3-Dimensional Retention Modelling of Gradient Time, Ternary Solvent-Strength and Temperature of the Reversed-phase Gradient Liquid Chromatography of a Complex Mixture of 22 Basic and Neutral Analytes using DryLab® 2010

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The present paper describes a multi-factorial optimization of three critical HPLC method parameters, i.e. gradient time (t_G), temperature (T), and ternary composition (B1:B2) based on twelve experiments for the separation of twenty-two pharmaceutically relevant analytes. Examining the effect of these experimental variables on critical resolution and selectivity was carried out in such a way as to systematically vary all three factors simultaneously. The basic element is a gradient time–temperature (t_G –T) plane, which is repeated at three different ternary compositions of eluent B between methanol and acetonitrile. The so-defined volume enables the investigation of the critical resolution for a part of the Design Space of a given sample. Multi-dimensional robust regions were successfully defined, graphically depicted and verified. The paper highlights the applicability of this approach for the rapid development of high quality robust LC methodologies.

Keywords: Ternary solvent-strength gradient chromatography; Reversed-phase LC: Computer modelling software; 3-Dimensional model; Robustness of HPLC methods; Method development; Optimization; Quality by Design; Design Space; Validation; QbD; ICH Q8

Introduction

The use of Quality by Design (QbD) and Design Space (DS) principles [1] is becoming increasingly popular within the pharmaceutical environment. Regulatory authorities, including the Federal Drug Administration (FDA) and International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), are actively promoting and demanding the application of these risk-based approaches to drug development in order to ensure a systematic approach in developing analytical methods.

QbD principles, which will now be required for New Drug Applications (NDA), are designed to build in quality from the earliest stage (and every subsequent stage) of the drug discovery process. The complete information, understanding and transparency of a process relating to risk assessment will now be required for all New Drug Applications – this should speed up the approval processes and, hopefully, eliminate late stage failures.

The move towards QbD in the field of chromatography is a logical consequence of the way in which many HPLC methods have traditionally been developed and validated using a trial and error process. The end result of applying QbD principles to chromatographic method development is an increased understanding of the influence of the chromatographic operating parameters on the analytical measurement (i.e. chromatographic selectivity, critical resolution, etc), which is achieved through sound science and quality risk management. The final output is the rapid development of chromatographic methods of proven quality and robustness.

L.R.Snyder and his team utilized computer modelling to predict chromatographic retention behaviour and to provide the chromatographer with optimum separations with a minimum number of input experiments [2]. In the meantime the new technology has become a commonplace tool for the modern chromatographic method developer [3]. This type of HPLC retention prediction and modelling software such as DryLab®2010 (Molnár Institute, Berlin, Germany),

AutoChrom (Advanced Chemistry Development, Toronto, Canada), ChromSword (Merck KGaA, Darmstadt, Germany) and Fusion (S-Matrix, Eureka, CA, USA) also support the principles of QbD in that the software enables the systematic design of experiments leading to an increased comprehension and understanding of the influences of the chromatographic operating parameters on the separation.

In the RP-LC arena, retention modelling has been mostly employed in the separation of small molecular pharmaceuticals including synthesis impurities and degradation products of widely differing polarities [4-11]. However, the approach has been also successfully used for peptides and proteins [12,13], oligonucleotides [13], metabolites [10,14], complex plant mixtures [15-20], environmental pollutants [21-23].

A major reason for the extensive use of these retention modelling packages within modern method development laboratories resides in their excellent prediction accuracy for analyte retention and resolution [4,24-26] 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 addition to the 1-dimensional modelling (or one factor at-a-time, OFAT) described above, which help to understand peak movements, some software packages can now accurately perform 2-dimensional modelling, i.e. simultaneous variation of any two-separation variables for a chromatographic procedure. The 2-dimensional (2-D) approaches have a much more pronounced effect on the separation selectivity than the additive effect of the two individual variables [27]. Examples include gradient time (t_G) vs pH, percentage organic in the mobile phase (%B) vs pH, t_G vs temperature (T), ionic strength vs temperature among many others possible combinations and show excellent results with U(H)PLC and sub-2-µm columns in industrial analysis by the group Fekete and Fekete at Richter Pharma [3, 31,32]. The 2-D technology has recently been extended to 3-D modelling [28] where it has been elegantly shown that, based on only twelve input experiments and after building the Resolution Cube, the chromatographer can investigate in excess of 10^6 (=1 million) virtual chromatograms with extremely high precision and can evaluate the interactions of t_G, temperature (T) and ternary composition or t_G -T-pH, or other combinations and find the best separation in seconds. A resolution space can be presented in a virtual mode which allows the establishment of robust HPLC methods, and their visualization, in an extremely efficient way.

