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Journal of Chromatography A, 1121 (2006) 219–227
JOURNAL OF CHROMATOGRAPHY A
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Retention modelling in ternary solvent-strength gradient elution reversed-phase chromatography using 30 mm columns

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Received 22 September 2005; received in revised form 7 April 2006; accepted 10 April 2006
Available online 18 May 2006

Abstract

An optimization strategy for ternary solvent-strength gradient elution RP chromatography is described in which a two-dimensional model of gradient time (2 levels) against ternary proportions of organic modifiers (4 levels) was constructed. From the resolution surface the optimum ratio of organic modifiers could be selected. Excellent retention time and acceptable peak width and resolution simulations were obtained. The separation could be further optimized from the same input data by using a standard one-dimensional model in order to optimize for gradient slope, duration and shape. Excellent retention time and acceptable peak width and resolution simulations were obtained (<1, 2 and 6% error, respectively).

Keywords: Computer optimization/prediction; Ternary solvent-strength gradient chromatography; Rapid reversed-phase LC analysis; Short columns; Computer modelling software

1. Introduction

The use of computer simulation software to predict retention behaviour and to optimize chromatographic separations has now become a pivotal tool for the chromatographic method developer [1]. Commercially available computer modelling/prediction software packages, while originally designed for modelling analytical scale reversed-phase LC separations, have now been expanded into such areas as capillary electrophoresis [2], gas chromatography [3–6], ion pair chromatography [7], scale up from analytical to preparative scale separations [8], enantiomeric separations by chiral LC stationary phases [9], ion chromatography [10] and as an education tool [11].

In the RP-LC arena, the use of computer modelling packages has found greatest success not only in the separation of small molecule pharmaceuticals including synthesis impurities and degradation products of widely differing polarities [12–19], but also peptide/tryptic digests and protein mixtures [20–23], oligonucleotides [23], metabolites [18,24], complex mixtures of active compounds from plant origin [25–30], environmental pollutants [31–33] and robustness validation of LC methodologies for routine QC analysis [34].

One of the major reasons for the widespread use of these separation design packages resides in their excellent prediction accuracy for analyte retention and resolution [10,12,35,36] and the flexibility of the software, which can be used to model isocratic or gradient separations as a function of variables such as percentage organic, gradient time, gradient steps, pH, temperature, ion pairing reagent concentration or ionic strength in a continuous way.

The use of computer modelling is extremely attractive, as only limited input data is required in order to rapidly obtain accurate optimum separation conditions. In many cases the rate limiting stage of the process is peak tracking/assessment. The simplest way is to use peak areas. However, a more elegant approach is to use diode array UV spectrometry in conjunction with extracted ion mass spectrometry.

In addition to the one-dimensional modelling described above, some software packages can now accurately perform two-dimensional modelling, i.e. simultaneous variation of any two-separation variables for a chromatographic procedure. Examples include gradient time (t_G) versus pH, percentage organic (%B)

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0021-9673/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2006.04.073

versus pH, t_G versus temperature and ionic strength versus temperature [1].

The modelling flexibility of these software programmes enables the chromatographer to develop their own/company method optimization strategies. A typical approach favoured by many chromatographers is to simultaneously model the selectivity of temperature and gradient steepness on a single selected RP column [37]. This two-dimensional approach has a much more pronounced effect on the separation selectivity than the additive effect of the two individual variables [38]. The t_G-temperature model is also the easiest to carry out automatically in an unattended mode.

The use of selectivity differences between the organic modifiers acetonitrile (MeCN), methanol (MeOH) and tetrahydrofuran (THF) in RP binary gradient chromatography is well documented [1,10]. Fig. 1 highlights the large selectivity differences that can be obtained using binary gradients of aqueous buffer and either MeCN, MeOH, THF, ethanol, 2-methoxyethanol or propan-2-ol in the separation of a multicomponent mixture containing 10 hydrophilic and lypophilic bases and two neutral compounds [39]. However, even with this comprehensive approach, certain separations can still remain intractable with binary gradient chromatography. Difficult separations can often be resolved when either ternary isocratic [35,39–44] or ternary gradient chromatography [45–47] is employed.

Numerous groups have produced theoretical descriptions of retention in linear binary gradient elution chromatography and these descriptions have been subsequently extended to include ternary gradients [45–47]. There are two types of ternary gradient chromatography defined by Jandera [49] as ternary solvent-strength and combined selectivity-solvent-strength as depicted in Fig. 2a and b. In the case of the former, the ratio of the concentrations of two organic modifiers is kept constant; whilst the sum of the two concentrations is changed (i.e. the solvent-strength increases, see Fig. 2a). In the latter case the ratio of the two organic modifiers and the sum of their concentrations changes simultaneously during gradient elution (see Fig. 2b).

This paper will deal exclusively with ternary solvent-strength gradient chromatography (where the ratio of the two organic modifiers is kept constant, see Fig. 2a); the effect of the sum of the two organic concentrations on the analyte retention is principally the same as in binary gradient chromatography. Therefore, a relationship similar to that employed in binary gradient chromatography can be modelled to describe the retention behaviour in ternary solvent-gradient chromatography [45].

Jandera [49,50] have previously reported a rational approach to the optimization of ternary solvent-strength gradient chromatography, however, to date there have been very few applications of its use. The strategy involved the selection of the appropriate ratio of the two organic modifiers in the ternary mobile phase composition followed by a gradient time/slope/shape optimization. Despite this elegant solution to the problem of "what two organic modifiers should be selected?" and "what will be their optimum ratio?" many workers still revert to a laborious trial and error solution to these problems.

