended such that, for the last eluting peak, $k' \gg 10$. At this point, the operator can choose to continue with the column or to restart the experiment either with a different column (stationary phase), alter the aqueous phase of the system, e.g. change the buffer pH, or to investigate the effect of increasing the operating temperature. Once A and Z have been calculated, the software begins an iterative process whereby methanolic solvent compositions are suggested, the user runs the sample and inputs the retention time of the last peak to the program. Based on the results, adjustments to the suggested solvent composition are made, and the process is

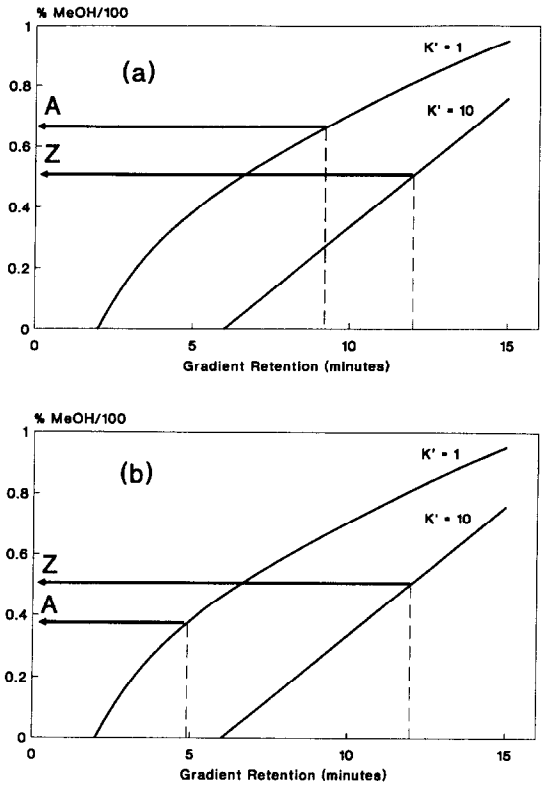

Figure 1
A graphical presentation, using theoretical data, of part of the 'expert' calculation built into the PLANE software that determines appropriate limits for the isocratic methanol–water binary solvent composition that is derived from the results of the methanolic gradient analysis. (a) $A > Z$: isocratic separation under the run-time constraints applied is possible. (b) $A < Z$: isocratic separation under the run-time constraints applied is not possible. (See text for further details.)

iterated until an appropriate composition is determined. Once this is achieved, theoretically equivalent acetonitrile and THF binary compositions are suggested using the solvent transfer rules (see refs 3 and 4 for further details). Again, an iterative process, if necessary, is begun until the appropriate values are determined.

Since the objective of the experiment was to compare the performance of the two systems with respect to their capacity to predict the optimal solvent composition for a separation, chromatographic data generated from the same solvent compositions were used in both systems. The iso-eluotropic solvent plane was defined using the PLANE software and the 10 solvent compositions (Fig. 2) were blended using the respective quaternary pump and integrated solvent proportioning valve system for both System I and II.

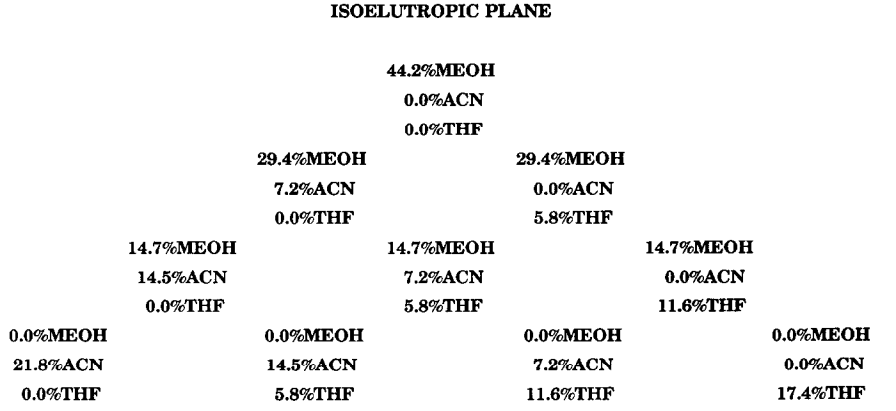

Figure 2
The 10 solvent compositions used to define the iso-eluotropic solvent plane, as determined using the PLANE software. The figures represent the percentage of each component in the mobile phase at each point, with water making the composition up to 100%. Chromatograms were run using each of these same 10 compositions in both chromatography systems.