This present paper investigates the application of the DryLab®2010, 3-D multi-factorial optimization modelling software of three critical HPLC method parameters, i.e. gradient time (t_G), temperature (*T*) and ternary composition (B_1:B_2), (where the ratio of the two organic modifiers is varied) based on 3x4 experiments in the separation of 20 pharmaceutically relevant basic molecules and 2 neutral compounds as controls. Examining the effect of the experimental operating variables on critical resolution and selectivity was carried out in such a way as to systematically vary all three factors simultaneously. The basic element was a gradient time–temperature (t_G–*T*) plane, which was repeated at three different ternary compositions of eluent B between methanol and acetonitrile. The so-defined volume enables the investigation of the critical resolution ($R_{s,\,crit}$) for a part of the Design Space of this complex pharmaceutically relevant sample mixture. 3-D modelling offers visual support of the Design Space which generates more flexibility and establishes more robust HPLC regions for utilisation. Multi-dimensional robust regions can be successfully defined and graphically depicted. The use of multi-factorial approaches to HPLC method development will undoubtedly result in a reduction in development costs associated with trial and error, generate highly robust methods and enable smoother method transfer between different laboratories in a global economy.

2. Experimental

2.1 Chemicals, compounds and reagents

Acetonitrile (AN) and methanol (MeOH) (both HPLC grade) were supplied by Lab-Scan Analytical Sciences (Gliwice, Poland). HPLC grade water was provided by Romil Ltd. (Cambridgeshire, UK). Amiloride hydrochloride, benzylalcohol, benzylamine hydrochloride, (S)-(+)-chlorpheniramine maleate, desipramine hydrochloride, diphenhydramine hydrochloride, (±)-nicotine, nortriptyline hydrochloride, oxprenolol hydrochloride, phenol, pindolol, procainamide hydrochloride, quinine, salbutamol hemisulphate, benzyltrimethylammonium chloride, doxepin hydrochloride (85:15 *E*:*Z*-isomer distribution) and terbutaline hemisulfate were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK). Quinoxaline was supplied by Acros Organics (Geel, Belgium). ARC 68397, ARD 12495 and remacemide hydrochloride were a generous gift from Astra Zeneca R & D Charnwood (Loughborough, UK). Individual stock solutions of pindolol, benzylalcohol and quinoxaline were prepared at a concentration of 0.5 mg/mL in (AN:H_2O) (1:1)(V:V), Quinine was prepared at a concentration of 0.5 mg/mL in 20 mM potassium dihydrogen-phosphate pH 2.7 in (AN:H_2O)(20:80)(V:V), ARC 68397 was prepared at a concentration of 0.5 mg/mL in 20 mM potassium dihydrogen - phosphate pH 2.7 in H_2O and all other compounds were prepared at a concentration of 0.5 mg/mL in H_2O. A mixture of the 22 compounds was prepared by mixing equal volumes (50 µL) 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, UK) equipped with a binary pump, a vacuum degasser, cooled autosampler, temperature controlled column compartment and a diode array detector. Data acquisition was performed using the Agilent ChemStation.

2.3 High Performance Liquid Chromatography (HPLC)

Eluent A: 20 mM KH_2PO_4 pH 2.7 in H_2O.

Eluent B: consisted of 3 different eluents

B1: 20 mM KH_2PO_4 pH 2.7 in MeOH : water (65 : 35 V/V)

B2: 20 mM KH_2PO_4 pH 2.7 in AN : water (65 : 35 V/V)

B3: 20 mM KH_2PO_4 pH 2.7 in MeOH : AN : water (32.5 : 32.5 : 35 V/V)

Gradient range: 5->100% eluent B in all experiments

At least 20 column volumes (ca. 30 mL) of the appropriate mobile phase were flushed through the column prior to commencing the testing. ACE 3 C18, 3 µm, 150 × 4.6 mm columns were supplied by Hichrom Ltd. (Reading, UK). All analyses comprised of duplicate 5 µL injections. Other conditions included: flow rate of 1.0 mL/min and detection at 214 and 254 nm. The system dwell volume was experimentally determined as 1.18 mL. Gradients of 15, 30 and 45 min (5%B to 100%B which equates to 3.3 to 65 % total organic) were performed using the different mobile phase compositions as described above. Each gradient run was performed at 40 and 60°C. A typical solvent-

strength gradient profile is shown in Table 1. Peak tracking was accomplished with PeakMatch®, 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, were 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. Alternatively, Analytical Instrument Association file extensions (AIA files) were exported from the ChemStation data capture programme into Peak Match® for peak tracking purposes.

Time (min)	%B
0	5
30	100
35	100
36	5
51	5

Table 1. Example of a typical gradient input conditions employed (30 minute gradient shown).

2.4 Software

2.4.1 Chromatography modelling and prediction software

Peaks were identified and aligned based on peak areas using the PeakMatch® software (v. 3.6.3, Molnár-Institute Berlin, Germany), which became part of DryLab®2010, having user friendly tools, such as peak turnover and peak splitting functions, which greatly reduce the ubiquitous problem of peak misassignment. Virtual experimentation with HPLC runs ("modelling") was performed in DryLab®2010 v. 3.9, (Molnár-Institute) including a recently developed 3-D-device for Design Space visualisation. Predictions were compared with the original experiments to control the validity of the modelling process. Generation of 3-D resolution models was carried out with a new proprietary algorithm.