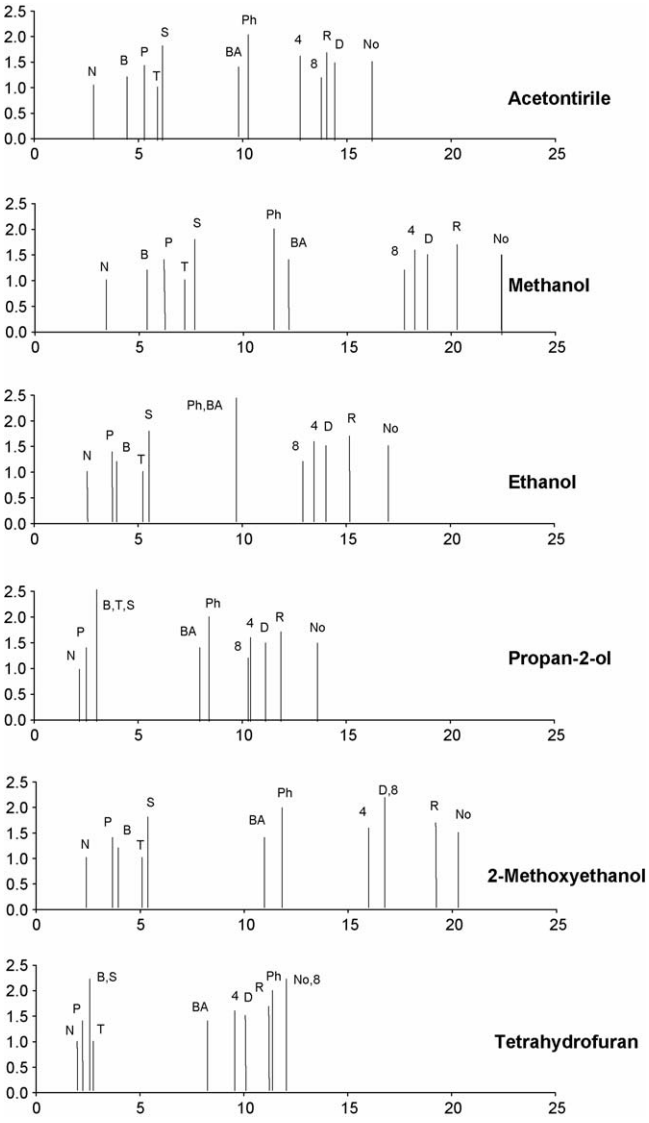

Fig. 1. The effect of organic modifier in binary gradient elution chromatography on the separation of 10 basic analytes and two neutral components. Hypersil GOLD C18 150 mm × 3 mm, 5 μm, 60 °C, 0.43 mL/min, t_G = 20 min, 5 min hold, gradient range 3.3–65% organic, 10 mM KH_2PO_4 pH 2.7 buffer. Peak identification, N, nicotine; B, benzylamine; P, procainamide; T, AR-D080301; S, salbutamol; BA, benzylalcohol; Ph, phenol; 4, AR12495; 8, AR-C68397; R, AR-R12924; D, diphenhydramine; No, nortripyline. For analyte structures see Ref. [48].

This paper seeks to evaluate the use of the "Jandera" approach to the optimization of ternary solvent-strength gradient chromatography using a commercially available chromatography modelling software package to rapidly and accurately predict and optimize the separation of an eight-component mixture using the increasingly popular approach of employing a short column (30 mm × 3 mm) operated at elevated temperature (60 °C) and high linear flow velocities (2 mL/min). The paper will discuss the applicability of simulation software to accurately model the retention, peak width behaviour and resolution using a two-dimensional model of % MeOH in MeCN versus gradient time. Once the appropriate ratio of MeOH in MeCN had been selected the model was used to optimize the duration,

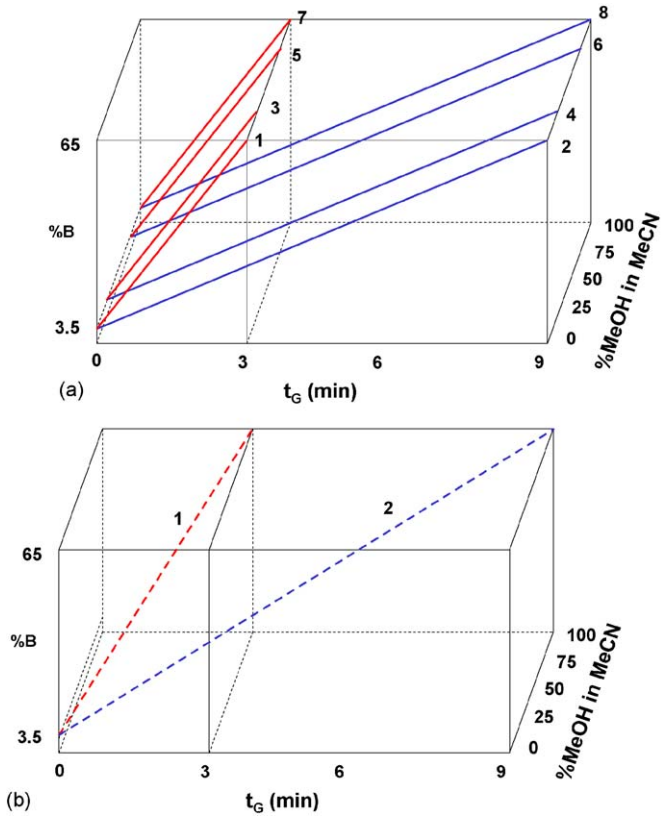

Fig. 2. Comparison of ternary gradient elution chromatography modes. (a) Graphical plot of the model t_G vs. ternary solvent-strength compositions with 2×4 experiments, running eight gradients from 3.3 to 65%B, with mobile phase B consisting of mixtures of MeOH in MeCN in the following compositions 0, 25, 75 and 100% MeOH in MeCN. Gradient runs are numbered to correspond to those described in Table 2. (b) Graphical plot of a model t_G vs. ternary combined selectivity-solvent-strength composition, running two gradients (run 1 and 2 depict 3 and 9 min gradients, respectively) from 3.3 to 65%B, initial mobile phase B consisting of 0% MeOH in MeCN and the final mobile phase B consisting of 100% MeOH in MeCN.

slope and the shape of the gradient profile. The predicted retention times, peak width and resolution were compared to those obtained experimentally.

