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Article

A Binary-Like Approach for the Computer Assisted Method Development of Isocratic and Programmed Ternary Solvent Elutions in Reversed-Phase Liquid Chromatography

J. García-Lavandeira, J.A. Martinez-Pontevedra and R. Cela*

Dpto. Química Analítica, Nutrición y Bromatología. Instituto de Investigación y Análisis Alimentarios. Universidad de Santiago de Compostela. Spain

*Author to whom correspondence should be addressed: email: rafael.cela@usc.es

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A specific tool to enable exploration of multisolvent isocratic and programmed elutions for the computer assisted method development of reversed-phase liquid chromatography separations is described. The tool is purposely identical to those used in the optimization of binary solvent systems, which are by far the most commonly used by chromatographers. Existing data from failed binary solvent optimization processes are reused to explore ternary solvent systems with a few additional isocratic and programmed runs. This allows the development of efficient retention models for ternary systems, although the work of the chromatographer remains identical to that for optimization of binary systems. The retention models are used to develop an unattended optimization process and finally, the chromatographer selects the most satisfactory solution for testing and implementing in routine analysis. The process is exemplified with a mixture of 12 compounds that cannot be separated satisfactorily in aqueous binary solvent systems with methanol and acetonitrile as modifiers.

Introduction

After several decades of development, reversed-phase liquid chromatography (RPLC) has become the most commonly used chromatographic mode in LC. Today, most laboratories use RPLC for research, routine analysis, and quality control, and the number of RPLC trials made daily all over the world continues to increase. Nevertheless, some practical possibilities enabled by modern LC instruments are only scarcely used. One of these rarely used features is multisolvent elution. For some types of elution, ternary solvent mixtures have been recognized as powerful tools that enable separations that cannot be achieved with binary solvent systems. The lack of availability of pumps that allow the easy handling of more than two solvents is a factor, but also the increased complexity in the optimization of this type of elution and the need to apply computer-assisted method development (CAMD) tools to deal efficiently with these separations may explain the reluctance of many chromatographers to use the feature. Trial and error approaches are inefficient and discouraging when considering the usual runtimes in RPLC. In addition, the experimental development of most formal optimization strategies becomes prohibitively time-consuming.

A few years ago Martinez-Pontevedra et al. (1) developed a general approach for ternary gradient separations. In this approach, the search space is not restricted, so the entire solvent triangle is optimized (or at least the maximum area feasible in experimental terms) by a computerized approach based on a powerful evolutionary algorithm (2) working on an islands model (3, 4). Ternary gradients are optimized by transforming the program shape into a stepwise equivalent shape (5); thus, the parameters of the gradient shape can also be introduced as variables in the optimization space. As a consequence, not only the entire solvent triangle may be explored, but also the gradient shape may be considered in the optimization process. This approach provides an extremely powerful procedure for optimizing ternary elution gradients that can eventually resolve very complex sample mixtures. The price to this approach, however, is the need of a considerable number (between 12 and 18) of priming experiments. In general, the chromatographer cannot have the guarantee that the optimum finally found in any optimization approach will really fulfill its needs, so the amount of priming work required must be drastically reduced, or the results can be disappointing.

In general, to limit the number of priming experiments, the optimization of mobile phases containing more than two solvents is developed by means of a drastic reduction in the search space. The solvent composition area to be explored is bound by binary isoelutropic mobile phases. In this way, not only the number of priming experiments may be reduced to reasonable numbers, but also reliable linear or quadratic retention models are produced because the reduction in the area explored (6–13). This type of approach makes use of binary retention models to predict the most favorable solvent composition space (9, 10) and then builds a specific retention model under ternary conditions inside the constrained area. Several examples of successful separations developed by this approach for more or less complex sample mixtures have been reported using ternary isocratic elutions (11), isocratic multisolvent programmed elutions (12), and ternary gradient elutions (13). The clear advantage of this type of approach is the reduction of the initial experimental effort. The price is the possibility of convergence in suboptimal solutions because the a priori selection of a restricted solvent-composition area (sometimes a straight line) in the triangle to be explored. On one hand, the retention inside the triangle may be substantially different from that at the binary edges. On the other, the possibility of a better solution outside the selected solvent composition area cannot be excluded except by exploring the whole experimentally accessible solvent triangle. Thus, the process of search space selection is critical, and the works of Heinisch and coworkers (9–11, 13) have provided the best approaches up to now.

The balance between brute force approaches exploring the entire triangle, and those that involve critical decisions from the beginning should be established in terms of the complexity of the separation problem and whether or not there is evidence of promising changes in selectivity between the different binary mobile phases. On the other hand, the evidence in day-to-day work at chromatographic laboratories is that most chromatographers find computerized tools for optimizing ternary solvent mixtures rather complex and are somewhat resilient to start the optimization processes for these solvent mixtures after failing to develop binary solvent elutions. In general, the impression is that there is a low chance of success associated with a large amount of effort. Additionally, many chromatographic systems running in research and routine laboratories are based on binary pumps. Thus, a convenient tool for ternary solvent elutions optimizations should allow: (*i*) the development of an automated optimization process after failing in the development of binary solvent elutions, (*ii*) using a limited number of priming experiments, which means using approaches that restrict the explored area inside the solvent triangle, (*iii*) reusing the available data and information gathered in developing the failed binary solvent elutions, (*iv*) ability to handle any type of gradient shape or programmed elution mode as provided by the available instrumentation, and (*v*) have a simple and familiar interface (e.g., the one in CAMD tools for binary solvent elution separations) thus helping eliminating the barriers raised by the intrinsic difficulties of ternary elutions.