Experimental

Two chromatographic systems were used, one system for each software package. In System I, the ICOS software (Version 1.0) was run on an HP 9000 Series 300 (Chemstation) computer. The HP 1090M Series II instrument, fitted with a PV5 quaternary pumping system, was operated by the same computer, using a Pascal-based operating software (version 5.3) (Hewlett–Packard, Novi, MI, USA). Sample injection volume was 20 μl. A 2 nm slit was used in the diode array with a 13 μl flow cell, and the pilot signal was set at 220 ± 2 nm, with the reference signal set at 450 ± 50 nm. Spectral data was collected from 220–400 nm using 2 nm increments. In System II, the DIAMOND software was run on a WIN 486 computer, configured with 8 MB RAM and additional operating boards. The Unicam Integrated Chromatography System (UICS version 1.0) control and data collection software was resident on the same computer, and was operated under a Windows 3.0 environment. The instrumentation consisted of a Crystal 240 diode array detector, Crystal 200 quaternary pump and a Unicam fixed volume injector autosampler (PU 4247) fitted with a 20 μl loop (Unicam Analytical Systems, Boston, MA, USA). The diode array detector was configured with a 0.15 mm slit and a 8 μl flow cell. Spectral data was collected from 220 to 383 nm using a 1.3 nm increment.

The columns used were Zorbax SB-phenyl (250 × 4.6 mm i.d.) (MacMod Analytical, Chadds Ford, PA, USA). Column serial number UU 1345 was used with the HP 1090M system, and column serial number UU 1128 was used with the Unicam system. The columns were operated at ambient temperature with identical mobile phase flow rates of 1 ml min^{-1}. Methanol, acetonitrile, tetrahydrofuran (THF) and water (all HPLC grade) were obtained from Burdick and Jackson (Muskegon, MI, USA). Phenol was obtained from Mallinckrodt (St Louis, MO, USA), the aminopyridine (AP) from Aldrich (Milwaukee, WI, USA) and other chemicals were obtained in-house.

Results and Discussion

Table 1 and Fig. 3 outline the different processes involved in the data collection, interpretation and prediction of the optimum solvent composition in the ICOS and DIAMOND systems. Figure 3 provides an expanded overview of the actual steps involved in the two optimization processes. Since two columns were to be used, one per chromatographic system, it was necessary to compare the performance of the columns. Similar samples were chromatographed in System I using both columns, and a comparative set of the resultant chromatograms is presented in Fig. 4. The mobile phase composition chosen for the comparison represented the mid-point in the iso-eluotropic plane. Based on this limited study, it was concluded that the differences observed in the retention times observed with each of the columns was within the expected range for inter-assay and inter-

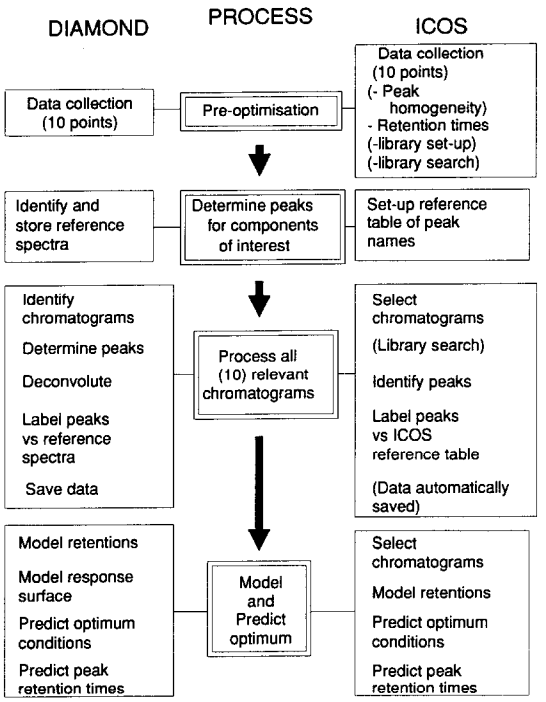

Figure 3
A comparison of the various steps involved in determining the optimum conditions using either the ICOS or the DIAMOND software packages. Those activities enclosed within brackets are optional steps.

column variation. The differences in the peak shapes observed between the columns would not be detected by the optimization algorithms used; since they operate primarily on retention time data. Subsequent to the work described in this paper, for an expanded series of compounds, the mobile phase was further optimized to incorporate a buffer at pH 3.5 and triethylamine as a modifier. These modifications did not significantly alter the relative retention times of the compounds, but the resulting peaks were sharper and more symmetrical, with improved column-to-column reproducibility.

ICOS software

Using the lattice search sub-routine of the ICOS software the system was set up to collect chromatographic data under the same 10 solvent compositions as were used in the DIAMOND system. The first step in the retention modelling involved the selection of a series of chromatograms that lie in a straight line across the iso-eluotropic solvent plane. Once these data sets have been identified, the retention times of the various components of interest, defined as those components identified in the ICOS standard (which is simply a table of reference names) are manually input into the database by the operator. At this point, peak spectra can be extracted and compared with those in a spectral reference library for identification purposes, or, if the spectra are suitably unique, those that were printed out as part of the post-run analysis report can be used as points of reference. The library needs to be created outside of the ICOS shell. An example of the peak identification, data presentation and input is provided in Fig. 5, for the four sets of data that were collected on the THF–acetonitrile–water edge of the iso-eluotropic plane.