2. Experimental

2.1. Chemicals, compounds and reagents

MeCN and MeOH (HPLC grade) were supplied by Romil Ltd. (Cambridgeshire, UK). Water was provided by a Milli-Q-plus 185 ultra pure water system (Molsheim, France). The structure of the AZ compound and its potential synthetic impurities (1–7) are proprietary information. Individual stock solutions of the AZ compound (**AZD**) and its potential synthesis impurities (1–7) were prepared at a concentration of 0.5 mg/mL in 1:1 (v/v) MeCN/water, a mixture of the eight AZ compounds was prepared by mixing equal volumes of the individual solutions.

2.2. Instrumentation

HPLC separations were performed on an Agilent Technologies 1100 LC with ChemStation v. 9.03 LC software (Agilent Technologies, Cheadle, Cheshire) equipped with a quaternary pump, a vacuum degasser, cooled autosampler, temperature controlled column compartment, diode array detector and Agilent 1100 (SL) mass selective detector (MSD). Data acquisition was performed using the Agilent ChemStation.

2.3. Liquid chromatography

The four lines of the quaternary pump consisted of mobile phases A: 500 mM ammonium acetate pH 4.5; B: water; C: MeCN and D: MeOH. At least 10 column volumes of the appropriate mobile phase were flushed through the column prior to commencing the testing. The 3 μm BetaBasic C18 30 mm × 3 mm column was new as supplied by the manufacturer (Thermo, Runcorn, UK). All analyses comprised duplicate 5 μL injections. Other conditions included: flow rate of 2 mL/min, thermostatted oven operating at 60 °C, detection at 254 nm and total ion scanning with the MSD (range 100–700 Da). MS conditions consisted of electrospray positive ionisation, 70 V fragmentor voltage, 350 °C gas temperature, 12 L/min drying gas flow, 35 psig nebuliser pressure and a 3000 V capillary voltage. The first disturbance of the baseline on the injection of methanol was used as dead time marker. The system dwell volume was experimentally determined as 1100 μL. Gradients of 3 and 9 min (3.3–65% total organic) were performed using 0, 25, 75, 100% MeOH in MeCN (v/v). A typical ternary solvent-strength gradient is shown in Table 1. Peak tracking was accomplished primarily by extracted single ion monitoring in conjunction with DAD spectroscopy and comparison of UV spectral matches with a pre-constructed spectral library from individual analyte injections. Integrated data, which included retention time, peak area and peak width at half-height, was exported into Microsoft Excel and arranged in a table, in which one peak was located with all its data in one single horizontal line. The table was then copied and pasted into the simulation software.

2.4. Software

2.4.1. Chromatography simulation/prediction software

Modelling was performed using Drylab 2000 plus version 3.50 (Molnar-Institut Berlin, Germany). The ternary solvent-strength gradient model used a 2-variable model consisting of gradient time (two runs, 5% extrapolation limit, no parameter transformation, retention time to retention factor transformation with a retention time linear spline model) and percentage MeOH in MeCN (four runs, 5% extrapolation limit, no param-

Table 1
Typical ternary solvent-strength gradient conditions for a 9-min gradient using a 25:75 (v/v) proportion of MeCN:MeOH

Time (min)	%A	%B	%C	%D	Total % organic
0	10	86.7	0.8	2.5	3.3
9	10	25	16.3	48.7	65
10	10	25	16.3	48.7	65
11	10	86.7	0.8	2.5	3.3

Eluents A: 500 mM ammonium acetate pH 4.5; B: water; C: MeCN; D: MeOH. Post time = 10 column volumes + dwell time (i.e. 5 min).

eter transformation, retention time to retention factor transformation with a retention time linear spline model), a peak width to efficiency transform was employed and gradient/column optimization were selected. A dead time of 0.13 min was determined at a flow rate of 2 mL/min. A pore diameter of 10 nm, A-value of 0.80, extra-column volume of 0.032 mL and a time constant of 0.1 s were used throughout the modelling.

2.4.2. log D and pK_a predictions

Predications of log D and pK_a were calculated using Advanced Chemistry Development software programme version 6.0 (Toronto, Canada).

3. Results and discussion

3.1. Background

Previous retention modelling and optimization studies had already proven that a range of C18 column chemistries (i.e. BetaBasic C18, Symmetry C18, ACE C18) failed to separate the AZ compound (**AZD**) and its seven potential synthesis impurities (impurity peaks 1–7) using a binary gradient composition of MeCN/50 mM ammonium acetate pH 4.5 on 30 mm × 3 mm columns at a flow rate of 2 mL/min and 60 °C. A mobile phase pH of 4.5 was selected, based on log D and pK_a estimations as calculated using ACD software, in order to chromatograph the compounds in their ion-suppressed mode.

The separation selectivity term in the resolution equation can be dramatically influenced by many parameters such as changing the gradient time and gradient shape/slope, stationary phase chemistry or by exploiting differing analyte–mobile phase interactions by changing the type of organic modifier in the mobile phase. While the approach of changing the stationary phase chemistry may seem attractive, many non-C18 phases possess problems of column bleed, low column life times and poorer batch-to-batch reproducibility than standard monomeric bonded C18 phases. Hence, the latter approach of selectivity enhancements afforded by ternary solvent-strength gradient elution chromatography (using varying MeOH and MeCN compositions with the BetaBasic C18 column) was investigated as a complimentary method development/optimization approach.

3.2. Development of a two-dimensional model of percentage of MeOH in MeCN versus gradient time

Scouting gradients used for input data are typically run from low to high % organic modifier in order to be sure of retaining polar and eluting non-polar analytes. The AZ compound and its impurities all possessed fairly high log D values between 1 and 4 at pH 4.5 hence the initial % organic could have been higher in order to utilize the whole of the gradient run more effectively. However, in order to prove the strategy was applicable to samples of unknown lipophilicity and so simulate a real life situation, the wide gradient range approach was employed.