In this paper, a system is presented that allows optimization of ternary solvent elutions of any kind without exiting from the conventional CAMD environment used for binary solvent elutions. We called this approach Pseudo-3D (a name derived from the term pseudo-ternary solvent which is used frequently in this context), because the chromatographer will work every time with computer applications used for binary gradients; although in fact, the system is handling ternary isocratic and programmed elutions of any kind and shape. This approach makes use of the quickest option of defining a restricted area in the search space to be explored, thus limiting the work involved in developing the required ternary retention model to a few additional experiments. Moreover, the CAMD process is fully automated using evolutionary algorithms as the optimization engines, which provides the chromatographer with the optimal solutions in a matter of seconds.

Experimental

Apparatus

The chromatographic system used in the study was a Waters Alliance 2695 separation module with low pressure mixing quaternary pump, autosampler and column oven (Waters Co., Milford, MA). The instrument was equipped with a photodiode array detector (Waters). The instrument has a dwell volume of 1.00 mL and an extra-column volume of 0.09 mL and was controlled by the Empower 2 software (Waters).

The analytical column was a Halo C18 column (Advanced Materials Technology Inc., Wilmington, DE) of length 75 mm, i.d. 4.6 mm and particle size, 2.7 microns. The guard cartridge system was a Gemini C18 ODS Octadecyl (Phenomenex Inc., Torrance, CA) of length, 4 mm and i.d., 2.0 mm.

Reagents

A conventional polarity mixture containing 12 compounds was considered in the study and included: benzene, toluene (Merck, Darmstadt, Germany), benzophenone, methylparaben, ethylparaben, propylparaben, butylparaben, 2,4-dimethylphenol (Sigma-Aldrich Quimica, Madrid, Spain), diethylphthalate, dimethylphthalate, *p*-cresol and phenol (Fluka Analytical, Buchs, Switzerland). The mixture was eluted with methanol–water and acetonitrile–water binary mixtures in isocratic and gradient modes, as well as in ternary (methanol–acetonitrile–water) isocratic and programmed elutions. HPLC solvents (methanol and acetonitrile) were supplied by Merck. Ultrapure water was produced in the laboratory with a Milli-Q gradient system (Millipore, Bedford, MA).

Chromatographic optimization and data handling

Chromatographic data were acquired and handled with an LC workstation. Isocratic retention times, peak widths, and symmetry factors were copied and pasted into the PREGA database module. All further data handling during the optimization processes was carried out by use of the PREGA v 6.0 software package (5, 14).

All software applications used for CAMD processes were developed in the author's laboratory and written in Delphi 2007 for Win 32 R2 version. Software modules in PREGA V 6.0 enable handling of isocratic retention data and construction of binary retention models to be used in the fully automated optimization of isocratic and binary gradient separations, with any kind of gradient shape (linear, curved, multilinear, and stepwise). Optimization processes were developed with evolutionary algorithms that utilize both conventional critical resolution and chromatographic response functions (CRFs) as objective functions in the search for the optimum. Pareto optimality (15) and transfer routines (16) between instruments have also been implemented as options. All PREGA modules, including the specific module for exploring ternary solvent mixtures presented here can be downloaded free at http://www.usc.es/gcqprega. Only the relevant PREGA functions in the context of the present study will be discussed below. Full details and helping materials can be obtained at the web address provided.

Results and discussion

General statement of the problem and retention models

When working with multisolvent systems it is advisable to refer to programmed and non-programmed elutions rather than to isocratic and gradient modes. According to the pioneering classification of Glajch and Kirkland (17), there are four possible modes of elution in multisolvent reversed-phase liquid chromatography: simple isocratic (SI), isocratic multisolvent programming (IMP), isoselective multisolvent gradient elution (IMGE), and selective multisolvent gradient elution (SMGE). In IS and IMP, the solvent strength of the mobile phase remains constant or nearly constant, although the composition may vary (IMP) or not (SI). If solvent strength is varied during the elution, the term gradient is appropriate. However selectivity of the mobile phase may remain approximately constant (IMGE) or not (SMGE); the latter is the more complex and general type of

elution. Jandera (6, 18) developed the theory of ternary solvent gradients and assigned the names ternary solvent strength to IMGE gradients and combined selectivity-solvent-strength to SMGE elutions. When only binary solvent elutions are considered, the elution modes available are the two most traditionally named: isocratic and gradient elutions.

From a practical point of view, during method development, the use of ternary solvent elutions (experimentally or by computer-assisted simulation) should follow evidence of failure of binary isocratic and gradient programs, because whatever the approach, optimization of ternary solvent elutions is much more complex and demanding than optimization of the binary counterparts. Any practical computer-assisted method development tool must consider a rational method of developing the separations that can be safely constructed by following the widely accepted practical guidelines for RPLC developed by Snyder et al. (19).

In order to explore the possibilities of the described elutions, the computer assisted system needs a retention model describing the analyte's retention behavior in the ternary solvent mixture. Retention modeling can be developed by grid search (20) or by interpretive methods (21–23). The latter are widely accepted because the number of priming experiments is small as compared with grid search procedures. In the case of binary elutions, linear or quadratic mathematical models allow in most cases an accurate enough representation of the retention for peaks. For ternary solvent systems, it is possible to model the whole search space using the Shepard algorithm (24) on transformed retention data (1/square root of retention data), although the number of priming experiments (between 12 and 18) is too high in most practical circumstances (1). Other approaches select an isoeluotropic plane, allowing all the peaks eluting in a reasonable time (e.g., retention factors -k- bellow 10). Retention data collection in this plane and the adjustment of quadratic or piecewise quadratic functions (7) provides models allowing the prediction of retention under ternary compositions. Euerby et al. (12) have shown that retention can be modeled using a 2×4 experimental design of two variables: the gradient time and the percentage of one organic modifier provided the ratio between both organic modifiers is kept constant, whilst the sum of the two concentrations is increased. This means that this approach applies only to IMGE or ternary solvent strength elutions.