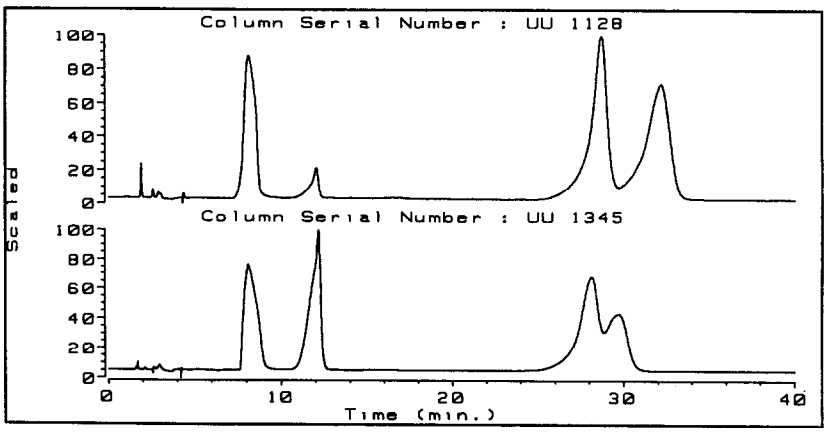

Figure 4
Comparison of the separation obtained on the two Zorbax SB-phenyl columns that were used in the two chromatographic systems. The comparison was run on System I. A sample containing the same four components was chromatographed on both columns, the difference in peak intensities reflect the differences in the sample composition. The mobile phase composition used for both analyses was methanol–acetonitrile–THF–water (14.7:7.2:5.8:72.3, v/v/v/v). (See text for other chromatographic details.)

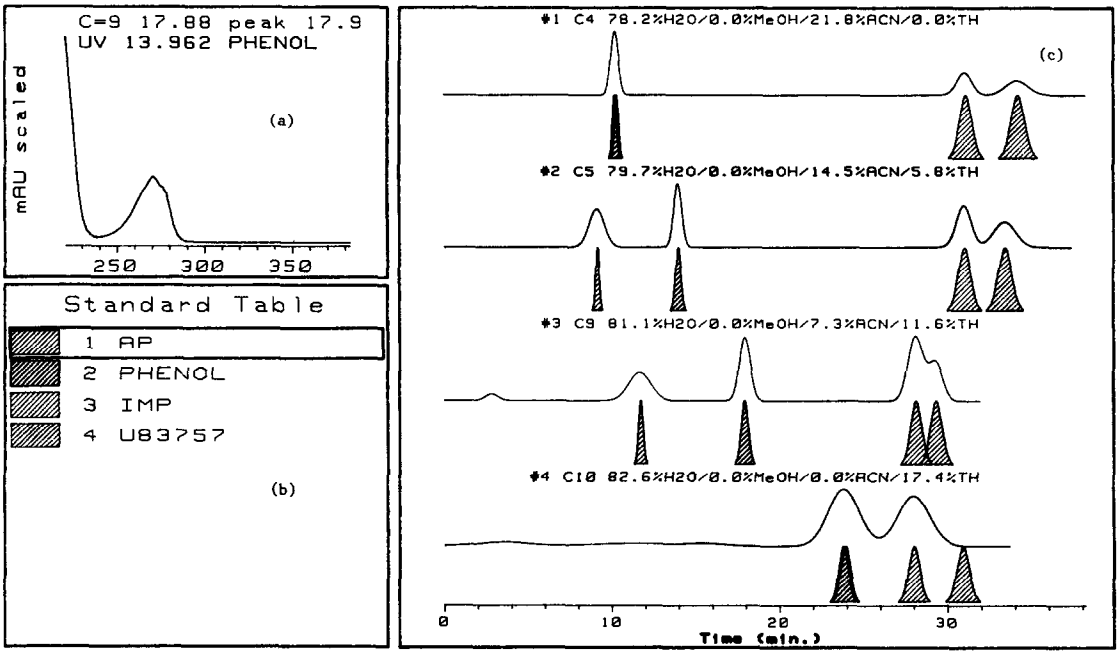

Figure 5
An example of the data input screen for the ICOS retention modelling. The four chromatograms are drawn from the four data sets that represent the THF–acetonitrile–water edge of the iso-eluotropic plane. (a) Spectral library match of phenol peak; (b) standard table; and (c) retention time data input, colour coded with the components in the standard table. The mobile phase compositions are given above each chromatogram. (Cx represents the order in which the data was initially collected.)

Once all the appropriate chromatographic data sets have been interrogated and the relevant data input into the database, the system's algorithms will process the data and suggest an optimal solvent composition, based on two criteria [15, 16]. During the retention modelling, the algorithm assumes a linear relationship between log k' and mobile phase composition. If retention times are used in the calculations instead of k' values, this relationship becomes curvilinear. However, since the actual data points are relatively close in space, the graphical presentation of the interpolation appears to be linear. R_s, min depicts the resolution between the least resolved pair of peaks. R_s, rp is a relative resolution product that reaches a minimum when all of the peak pairs are equally well resolved. The results of the retention modelling for the THF–acetonitrile–water edge of the iso-eluotropic plane is presented in Fig. 6. In this presentation it can be seen that the four components follow two characteristic trends as the mobile phase is changed from THF–water to acetonitrile–water. The two sets of peaks also show opposite separation potnetials — as one pair separates, the other pair begins to co-elute, and *vice versa*. This was also seen on the other two edges of the iso-eluotropic plane. This type of presentation of the data was determined to be a highly efficient means of overviewing the chromatographic characteristics of all of the components of interest simultaneously. The results of the retention modelling along the acetonitrile–methanol–water edge are also presented in Fig. 7, along with a comparison of the theoretical and actual results obtained from running the predicted optimal solvent composition for R_s, min.