The software "Drylab 2000 plus" is widely used within the pharmaceutical and chemical industries for HPLC method development and for the routine method control; hence it was selected in order to evaluate the approach of ternary solvent-strength gradient elution chromatography. The software package allows the development a two-dimensional model for the two variables of gradient time and % MeOH in MeCN, the model accepts 6–16 experimental input runs (i.e. 3 and 9 min gradients run at 2–8 differing ratios of MeOH in MeCN. We selected a 2 × 4-model with 0, 25, 75 and 100% MeOH in MeCN (see Table 2). Peak assignment of the eight experimental input gradients was achieved by using MS extracted ion monitoring. After this task was complete the peak assignment, retention times and peak areas were entered into the data entry section of the software programme. The dwell volume of the quaternary LC system had previously been determined by running three gradients at 3, 6 and 9 min and developing a retention model from the 3 and 9 min runs and then predicting, what the retention times should be for the 6 min run, the dwell volume in the data entry section was then adjusted manually until the predicted and actual retention times matched with the least deviation.

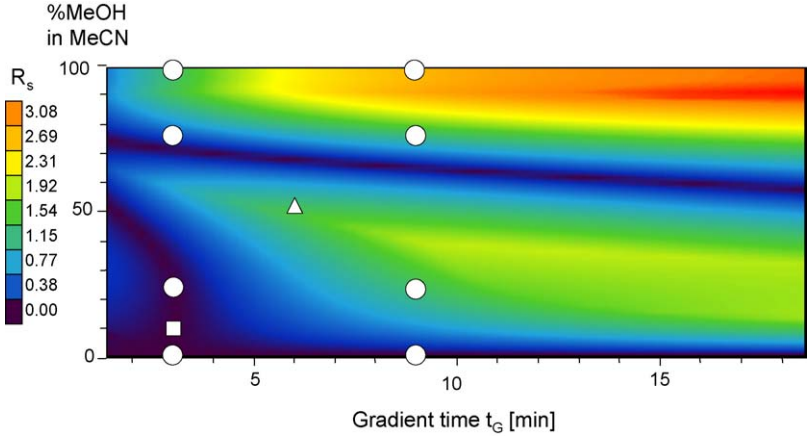

Fig. 3. Two-dimensional resolution map of the proportion of MeOH in MeCN against gradient time for the separation. White circles = experimental input values, white square = conditions corresponding to Fig. 4, white triangle = conditions corresponding to Fig. 5. In Fig. 4 we see only six peaks, as there are two co-eluting pairs. These peaks are well resolved in Fig. 5 and eight peaks can be resolved.

Table 2
Eight experimental input runs for the retention modelling

Compound	Gradient 1: t_G = 3 min, 0%MeOH			Gradient 2: t_G = 9 min, 0%MeOH		
	Retention time (min)	Peak area	Peak width (min)	Retention time (min)	Peak area	Peak width (min)
1	1.617	349.37	0.0415	2.808	353.40	0.0511
2	1.617	349.37	0.0415	2.808	353.40	0.0511
3	1.753	185.88	0.0417	3.159	189.51	0.0511
4	1.999	487.72	0.0411	4.013	487.34	0.0493
AZD	2.193	230.16	0.0424	4.501	230.10	0.0513
5	2.400	246.75	0.0424	5.023	248.35	0.0533
6	2.566	1001.24	0.0548	5.359	271.12	0.0548
7	2.566	1001.24	0.0548	5.463	723.60	0.0552
	Gradient 3: t_G = 3 min, 25%MeOH			Gradient 4: t_G = 9 min, 25%MeOH		
	Retention time (min)	Peak area	Peak width (min)	Retention time (min)	Peak area	Peak width (min)
1	1.766	353.72	0.0452	3.131	249.63	0.0528
2	1.766	353.72	0.0452	3.256	109.28	0.0518
3	1.896	187.75	0.0421	3.506	190.65	0.0541
4	2.239	485.96	0.0420	4.615	487.43	0.0520
AZD	2.445	221.80	0.0428	5.150	222.65	0.0548
5	2.647	215.97	0.0431	5.675	211.84	0.0567
6	2.785	220.26	0.0439	6.034	229.87	0.0581
7	2.846	713.21	0.0439	6.189	700.24	0.0583
	Gradient 5: t_G = 3 min, 75%MeOH			Gradient 6: t_G = 9 min, 75%MeOH		
	Retention time (min)	Peak area	Peak width (min)	Retention time (min)	Peak area	Peak width (min)
1	2.098	261.76	0.0436	3.842	265.10	0.0607
2	2.219	313.58	0.0472	4.294	108.21	0.0607
3	2.219	313.58	0.0472	4.222	204.67	0.0607
4	2.787	504.69	0.0430	6.031	503.40	0.0600
AZD	2.979	229.81	0.0444	6.578	209.51	0.0617
5	3.138	185.28	0.0439	7.037	173.25	0.0608
6	3.258	203.15	0.0442	7.365	195.79	0.0622
7	3.364	697.60	0.0433	7.631	685.59	0.0633
	Gradient 7: t_G = 3 min, 100%MeOH			Gradient 8: t_G = 9 min, 100%MeOH		
	Retention time (min)	Peak area	Peak width (min)	Retention time (min)	Peak area	Peak width (min)
1	2.233	239.92	0.0438	4.117	242.64	0.0642
2	2.454	64.44	0.0439	4.824	66.43	0.0658
3	2.323	191.88	0.0441	4.456	191.49	0.0633
4	2.994	496.20	0.0438	6.600	485.43	0.0622
AZD	3.164	259.99	0.0451	7.104	233.87	0.0633
5	3.303	260.25	0.0446	7.520	253.37	0.0625
6	3.409	298.62	0.0442	7.812	258.00	0.0667
7	3.553	737.59	0.0444	8.174	697.13	0.0642

From the two-dimensional model of % MeOH in MeCN and gradient time (t_G), the software was able to generate a resolution surface (see Fig. 3) from which the optimum % MeOH in MeCN could be determined. It was later found that increasing the number of input experiments by a factor of two (i.e. the addition of 6 and 27 min gradients at 0, 25, 75 and 100% MeOH in MeCN) was not necessary to improve the predictive capabilities of the model.