The utilization of binary retention data to predict ternary ones has been exploited by Pappa-Louisi and coworkers (8), using equations in three parameters. Another different approach using also the binary data information was previously developed by Heinisch and coworkers (9-11, 13). In that case, the key idea of resorting to ternary solvent when binary ones have failed is clearly stated and used to define the optimization strategy. A selection criterion, namely the CRIT, is calculated to establish, if they exist, the location of binary solvent compositions, allowing the separation of each pair of solutes over a required resolution value. If it is not the case for at least one possible pair of solutes, it may be concluded that the optimization research within this ternary composition area is likely to be unprofitable. On the contrary, given a favorable CRIT values margin, the region can be defined and modeled via new experiments, allowing in the best case the finding of an optimum separation.

The approach described here also departs from binary data in cases where binary solvent systems failed in the search for a satisfactory separation. Instead of defining a particular criterion to select the favorable binary compositions, the chromatographer may choose among a variety of published chromatographic response functions (25, 26) including the usual critical resolution function to be modified to take into account the total analysis time. Moreover, all the calculations use binary or pseudo-binary retention data running into conventional binary solvent optimization software, thus simplifying the user interface because the chromatographer would always work in the same environment, although allowing the full use of chromatographic resources and experiments. This means that in the described approach all retention modeling use the same binary solvent approach belonging to a general interpretive computer-assisted method development strategy named PREGA, which was developed for reversed phase binary solvent separations (5, 14–16, 27–32).

The PREGA optimization process is the following: first, some isocratic retention data for the compounds in the problem mixture are used (or reused from retention databases corresponding to the same column and modifier, if available) to develop an isocratic retention model individually for each peak. This retention model considers as variables the mobile phase composition, the flow rate, and temperature, and is re-calibrated by means of some experimental gradient runs (usually not more than 2 or 3) with different conditions (modifier proportions, runtime and any kind of shape).

Once the retention model appears sufficiently robust and reliable for all peaks, the model is used to optimize the separation. In PREGA the optimization process runs via a fast panmictic evolutionary optimization algorithm (2, 3) that maintain a fixed size population of solutions (elution gradient programs) competing for survival, affected by the pressure of selection, crossover, and mutation genetic operators (2, 33). This process finally proposes to the chromatographer one (or several in the case of Pareto optimality approaches) optimal solutions. The participation of the chromatographer in this stage of the process may be restricted to the study of the final optimum solutions selected by the application and, in case of promising results, the experimental verification of those solutions. However, the optimization process may become interactive and the chromatographer may take several decisions and configure the whole process according to their goals. The entire process (except obviously the experimental acquisition of the priming data) may be developed in less than 3–5 min, thus providing a rapid insight into the possibilities of resolving the sample mixture with the binary solvent system tested. This process can be developed for other binary solvent mixtures if the first one tested is unable to provide the desired separation. Further details about the CRFs are available, the method used by PREGA for developing their retention models and the way the chromatographer takes decisions in PREGA can be obtained http://www.usc.es/gcqprega.

Considering ternary solvent systems

If two binary solvent combinations (water–S_1 and water–S_2, or any two pairs of combinations with a common solvent) have been tested and no satisfactory binary isocratic or gradient