DIAMOND software

The UICS operating software was configured to collect data at the 10 solvent compositions that were determined by the PLANE software. The conditions were run in the same order as with the ICOS system.

The first step in the retention modelling is to locate a suitable series of potential reference spectra, ideally from a single chromatographic run, but this is not an absolute requirement. A spectral reference library is set up, with DIAMOND, containing the spectra of interest. Each entry in the reference library includes

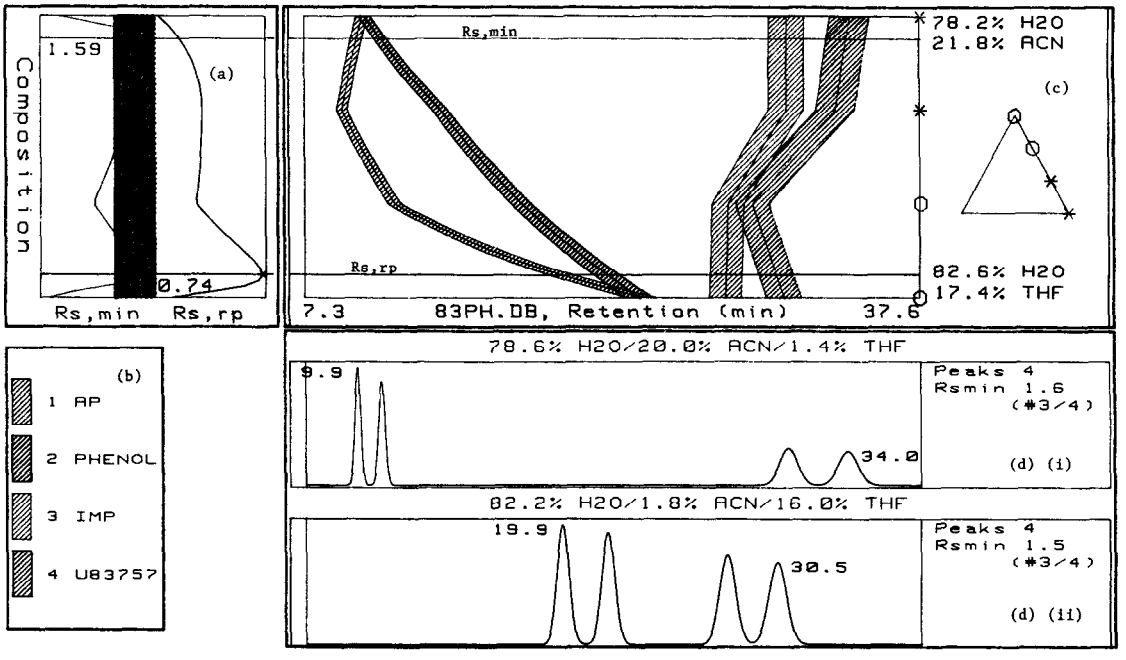

Figure 6
The results of the retention modelling for the THF–acetonitrile–water edge of the iso-eluotropic plane. (a) R_s, min and R_s, rp graphs; (b) colour coded listing of the components in the standard table to facilitate peak identification in the retention modelling presentation; (c) presentation of the characteristic retention of each of the components. The triangle indicates the position on the iso-eluotropic plane from where the data sets were derived; (d) predicted optimal separations and conditions, as determined by the (i) R_s, min and (ii) R_s, rp values.

information on the spectral identity, the chromatographic peak volume from which it was derived, retention time and concentration information in arbitrary units. For subsequent library searches weighting factors are applied to the library as a whole, not to the individual components. These factors can be varied by the user to favour the spectral, concentration and retention time matches.

For each of the 10 chromatograms in turn, the spectrochromatographic data were interrogated over user-defined limits and a composite chromatogram extracted. After dividing the chromatogram into segments, usually separated by baseline sections, the position of potential and real peaks are determined, as derived from the second derivative of the chromatogram. The extent of the subsequent deconvolution of the various sections can be determined by the user. This was followed by spectral extraction using principal component analysis and iterative target transfer factor analysis (PCA and ITTFA; see refs 17–20, 22 for more details). At this point the operator may view the extracted spectra. The mathematical algorithms that enable the user to determine possible combinations of component reference spectra in the extracted spectra can prove to be exceptionally useful at this point. If the extracted spectra appear to be unreal or unreasonable the deconvolution–extraction process may be iterated using different criteria. Once a decision has been made to accept a set of extracted spectra, they are matched against those in the reference library, using appropriate (user-defined) weighting factors. Once again, the operator may choose to accept or reject the assignments, and either iterate the process or manually input known retention data. The retention times for the components of the reference set are then stored in the database.