The extra-column volume factor and the column plate number, respectively were adjusted manually so that the critical resolution for the simulated input data matched that of the experimental data (i.e. 3 and 9 min gradient runs at 0 and 100% MeOH in MeCN typically resulted in <5% error in critical resolution). Once this has been performed, acceptable agreement was obtained over the resolution surface for predicted versus experimental resolution values.

To establish the accuracy of the model, numerous simulations from a variety of positions within the resolution map were compared to experimentally derived chromatograms (see Figs. 4 and 5 for typical examples). The predicted reten-

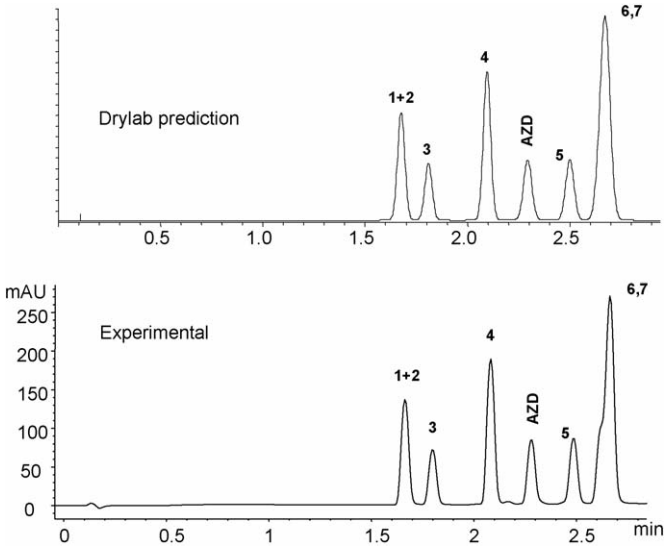

Fig. 4. Predicted vs. experimental chromatograms for a 10% MeOH in MeCN mobile phase composition (white square on Fig. 3), gradient time of 3 min and 3.3–65% total organic gradient.

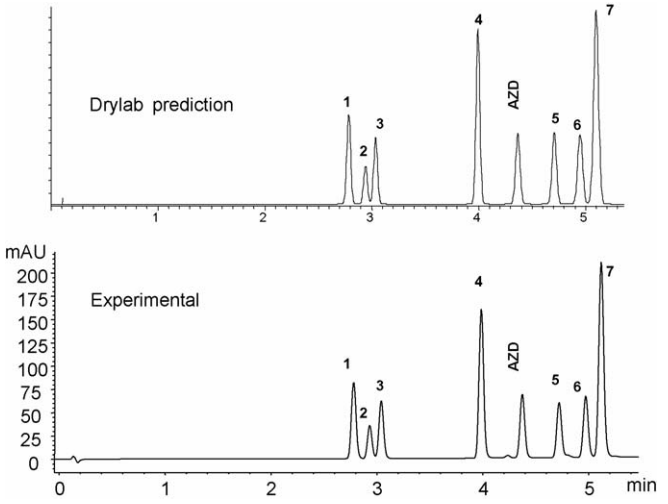

Fig. 5. Predicted vs. experimental chromatograms for a 50% MeOH in MeCN mobile phase composition (white triangle on Fig. 3), gradient time of 6 min and 3.3% to 65% total organic gradient.

Table 3
Experimental retention times and peak width vs. predicted from the two-dimensional ternary solvent-strength model; gradient range 3.3–65% organic 1:9 (v/v) ratio of MeOH/MeCN and a gradient of time of 3 min used

Compound number	t_G = 3 min (10% MeOH in MeCN)							
	Retention time (min)		Difference (min)[a]	% error[b]	Peak width (min)[c]		Difference (min)[a]	% error[b]
	Experimental	Predicted			Experimental	Predicted		
2	1.665	1.673	−0.008	−0.48	0.0419	0.0417	0.002	0.48
1	1.665	1.674	−0.009	−0.54	0.0419	0.0422	−0.003	−0.71
3	1.799	1.808	−0.009	−0.50	0.0419	0.0424	−0.005	−1.18
4	2.083	2.090	−0.007	−0.33	0.0417	0.0417	0	0
AZD	2.281	2.289	−0.008	−0.35	0.0424	0.0422	0.002	0.47
5	2.487	2.495	−0.008	−0.32	0.0427	0.0428	0.001	−0.23
6	2.664	2.651	0.013	1.33	0.0427	0.0431	0.003	−0.93
7	2.664	2.675	−0.011	−0.41	0.0427	0.0432	−0.005	−1.16

[a] Difference = experimental − predicted retention time.
[b] % error = [(experimental − predicted)/predicted] × 100.
[c] Determined at half height.

Table 4
Experimental retention times and peak width vs. predicted from the two-dimensional ternary solvent-strength model; gradient range 3.3–65% organic 1:1 (v/v) ratio of MeOH/MeCN and a gradient of time of 6 min used

Compound number	t_G = 6 min (50% MeOH in MeCN)							
	Retention time (min)		Difference (min)[a]	% error[b]	Peak width (min)[c]		Difference (min)[a]	% error[b]
	Experimental	Predicted			Experimental	Predicted		
1	2.781	2.787	0.004	−0.22	0.0487	0.0512	−0.025	−4.88
2	2.933	2.911	0.022	0.76	0.0483	0.0487	−0.004	−0.82
3	3.041	3.042	−0.001	−0.03	0.0491	0.0510	−0.009	−3.73
4	3.986	3.987	−0.001	−0.03	0.0487	0.0495	−0.008	−1.62
AZD	4.374	4.376	−0.002	−0.05	0.0493	0.0504	−0.011	−2.18
5	4.722	4.726	−0.004	−0.08	0.0504	0.0513	−0.009	−1.75
6	4.973	4.958	0.015	0.30	0.0511	0.0517	−0.006	−1.16
7	5.119	5.125	−0.006	−0.12	0.0507	0.0528	−0.021	−3.98

[a] Difference = experimental − predicted retention time.
[b] % error = [(experimental − predicted)/predicted] × 100.
[c] Determined at half height.