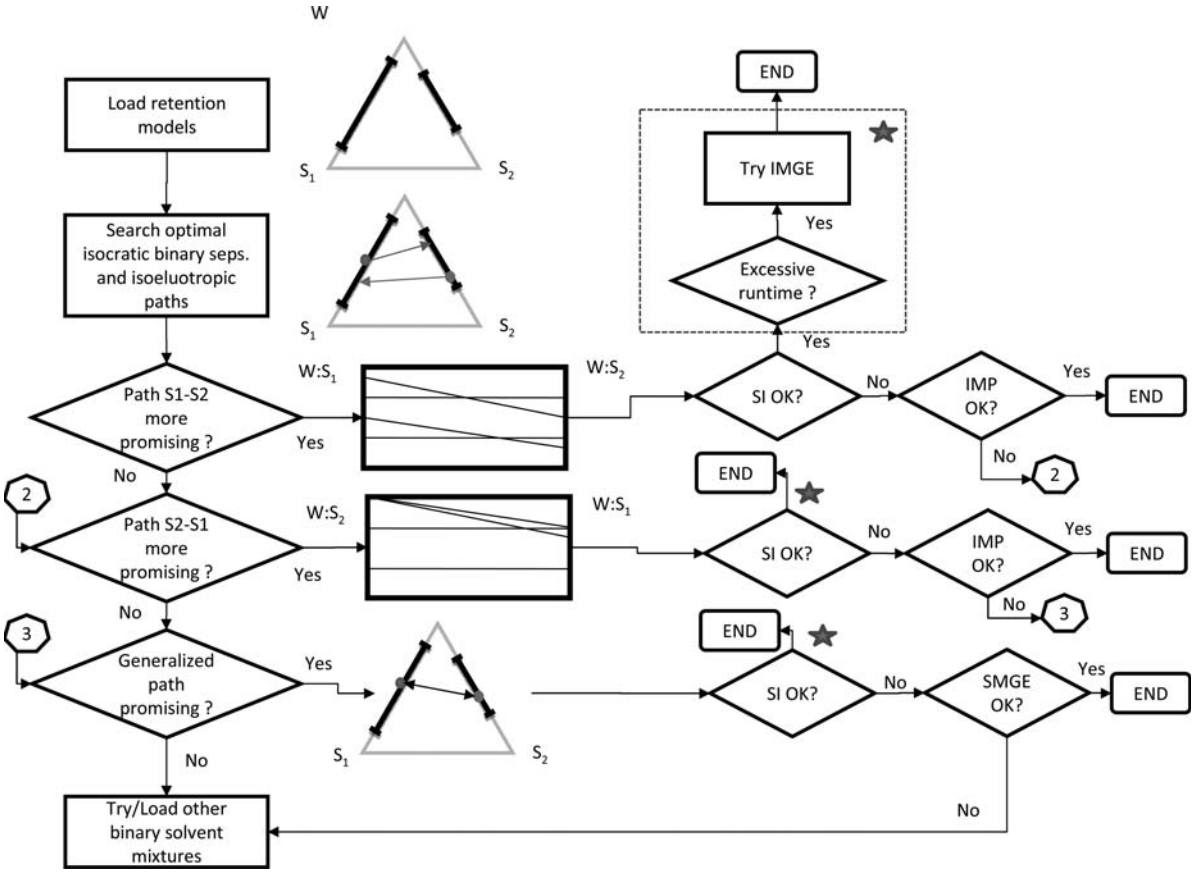

Figure 1. General flowchart for pseudo-3D modules in PREGA computer-assisted method development for RPLC with ternary solvent systems.

separation has been obtained, then a ternary combination may provide a better solution if some selectivity changes are evident in both solvent systems, particularly when the changes in selectivity affect the critical of poorly resolved pairs of peaks in the mixture. Thus, the decision as regards to whether the ternary solvent mixture should be explored or not, should be based on criteria that take into consideration the modifications in selectivity. In PREGA, this process is developed without any need for additional experiments, as outlined in the diagram in Figure 1.

Firstly the binary retention models are loaded to give a visual cue about the system to be explored. Note that although a solvent triangle is used for graphic depiction of the loading of the retention models, the chromatographer will always work with a binary solvent type environment. This is why we called the system Pseudo-3D. This graphical support may be used to load two or three solvent pairs if needed. Automatic checking of the solvent consistency (e.g., the existence of a common solvent in each two binary solvent mixtures loaded) avoids the need for interpretation of the triangular diagram for the chromatographer. Note also that PREGA is not restricted to work with constant strength solvent triangles. The retention models are charged as such, to cover the range of modifier proportions that have been explored during the binary solvent optimization stages in PREGA. From this moment, the system explores the several possible paths in which ternary isocratic or programmed elutions may provide enhanced possibilities of a satisfactory separation. Firstly, the optimal isocratic separations with each binary solvent mixture are located and connected to an isoeluotropic composition in the opposite binary system. It should be noted that the optimal isocratic separations are located according the optimization criteria selected: CRFs, critical resolution, and pareto optimality; although they can be selected manually by the chromatographer. At this point, the chromatographer knows quite well how the binary separation system will proceed because he/she has studied these possibilities while developing the models for those binary solvent systems. The most convenient objective function and conditions are therefore easily selected and, of course, several can be studied successively if needed for comparative purposes. Secondly, the isoeluotropic opposite point in the triangle is located on the basis of empirical and retention model data available and not on theoretical relationships (34, 35). This makes the system also applicable in cases of solvent combinations that do not accurately follow the theoretical transfer rules.

Thus, at this point two paths are defined in the solvent triangle (see the scheme in Figure 1) and enable exploration of simple isocratic (SI) elutions as well as isocratic multisolvent programmed elutions (IMP) along these paths. Although the exact retention behavior of peaks inside the paths is not known at this time (which is why the use of critical resolution maps is unadvisable at this stage), the differences in selectivity at the binary extremes may provide a clear insight into which of the two paths is most promising, if any. This decision is

helped by the inspection of the corresponding simulated chromatograms and selection of the critical pairs at both sides of the path, although the time constraints may also be important because some CRF formulations, depending on the values assigned to runtime weighting coefficients, may suggest large inconvenient runtimes.

In the event that one of these paths exhibits sufficient differences in selectivity to make further exploration advisable, the Pseudo-3D path must be completed by carrying out some (two or three) additional experimental isocratic runs along this route. A couple of gradient elutions also using the solvent path are used to validate the retention model. These gradients can be handled as binary ones if we consider the solvent compositions at the path extremes as two different solvents that will be mixed during the gradient (e.g., in the case the chromatographic system available have a binary pump). These additional data (the exact location of these new experiments is suggested by the system) are used to construct a new pseudo-ternary retention model that will be handled in the same way as the original binary solvent models tested.

Good isocratic elutions inside this new retention model can be further improved in the case of excessive retention for the last eluted peaks by developing isoselective multisolvent gradient elutions (IMGE), from the point of the optimum separation, following a vertical path of increasing strength but equivalent selectivity in the solvent triangle (see Figure 1).

IMP elutions can also be explored following traces inside this path of variable length and shape. Because the system is managed by the same optimization engine applied for binary gradient separations, any kind of programmed elution shape (linear, curved–if allowed by the available instrumentation–multilinear or stepwise) can be explored for the ternary solvent system. As far as we know, no other commercial or academic computer-assisted method development system for RPLC can explore IMP elutions with such versatility.

If convenient, the second path can be explored in exactly the same way as described, and only 2–3 new isocratic and gradient runs are needed to fully explore these additional possibilities.