Once all 10 chromatographic data sets have been interrogated and the retention information stored in the database, the retention maps for each of the components of interest were synthesized using piece-wise quadratic modelling (see ref. 22 for further details). The retention maps for all the relevant components are combined to produce the final resolution map, from which the global optimal separation conditions are derived. There are five methods available in the software for calculating this optimum. Of the five methods, 'smin' and 'rnt'

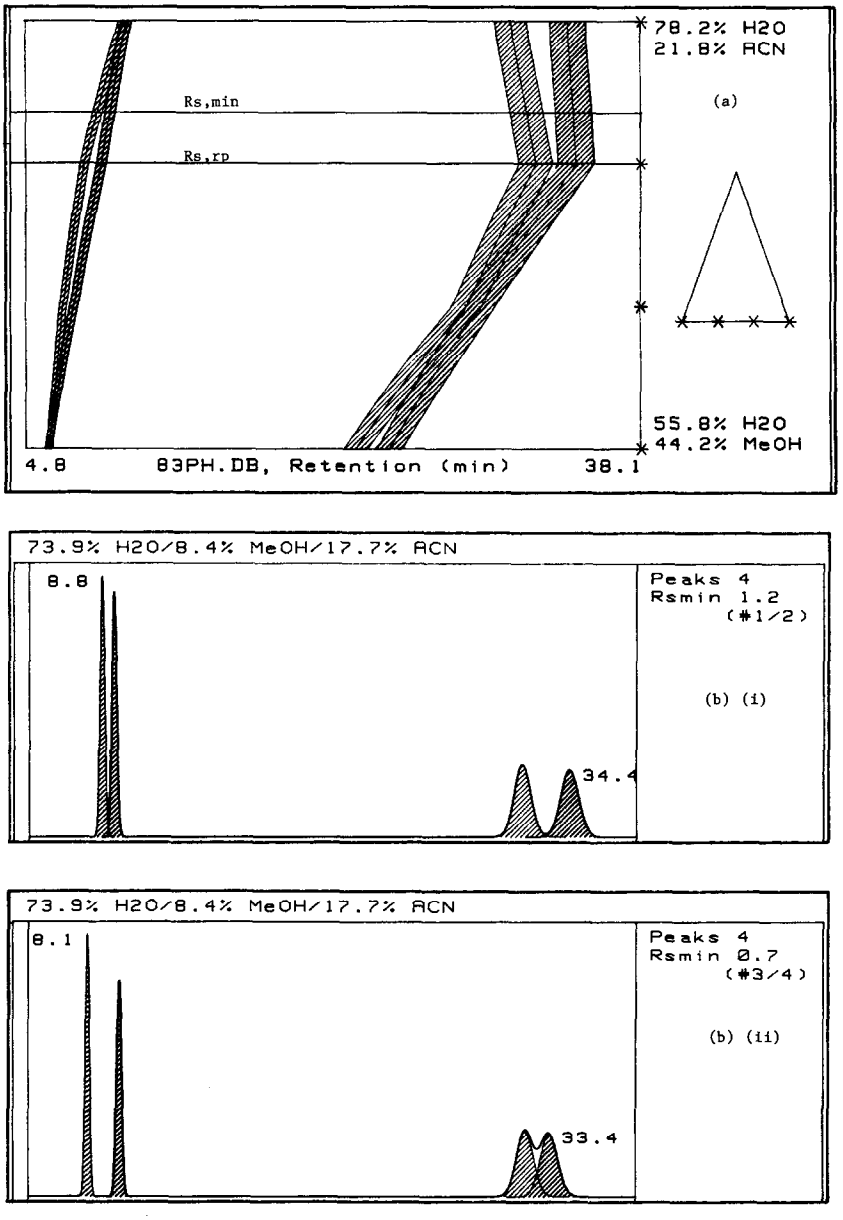

Figure 7
The results of the retention modelling for the methanol–acetonitrile–water edge of the iso-eluotropic plane. (a) Presentation of the characteristic retention of each of the components. The triangle indicates the position on the iso-eluotropic plane from where the data sets were derived. (b) A comparison of the simulated chromatograms derived from (i) the predicted optimal and (ii) the actual separation data obtained using the predicted optimal mobile phase composition calculated for R_s, min.

were thought to be similar to the functions used in ICOS. (See refs 3, 17–20, 22 for further information and subsequent references on these functions.) At this point, not all of the components in the reference library may be 'of interest', however, any component that is relevant to the optimization must have been a component in the reference set. Two of these retention maps are presented in Fig. 8 and the resultant resolution maps for the four components, as calculated using the 'rnt' function, are provided in Fig. 9. While the contour map (Fig. 9(a)) was thought to give the better global overview, and it could be used to synthetically track the movements of the various peaks using a mouse, the response surface presentation (Fig. 9(b)) provided a more direct visual judgement of the potential effect on assay ruggedness of slight changes in the composition of the 'optimal' mobile phase.