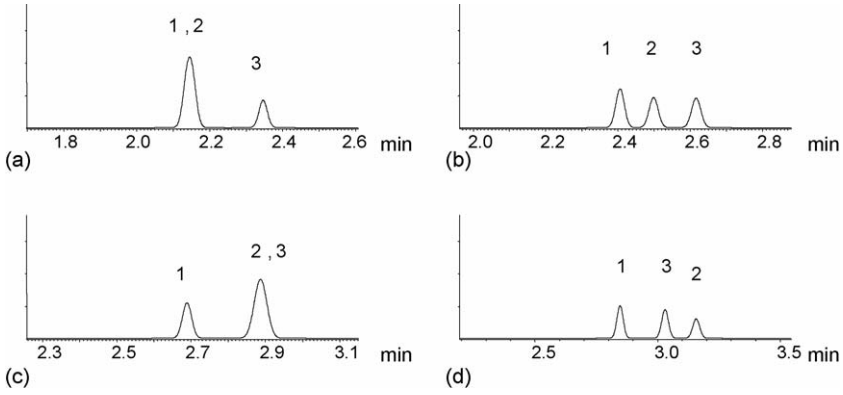

Fig. 6. Effect of percentage MeOH in MeCN and gradient time on the elution order of components 1–3: (a) 10% MeOH in MeCN gradient times <10 min; (b) 20–60% MeOH in MeCN gradient times >6 min; (c) 70% MeOH in MeCN gradient times <13 min; (d) 90% MeOH in MeCN gradient times >3 min.

tion times were in excellent agreement with the experimental ones, retention time errors were typically well below 1% (see Tables 3 and 4) which is well within the accuracy of quaternary LC pumps operating at high flow rates combined with rapid gradient profiles. There was only one result with an error >1% (compound 6 run at 10% MeOH in MeCN at a gradient time of 3 min), this could be explained in that compounds 6 and 7 co-eluted at low MeOH compositions and low gradient times, their retention times were entered with exactly the same value, hence there may have been larger relative inaccuracies (i.e. only a 0.013 min difference) in the retention time input data associated with this compound, which resulted in a less precise retention model. The development of high scanning MS detectors will greatly improve the precision the retention time determinations of closely eluting or co-eluting peaks and hence the accuracy of prediction software.

It is of interest to note the change in the elution order for compounds 1, 2 and 3; at short gradient times (<10 min) and low % MeOH compounds 1 and 2 co-elute whereas with 20–60% MeOH the elution order is 1, 2, 3, at 70% MeOH compounds 2 and 3 co-elute and at 90% the elution order is 1, 3, 2 (see Fig. 6a–d). This knowledge is invaluable as it allows the analyst the opportunity of eluting the minor impurity before the major component aiding quantification. The resolution map (see Fig. 3) showed a maximal resolution ($R_s \geq 4$) in the region of 90% MeOH in MeCN.

While the retention time predictive nature was excellent the prediction of the peak width and the critical resolution was less precise due to inaccuracies in the measurement of the basic parameters. Typically, peak width differences between predicted and experimental values were observed to be <5% (see Tables 3 and 4). The reported resolution accuracy of predictive software programmes, using standard LC columns and linear velocities, varies between 1 and 10% [1,12,35,36] depending on the application, the results obtained in the present study were shown to be typically <3% except for a few situations (see Table 5). The large apparent difference in resolution seen between compounds 5 and 6 with 10% MeOH in MeCN and a gradient time of 3 min and the early eluters with 50% MeOH in MeCN and a gradient of time of 6 min can be attributed to inaccuracies in retention times due to co-elution of the peaks in the input data. Inaccuracies associated with measuring very narrow peak widths (typically less than 2 s) and low retention times, which are generated using small columns, and rapid linear velocities may also be responsible for the higher errors in predicting resolution. Similar retention, peak width and resolution prediction accuracies have also been observed for the ternary solvent-strength modelling of a nine component mixture of acids, neutrals and phenols employing a Hypersil GOLD C18 column and ternary solvent-strength gradient mobile phase mixtures of MeCN and THF [39], results not shown.

3.3. Optimization of gradient slope using ternary solvent-strength gradient chromatography

The two-dimensional resolution map of % MeOH in MeCN versus gradient time highlighted the fact that optimum resolution was obtained at 90% MeOH in MeCN (see Fig. 3). Once

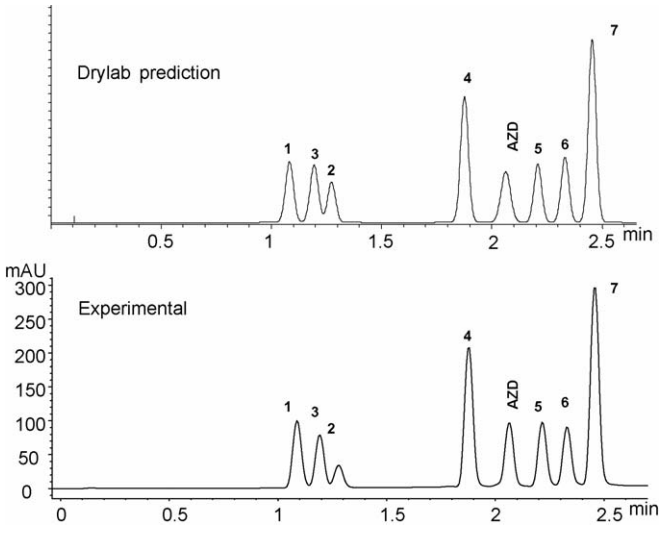

Fig. 7. Predicted vs. experimental chromatograms for a one-dimensional optimized separation using a 90% MeOH in MeCN mobile phase composition, gradient time of 2 min and 25–65% total organic gradient. At 0 min (quaternary mobile phase lines A: 500 mM ammonium acetate pH 4.5) 10%; (B; water) 65%; (C; MeCN) 6.5%; (D; MeOH) 58.5%. This optimized separation further increases the speed of the analysis compared to Fig. 5.