Another option is to explore that which we have called the *generalized path*, which is simply the pathway connecting both optima in the binary solvent systems. Note that both optima may have been obtained by application of different criteria (e.g., different CRFs or different weighting coefficients in the same CRF, or differently constrained solutions in the optimal Pareto front), according to the chromatographer's goals and decisions, in order to exploit fully the possibilities of both binary solvent mixtures. The generalized path may also provide good solutions in isocratic mode so that this type of separation would be always explored after completing the pseudo-binary retention model corresponding to this path, providing a rapid, easy exploration of the selective multisolvent gradient elutions (SMGE) by use of the same optimization engine developed for binary solvent mixtures.

This means that all possibilities of elution in multi-solvent systems can be explored efficiently in a fully automated manner. All experimental data obtained during the whole process is reused so that the work of the chromatographer is optimized, contrasting with the days or weeks of work usually devoted to the experimental development of ternary solvent elutions.

Table I
Isocratic Retention Volumes (mL) of Peaks in the Mixture to be Separated with Methanol and Acetonitrile as Modifiers Used to Build the Initial Retention Models

		Percentage of modifier in the mobile phase							
		Methanol				Acetonitrile			
Peak code	Compound	10%	40%	60%	80%	10%	30%	50%	70%
1	Benzene	27.71	6.04	2.17	1.11	25.49	6.02	2.06	1.16
2	Benzophenone		19.7	3.16	1.15		16.82	2.96	1.29
3	Buthylparaben		20.46	2.86	1.03		10.31	1.87	0.97
4	Diethylphthalate		11.23	2.04	0.9		10.17	2.13	1.07
5	Dimethylphenol		5.33	1.65	0.92	36.54	4.08	1.38	0.91
6	Dimethylphthalate		3.08	1.13	0.81	32.41	3.21	1.26	0.87
7	Ethylparaben		3.88	1.25	0.81	32.08	2.6	1.04	0.8
8	Methylparaben	22.54	2.05	0.98	0.77	10.79	1.57	0.88	0.75
9	*p*-Cresol	17.81	2.63	1.17	0.82	12.62	2.22	1.05	0.81
10	Phenol	5.85	1.54	0.94	0.77	4.91	1.46	0.92	0.77
11	Propylparaben		8.64	1.78	0.9		5.01	1.35	0.87
12	Toluene		14.11	3.61	1.38		12.64	3.09	1.42

Table II
Retention Times (min) for Peaks in the Mixture Eluted in Gradient Mode and Used to Calibrate the Retention Models

		Modifier in the mobile phase			
		Methanol		Acetonitrile	
Peak code	Compound	Gradient A	Gradient B	Gradient C	Gradient D
1	Benzene	9.52	9.51	7.97	8.51
2	Benzophenone	11.68	10.64	9.7	10.04
3	Buthylparaben	11.56	10.38	8.7	7.57
4	Diethylphthalate	10.7	9.68	8.85	8.43
5	Dimethylphenol	9.43	8.79	7.2	6.05
6	Dimethylphthalate	8.38	7.97	6.76	5.64
7	Ethylparaben	8.84	8.32	6.4	4.78
8	Methylparaben	7.05	7.26	5.17	4.2
9	*p*-cresol	7.32	7.68	5.75	4.79
10	Phenol	4.72	6.09	4.25	4.31
11	Propylparaben	10.32	9.35	7.6	5.89
12	Toluene	11.63	11	9.5	10.53

* Gradients A and C were linear gradients from 5% to 95% of modifier executed in 15 minwith a flow rate of 1.0 mL/min. Gradient B was a gradient from 5% to 95% of methanol in 30 min following a curve 3 in the Waters controller and a flow rate of 0.5 mL/min. Gradient D was a gradient from 54% to 91% of acetonitrile in 28 min following curve 3 in the controller, with a flow rate of 0.2 mL/min. All runs were executed at 40°C.

Case study

In order to exemplify how a Pseudo-3D separation is optimized, a polarity mixture including 12 compounds was used and tested first with binary solvent mixtures. To build the initial retention models for peaks in the mixture, some isocratic injections were made with different percentages of modifier. A pair of gradient runs was also developed with each modifier to re-calibrate the retention models. The experimental data used to build and re-calibrate the binary retention models are summarized in Tables I and II. Although it is evident from Table I that many isocratic retention data are missed because of excessive retention, the re-calibration of the retention model with complimentary gradient data allows modeling retention in the 5–95% modifier range accurately.

Using these retention models, several simulations were carried out with the different modules of the PREGA tool trying to develop good binary solvent separations. Flow rate was explored in the range 0.2–3 mL/min. The critical

resolution criteria and the chromatographic response function proposed by Berridge (36) were used as objective functions in that optimization process. The results were quite disappointing. The best isocratic separations did not resolve all the peaks in a reasonable time (75 min in the case of ACN and more than 150 min when using methanol). In elutions with methanol, the pair of peaks 1 and 5 as well as the pair of peaks 2 and 3 appears very difficult to separate except with longer runtimes and small flow rates of mobile phase, very far from the optimum flow rate values expected for the column in use. When using ACN, peaks 3 and 4 are usually strongly overlapped. Also gradient simulations show clearly unsatisfactory results, especially when methanol acts as the modifier. In this case, again pairs 1–5 and 2–3 appear overlapped, and only gradient runs using very long runtimes (>100 min) and small flow rates enable complete separation of the mixture. Acetonitrile performed somewhat better although clearly inadequate for quantitative purposes.