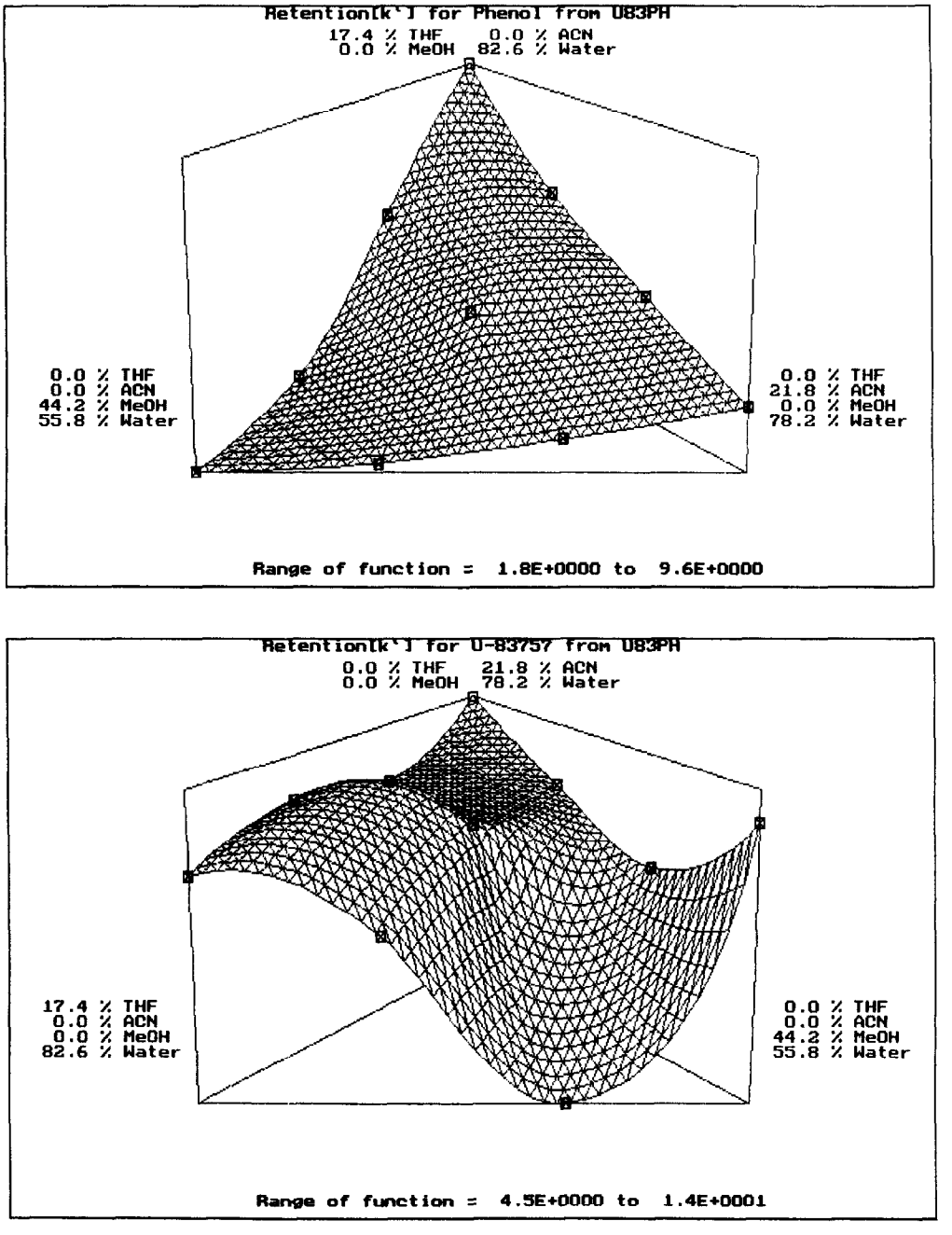

Figure 8
Retention maps for phenol and U-83,757 indicating the marked differences in the chromatographic characteristics of the two compounds. The phenol map is viewed towards the THF corner of the iso-eluotropic plane. The map for U-83,757 has also been rotated for clarity, and is presented with the view towards the acetonitrile corner.

Comparison of the optima predicted using ICOS and DIAMOND

The optimum predicted using the DIAMOND software was a true global optimum, whereas that predicted using the ICOS software was, by nature of its design, a linear or local optimum. With the information available in DIAMOND the interacting effect of all four solvents can be easily visualized. While the same information was available within ICOS, it was not so readily appreciated. However, the graphical presentation of the results within ICOS provided a more direct comparison of the elution characteristics of the individual components within a defined environment.