Table 5
Experimental resolution vs. predicted from the two-dimensional ternary solvent-strength model; gradient range 3.3–65% organic

Compound number	t_G = 3 min (10% MeOH in MeCN)				t_G = 6 min (50% MeOH in MeCN)			
	Resolution[a]		Difference[b]	% error[c]	Resolution[a]		Difference[b]	% error[c]
	Experimental	Predicted			Experimental	Predicted		
2, **1**	0.00	0.00	0.00	0	1.83 (1, 2)	1.46	0.37	25
1, **3**	1.89	1.86	−0.01	1.6	1.31 (2, 3)	1.51	−0.20	−13
3, **4**	3.98	3.95	0.02	<1	11.36	11.07	0.29	2.6
4, **AZD**	2.77	2.80	0.03	−1.1	4.65	4.58	0.07	1.5
AZD, **5**	2.84	2.85	−0.08	<1	4.10	4.04	0.06	1.5
5, **6**	2.16	1.83	0.09	18	2.90	2.85	0.05	1.8
6, **7**	0.00	0.00	0.0	0	1.70	1.66	0.04	2.4

[a] Determined at half height.
[b] Difference = experimental − predicted retention time.
[c] % error = [(experimental − predicted)/predicted] × 100.

Table 6
Experimental retention times and peak width vs. predicted from the one-dimensional ternary solvent-strength model; gradient time = 2 min, gradient range 25–65% organic (90% MeOH in MeCN)

Compound number	Retention time (min)		Difference (min)[a]	% error[b]	Peak width (min)[c]		Difference[a]	% error[b]
	Experimental	Predicted			Experimental	Predicted		
1	1.088	1.084	0.004	0.37	0.0458	0.0465	−0.007	−1.51
2	1.280	1.297	−0.017	−1.31	0.0421	0.0458	0.037	−8.08
3	1.193	1.206	−0.013	−1.08	0.0451	0.0455	−0.04	−0.88
4	1.878	1.887	−0.009	−0.48	0.0436	0.0439	−0.003	−0.68
AZD	2.064	2.073	−0.009	−0.43	0.0446	0.0440	0.006	1.36
5	2.216	2.223	−0.007	−0.31	0.0443	0.0440	0.003	0.68
6	2.330	2.347	−0.017	−0.72	0.0442	0.0442	0	0
7	2.457	2.466	−0.009	−0.36	0.0442	0.0446	−0.004	−0.90

[a] Difference = experimental − predicted retention time.
[b] % error = [(experimental − predicted)/predicted] × 100.
[c] Determined at half height.

the optimum organic ratio has been established the gradient editor menu can be selected where the separation can be optimized for gradient time, slope and shape. Using this approach it was feasible to generate a sub 3 min analysis for the parent compound (**AZD**) and its seven potential synthesis impurities by employing a simple linear gradient starting from 25% total organic and employing a gradient slope of 20% total organic (9:1 MeOH/MeCN) per minute over 2 min (see Fig. 7). Comparison of this predicted separation with the actual experimentally derived chromatogram highlighted a good agreement between the predicted and experimental values with errors of less than 1.5, 8 and 13% for retention time, peak width and resolution, respectively (see Tables 6 and 7). Discounting the early eluting peaks, the retention model is less precise due to inaccuracies of the input data as a result of co-eluting peaks, the resultant percentage errors for retention, peak width and resolution are <1, 2 and 6%, respectively.

Table 7
Experimental resolution predicted from the one-dimensional ternary solvent-strength model; t_G = 2 min, gradient range 25–65% organic (90% MeOH in MeCN)

Compound number	Resolution[a]		Difference[b]	% error[c]
	Experimental	Predicted		
1, **3**	1.35	1.55	−0.20	−12.9
3, **2**	1.17	1.18	0.01	<1
2, **4**	8.20	7.77	0.43	5.5
4, **AZD**	2.48	2.49	−0.01	<1
AZD, **5**	2.01	2.01	0	0
5, **6**	1.51	1.52	−0.01	<1
6, **7**	1.69	1.71	−0.02	−1.2

[a] Determined at half height.
[b] Difference = experimental − predicted retention time.
[c] % error = [(experimental − predicted)/predicted] × 100.

4. Conclusion

This paper has demonstrated the flexibility of commercially available computer simulation/prediction software in its ability to model/optimize the ternary solvent-strength gradient chromatographic separation of a drug candidate and its seven synthetic impurities. A two-dimensional retention model, describing the effect of gradient time and the ratio of MeOH:MeCN in the mobile phase on the separation, was constructed from eight experimental gradient runs. Excellent agreement of retention time and reasonable agreement of peak width and resolution predictions with those of experimental runs was achieved over the entire surface of the resolution map. Once the optimum composition of MeOH:MeCN had

been established, the separation could be optimized further, for gradient time and slope as in a standard one-dimensional gradient optimization protocol. Excellent retention time and acceptable peak width and resolution agreement was obtained between the predicted and actual experimental chromatograms.

Ternary solvent-strength gradient optimization using commercially available modelling software has been shown to be a powerful tool, which can be successfully employed in method development strategies for the resolution and optimization of intractable binary gradient elution separations. It is envisaged that ternary solvent-strength gradient elution RP chromatography, when used in combination with a limited number of stationary phases with orthogonal separation selectivity, will allow the separation selectivity term in the resolution equation to be fully exploited hence providing a powerful and comprehensive approach to the resolution of difficult mixtures.

Acknowledgement

The authors wish to thank ThermoHypersil (Runcorn, UK) for supplying the BetaBasic C18 column.