Preparing for exploration of ternary solvent elutions

At this point, the conclusion was that none of the modifiers tested is able to provide satisfactory separation of the mixture components (either in isocratic or in gradient mode) within a reasonable time range. Also, the small flow rates suggested by the simulation, CAMD would make advisable to change to a microbore column, but it was decided to explore ternary solvent elutions before introducing other changes in the chromatographic system. It is important to realize that no additional experimental runs were made to derive these conclusions. Retention models of both tested solvent systems indicated some differences in selectivity, thus, exploring ternary solvent mixtures formed by methanol, acetonitrile, and water may be worthwhile. To this end, the Pseudo-3D module in PREGA was applied by entering the two binary retention models available for the mixture. The same objective function (the CRF of Berridge) was used for both systems. Here, the application simply evaluates the best isocratic separation in each binary solvent system and gives a graphical view of the results, as shown in Figure 2. Binary solvent mixtures are represented as thick lines of length equivalent to the range of modifier modeled in the binary solvent. Two dotted lines connect the optimal isocratic conditions to the equivalent isoelutropic conditions with the other solvent system. These end points are calculated by use of the simulation outputs produced during calculation of the isocratic optima and thus depend on the most retained peak. Along these lines, isocratic elutions will provide almost constant runtime, enabling exploitation of the differences in selectivity promoted by the mixture of the three solvents. These differences can be roughly estimated by comparing the relative retention of peaks at both extremes of each isoelutropic path. However, not only isocratic elutions are of interest along these isoelutropic paths, and isocratic multisolvent elution programs (IMP) can be explored by simulating elution programs starting at any point inside the paths and using any program shape to exploit the existing differences in selectivity. Note that given the isoelutropic character of these paths, the programmed elutions can be executed in both directions, which sometimes provides interesting findings. To help identify programmed elutions along these paths in the triangle,

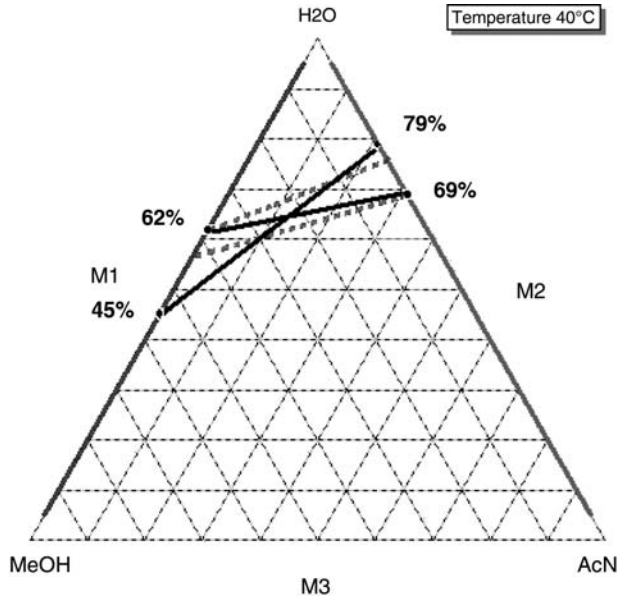

Figure 2. Isoelutropic and generalized paths (see text) defined departing from binary solvent data with methanol and acetonitrile as modifiers.

the solvent systems are labeled as M1, M2, and M3. In this way, we can define programmed elutions from M1 to M2 or vice versa.

In the case study, the optimum for the methanol–water solvent system appeared at 62% water (which corresponded to a 24% acetonitrile–water isoelutropic end mobile phase), whereas for the acetonitrile–water system side it occurred at 69% water (which corresponded to a 43% methanol–water isoelutropic end).

Additionally, a non-isoelutropic path can be defined by connecting both optimal binary solutions (the solid line in Figure 2 showing the path from 62% water–methanol to 69% water–acetonitrile), which we have called a "generalized path". Sometimes, both binary solvent systems produced very similar optimal solutions in terms of percentage water, as in the case studied here when Berridge's CRF is used as the objective function. This means that the region between both isoelutropic paths remains quite narrow, as shown in Figure 2. In such situations, it can be expected that the differences in retention behavior for peaks in the mixture will be relatively small when comparing the isoelutropic and the generalized paths. In these cases, only one of these paths, usually the generalized one should be selected for further study in order to reduce as far as possible the experimental work needed to continue the process. However, it should be noted that the starting and ending points in Figure 2 correspond to "optimal solutions" and thus strongly depend on the objective function chosen, and sometimes other objective functions provide "optimal solutions" that can be very different. The generalized path for the mixture considered when the critical resolution objective function (without strong constraints on runtime) was applied to each binary solvent mixture is also shown in Figure 2 (the solid line showing the path from 45% of M1 to 79% in M2). In that case, differences in retention between both optima are large and therefore (apart from searching for a good isocratic