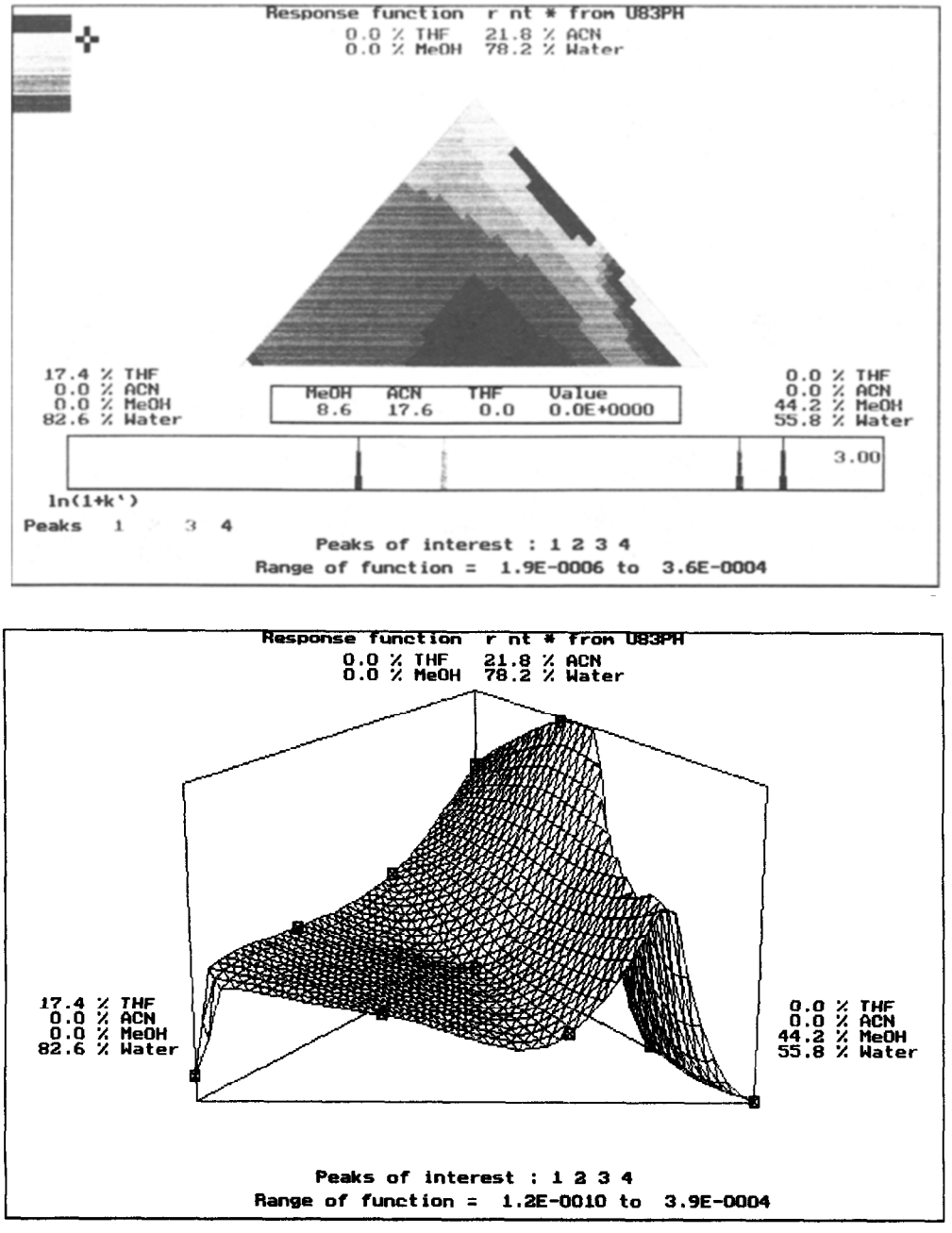

Figure 9
Final resolution map for the separation of the four components, calculated using the 'rnt' function (see refs 17–20, 22 for further details), presented as (a) a contour plot, and (b) a response surface. The plots have been rotated so that the viewpoint is towards the acetonitrile corner, to facilitate comprehension of the response surface presentation.

If the comparison is restricted to considering the results obtained on the methanol–acetonitrile–water edge of the iso-eluotropic plane, both systems predicted similar optimal mobile phase combinations. In ICOS, the methanol limits were defined as 14.7:8.4, v/v, with the respective acetonitrile limits of 14.5:17.7, v/v, which represent the limits for the two criteria R_s, rp and R_s, min. In DIAMOND the optimum was over the range 20.7:8.4, v/v methanol with 11.6:17.6, v/v acetonitrile, respectively. These differences were not considered to be significant, and for both systems the chromatographic trace obtained using the predicted optimal mobile phase was similar to the predicted separation (Figs 7 and 10).