References

[1] I. Molnar, J. Chromatogr. A. 965 (2002) 175.
[2] P. Haber, T. Baczek, R. Kaliszan, L.R. Snyder, J.W. Dolan, C.T. Wehr, J. Chromatogr. Sci. 38 (2000) 386.
[3] A. Jayatilaka, C.F. Poole, J. Chromatogr. 617 (1993) 19.
[4] L.R. Snyder, D.E. Bautz, J.W. Dolan, J. Chromatogr. 541 (1991) 34.
[5] J.W. Dolan, L.R. Snyder, D.E. Bautz, J. Chromatogr. 541 (1991) 21.
[6] D.E. Bautz, J.W. Dolan, L.R. Snyder, J. Chromatogr. 541 (1991) 1.
[7] R.C. Kong, B. Swachok, S.N. Deming, J. Chromatogr. 199 (1980) 316.
[8] T. Wennberg, J.P. Rauha, H. Vuorela, Chromatographia 53 (S) (2001) S240.
[9] M. Lammerhofer, P. Di Eugenio, I. Molnar, W. Lindner, J. Chromatogr. B 689 (1997) 123.
[10] I. Molnar, LC–GC Eur. 17 (2001) 231.
[11] R.L. Grob, E.F. Barry, S. Leepipatpiboon, J.M. Ombaba, L.A. Colon, J. Chromatogr. Sci. 30 (1992) 177.
[12] T.H. Hoang, D. Cuerrier, S. McClintock, M. Di Maso, J. Chromatogr. A. 991 (2003) 281.
[13] H.W. Bilke, I. Molnar, Ch. Gernet, J. Chromatogr. A 729 (1996) 189.
[14] R. Bonfichi, J. Chromatogr. A 678 (1994) 213.
[15] L. Wrisely, J. Chromatogr. 628 (1993) 191.
[16] H. Hofmann, I. Molnar, Pharm. Ztg. Wiss. 1 (1992) 137.
[17] P.A. Ryan, B.A. Ewels, J.L. Glajch, J. Chromatogr. 550 (1991) 549.
[18] R.G. Lehman, J.R. Miller, J. Chromatogr. 485 (1989) 581.
[19] J. Fulper, J. Chromatogr. 485 (1989) 579.
[20] J.W. Dolan, L.R. Snyder, LC–GC 17 (1999) S17.
[21] L.R. Snyder, in: W. Hancock (Ed.), New Methods in Peptide Mapping for the Characterization of Proteins, CRC Press, Boca Raton, Florida, 1996, p. 31.
[22] R.C. Chloupek, W. Hancock, B.A. Marchylo, J.J. Kirkland, B. Boyes, L.R. Snyder, J. Chromatogr. A 686 (1994) 45.
[23] A.J.J.M. Coenen, L.H.G. Henckens, Y. Mengerink, S. van der Wal, P.J.L.M. Quaedifleig, L.H. Koole, E.M. Meijer, J. Chromatogr. 596 (1992) 59.
[24] H. Fritsch, I. Molnar, M. Wurl, J. Chromatogr. A 684 (1994) 65.
[25] A.H. Schmidt, I. Molnar, J. Chromatogr. A. 948 (2002) 51.
[26] L. Van Heukelem, C.S. Thomas, J. Chromatogr. A. 910 (2001) 31.
[27] M.L. Hajnos, M. Waksmundzka-Hajnos, K. Glowniak, Acta Chromatogr. 12 (2002) 211.
[28] W. Markowski, K.L. Czapinska, Chem. Anal. (Warsaw) 42 (1997) 353.
[29] W. Metzger, K. Reif, J. Chromatogr. A. 740 (1996) 133.
[30] T.H. Dzido, E. Soczewinski, J. Gudej, J. Chromatogr. 550 (1991) 71.
[31] T.-Y. Liu, A. Robbat Jr., J. Chromatogr. 539 (1991) 1.
[32] W. Markowski, T.H. Dzido, E. Soczewinski, J. Chromatogr. 523 (1990) 81.
[33] D.J. Thompson, W.D. Ellenson, J. Chromatogr. 485 (1989) 607.
[34] I. Molnar, LC–GC Int. 10 (1997) 32.
[35] J.A. Lewis, L.R. Snyder, J.W. Dolan, J. Chromatogr. A 721 (1996) 15.
[36] R. Dappen, I. Molnar, J. Chromatogr. 592 (1992) 133.
[37] J.W. Dolan, L.R. Snyder, N.M. Djordjevic, D.W. Hill, D.L. Saunders, L. Van Heukelem, T.J. Waeghe, J. Chromatogr. A 803 (1998) 1.
[38] L.R. Snyder, J. Chromatogr. B 689 (1997) 105.
[39] F. Scannapieco, M.Sc. Thesis 2004, University of Strathclyde.
[40] J.L. Glajch, J.J. Kirkland, K.M. Squire, J.M. Minor, J. Chromatogr. 199 (1980) 57.
[41] J.W. Weyland, C.H.P. Bruins, D.A. Doornbos, J. Chromatogr. Sci. 22 (1984) 31.
[42] P. Schoenmaker, H.A.H. Billiet, L. de Galan, J. Chromatogr. 218 (1981) 261.
[43] M.R. Euerby, C.M. Johnson, S.C. Nichols, in: S. Görög (Ed.), Proceedings of the 5th Symposium on the Analysis of Steroids, Szombathely, Hungary, 1993, p. 213, Presented at the Fifth Symposium on the Analysis of Steroids, Szombathely, Hungary, May 1993.
[44] M.R. Euerby, J.A. Graham, C.M. Johnson, R.J. Lewis, D.B. Wallace, J. Pharm. Biomed. Anal. 15 (1996) 299.
[45] P. Jandera, L. Petránek, M. Kučerová, J. Chromatogr. A 791 (1997) 1.
[46] P. Jandera, J. Churáček, H. Colin, J. Chromatogr. 214 (1981) 35.
[47] J.J. Kirkland, J.L. Glajch, J. Chromatogr. 255 (1983) 27.
[48] B. Channer, P.U. Uhl, M.R. Euerby, A.P. McKeown, G.G. Skellern, D.G. Watson, Chromatographia 61 (2005) 113.
[49] P. Jandera, J. Chromatogr. 485 (1989) 113.
[50] P. Jandera, J. Liq. Chromatogr. 12 (1989) 117.