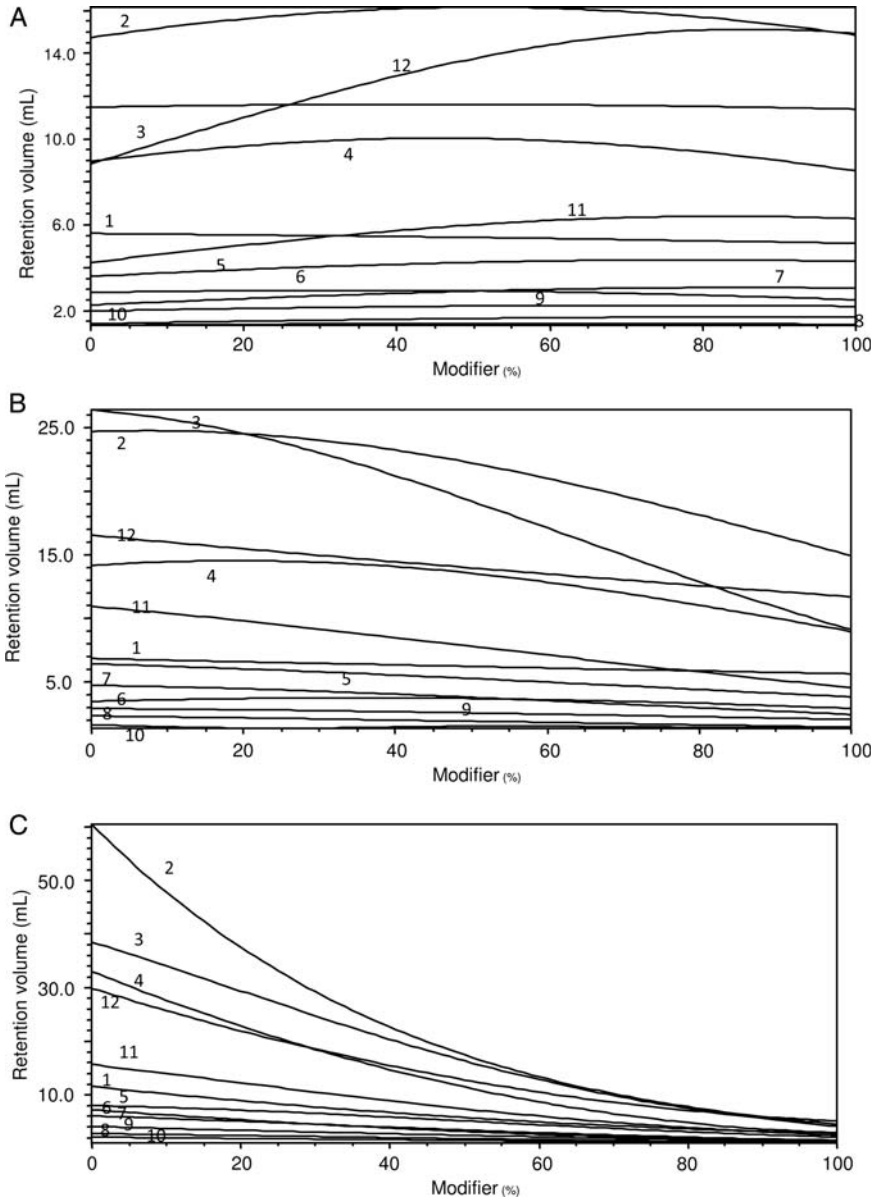

Figure 3. Retention models for isoelutropic path (A) and generalized paths (B) path from 69% water–acetonitrile to 62% water–methanol, and (C) path from 45% water–methanol to 79% water–acetonitrile, in Figure 2.

separation in this generalized path) the most obvious approach would involve exploration of selective multisolvent gradient elutions.

As already mentioned, the generalized paths are often the only ones experimentally explored. However, to clearly demonstrate the approach, also one isoeluotropic pathway was explored; the results are summarized in the following, and the retention models corresponding to these elution paths are shown in Figure 3. These retention maps were produced by running three isocratic elutions (at 25%, 50%, and 75% A) inside each path and the retention data obtained were used to build the pseudo-ternary retention models. We can handle each solvent system point inside these paths as binary mixtures of the solvents defined at the extreme points in the path. With binary pumps, these experiments are more easily handled by assuming one extreme (e.g. the departing point) means 100% of solvent A, where solvent A is the particular binary mixture corresponding to this point With a CAMD tool such as PREGA, the real ternary composition in these experiments is also available. This makes data collection easy with an apparatus based on three or four-solvent low pressure mixing pumps. As can be seen in the retention maps of Figure 3 in isoeluotropic paths, it is not unusual to observe non-linear retention behavior for some peaks.

Exploring Pseudo-3D simulation solutions

The possibilities of ternary isocratic elutions were explored by use of the retention models in Figure 3. The optimum isocratic separation encountered in the isoeluotropic M2 to M1 path in

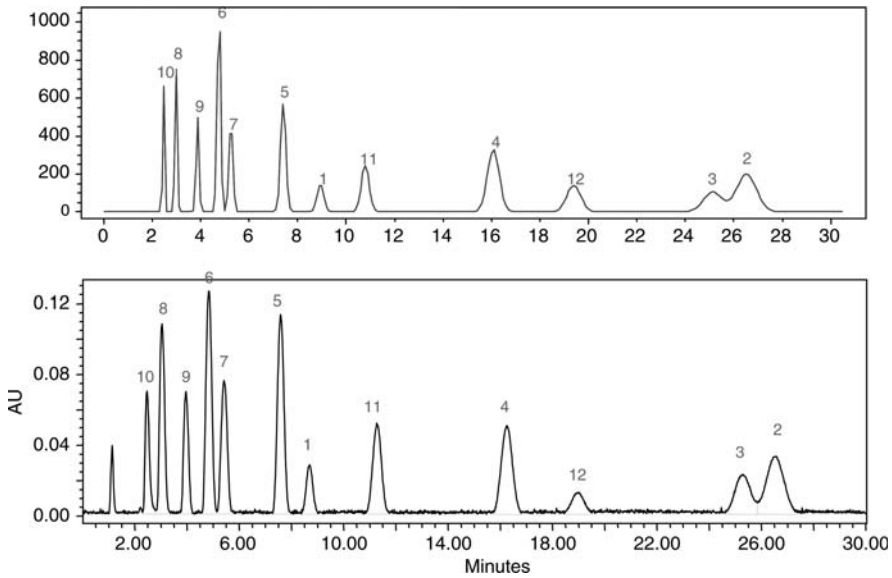

Figure 4. Optimum simulated and experimental isocratic ternary elution of components in the mixture. Composition of mobile phase: 7.7% acetonitrile, 32.3% methanol and 60.0% of water. Flow rate 0.6 mL/min. Temperature 40°C.