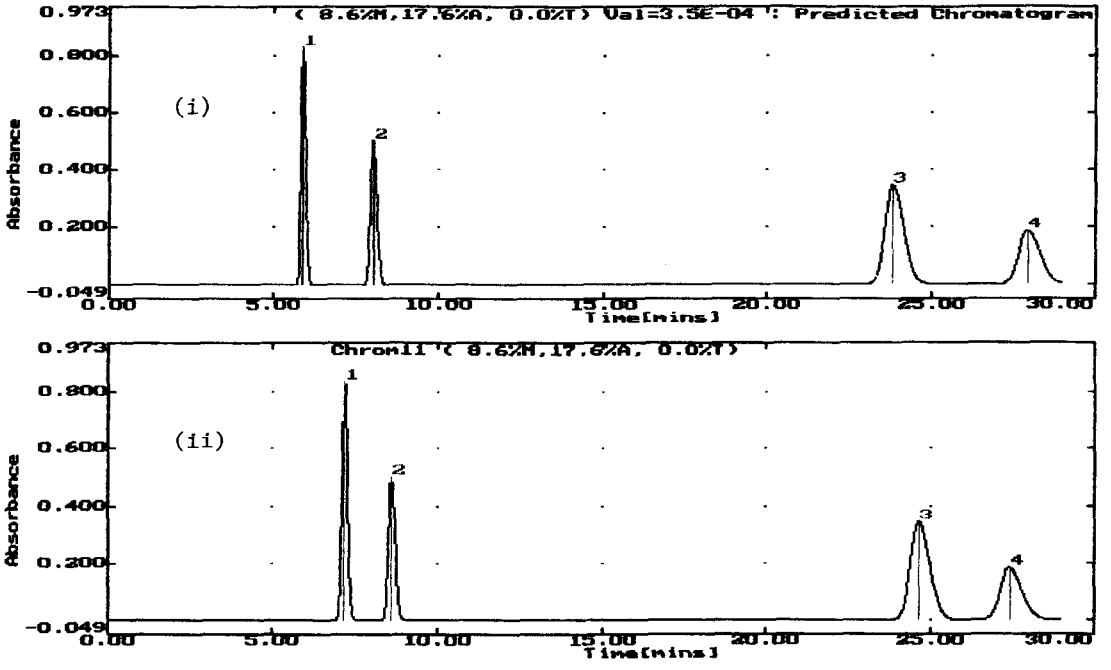

Figure 10
A comparison of the simulated chromatograms for the optimal separation, derived from the (i) response surface data and (ii) the actual retention times achieved using the predicted optimal mobile phase composition from the 'rnt' function in DIAMOND. The mobile phase composition was methanol–acetonitrile–water (8.6:17.6:73.8, v/v/v).

Conclusions

The systems described here provide two different approaches to the common challenge that most chromatographers encounter, namely how to maximize the information content of the data that have been collected. Each system approaches this issue differently, with differing degrees of complexity but not at the expense of maintaining a user-friendly interface. Both systems have the advantage of being only semi-automatic. This permits the operator to tailor the optimization to the needs at the time. This was particularly seen with the DIAMOND system, where multiple endpoints could be obtained from the same 10 chromatogram data set, dependent on which peaks in the reference set were determined to be 'significant' at the time. For example, an optimum could be generated wherein one particular component could be separated from all the others in the reference set, as in a potency assay or isolation experiment, or the separation of all the components could be optimized, as for an impurities assay.

The versatility and extent of user-control varies for both systems. While both systems are designed to be used by novices, the simplicity of operation does not preclude their use by those more experienced in the field. Such users may be able to extract and exploit the information available in different ways. For instance, if the data that was generated from the methanolic gradient is interpreted using a graphical function the feasibility and the constraints on an isocratic separation can be investigated. As a practical application, the type of information that was presented in Fig. 1, derived from DIAMOND's PLANE software, could be used as a means of rapidly screening a series of potential column types for suitability.

Both systems included methods for peak tracking, identification and homogeneity testing. The PCA/ITTFA methodology also enables a certain amount of peak purity testing to be performed. The information obtained following the application of the PCA/ITTFA process was also found to be useful for detecting unknown components, improving the deconvolution of composite peaks and for detecting changes in the spectra of the component due to sample age or environment [21]. However, both systems were limited to predicting an optimum based on data that was linked to identified components in the refer-

ence set. The appearance of compounds outside of the reference set in either system did not influence the predictions. In this way a certain amount of knowledge about the sample and general common sense is required of the operator. Nevertheless, both systems were versatile enough that information for unknown or unexpected components could be subsequently incorporated into the database and thereby influence the predicted optimum.

Most analytical laboratories will continue to have a need for experienced chromatographers. The role of these computerized systems should not be seen as direct competition. Rather, they are tools that enable the experienced analyst to fulful his or her role more efficiently, by extracting and using information from data sets that might not be intuitively obvious to the human expert. Apart from reducing analytical run times [20], our work with these systems has emphasized two issues. The first is the need to define the objective and understand the experimental design and limitations of the software. The second, often overlooked issue, is the need to know what components are in the sample and understand the complex interactions between those sample components and the varying environments to which they are subjected in the course of the experimentation [21]. For both systems, apart from the final optimization details, probably the most useful information that is provided was the graphical depiction of the estimate of the potential ruggedness of the optimized separation, with respect to changes in the mobile phase.

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