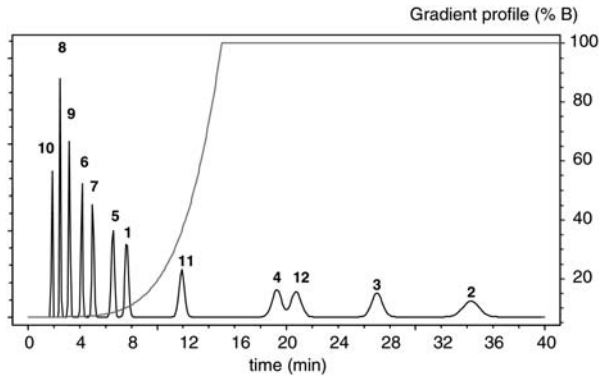

Figure 5. Simulated isocratic multisolvent programmed elution corresponding to a curve 10 from 35.3% MeOH–1.7% ACN–63.0% water to 24% ACN–76% water in 15 min. Flow rate 0.9 mL/min, temperature 40°C.

Figure 2 is shown in Figure 4 and compared with the experimental run in the proposed conditions. This separation corresponds to 7.7% acetonitrile–32.3% methanol–60% of water. In the generalized path, which starts at 62% of water, a quite similar separation, in terms of resolution between peaks and runtime, can be obtained using a mobile phase composed of 0.9% acetonitrile, 36.2% methanol, and 62.2% of water. The other isoeluotropic path was unable to provide satisfactory separation of all peaks in the mixture. Something similar occurred with isocratic separations in the generalized path from 45% M1 to 79% M2, where good isocratic separations only appeared at the price of unacceptably large runtimes.

Isocratic multisolvent programmed elutions (IMP) were also simulated along the isoelueotropic paths in Figure 2. Quite good optimal separations were obtained, although the runtimes were longer (35–40 min) than with the readily available optimum isocratic separation, and therefore these optimal solutions were not tested experimentally. As an example of this type of separations Figure 5 shows the optimal curved shaped IMP elution along the isoeluotropic paths. The study of the

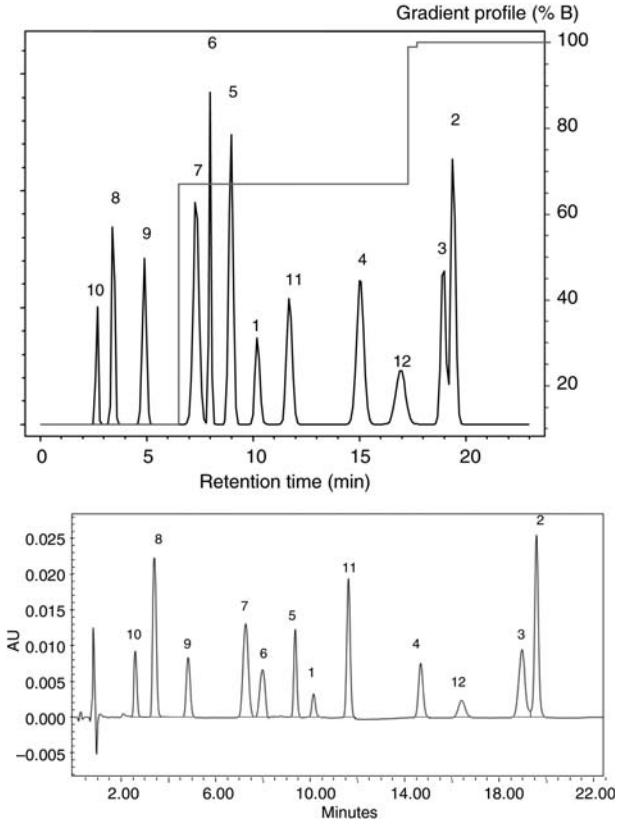

Figure 6. Simulated and experimental selective multisolvent gradient elution in the generalized path of 45% M1 to 79% M2 (see Figure 2). Elution program: stepwise from 18.7% ACN–6.1% MeOH–75.2% water to 6.5% ACN–38.0% MeOH–55.5% water at 6.5 min, then to 54.9% MeOH–45.1% water at 17.3 min. Flow rate 0.8 mL/min, temperature 40°C.

generalized path from 45% M1 to 79% M2 in Figure 2 provided the most satisfactory solutions in the study. The optimal linear and curved selective multisolvent gradient elutions allowed

separation of all the peaks in the mixture in less than 25 min. The optimum stepwise gradient showing the shorter runtime was selected as the best solution in the study and tested experimentally to check the practical validity of the CAMD tool developed. The excellent agreement between simulated and experimental chromatograms for that optimum is shown in Figure 6.

Conclusion

The use of specifically developed computer assisted method development tools may greatly alleviate the difficulties associated with the optimization of multisolvent elutions in reversed phase liquid chromatography, thus allowing exploitation of this powerful analytical tool for practitioners. If the CAMD tool is designed to limit the experimental work and to maintain a common interface with binary solvent optimization tools, the optimization of ternary solvent elutions becomes very easy and straightforward. Here we have developed and tested one such tool, specifically aimed to reuse all the data obtained in the failed optimization of binary solvent systems for easy exploration of all the separation modes available in multisolvent systems by selecting several promising elution paths in the solvent selectivity triangle for the system. Only a few experimental isocratic and gradient runs are needed to build accurate and robust retention models that allow the unattended optimization of separations in a few minutes. Finally, the most promising separation is processed to test the real validity of proposed solutions and the ternary solvent chromatographic procedure becomes ready to be applied in routine analysis.

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