2.5.1 Experiments for modelling

Four initial input data runs were acquired under the following conditions: Gradient times (t_G) of 15 and 45 min, temperatures of 40°C and 60°C, eluent A and B as described in section 2.3. The organic modifier in eluent B consisted of either MeOH, AN or mixtures of both: (MeOH:AN) (1:1)(V/V). 12 binary and ternary mobile phase conditions were chromatographed as shown in Fig. 7. Input data, 3 x 4 experimental runs were performed overnight. After the chromatograms were collected, they were exported into Peak Match® for peak tracking. The data were then finally transferred by one mouse click to DryLab®2010 (Fig. 2).

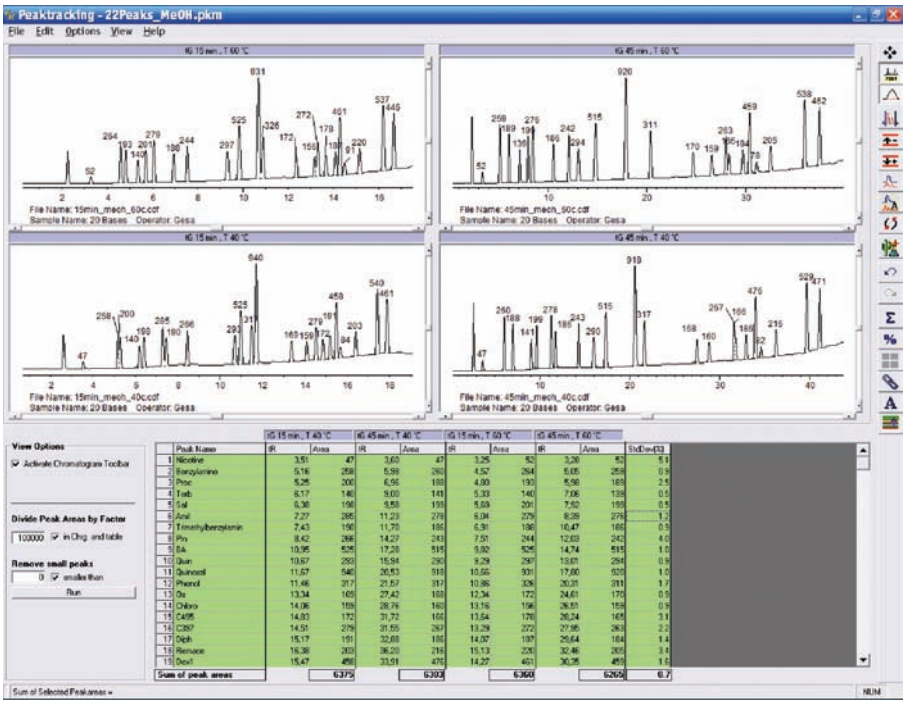

Figure 1. Screenshot of the experimental chromatograms and tabulated peak assignments in PeakMatch®: Retention times and peak areas were obtained using the following chromatographic conditions: Gradient times, temperature, column, gradient range, flow rate and eluent A as stated in the experimental section. Eluent B was B1 as described in section 2.3. Binary gradients for the MeOH-t_G–T plane are as follows: Chromatograms correspond on the left and right sides to t_G:15 min and 45 min respectively, whereas the top and bottom ones correspond to T: 60° and 40°C respectively.

3. Results and discussion

The approach of two dimensional modelling of gradient time and temperature is possibly the most widespread type of modelling and optimisation protocol performed within the pharmaceutical industry [30]. Acetonitrile is commonly used as the organic component of the mobile phase due to its low viscosity and UV cut off – however, recent shortages of AN and its associated high costs, have forced chromatographers to re-assess their need for AN. The use of selectivity differences of "ternary eluent systems", and hence varying chromatographic resolution and selectivity between the organic modifiers acetonitrile and methanol in RP binary gradient chromatography is well documented [3,24,29]. Several of the commercially available software packages can model the retention of analytes using gradient chromatography as a function of ternary mobile phase composition [29].

In a recent publication, Molnár *et al* [28] have shown that this approach can be extended to 3-dimensional modelling of t_G, temperature (T) and ternary composition or pH. Only twelve experimental input runs - two differing gradient times, two differing temperatures and three differing ternary eluent compositions have been shown to be necessary in order to reliably create 3-dimensional retention models of these factors within DryLab®2010.

This paper will highlight the use of this approach in the method development of the separation of a range of twenty pharmaceutically relevant bases and two neutral components of widely differing chemical/physical properties in a rapid and semi-automated fashion. The work flow involved the design of the experiments, automated collection of the input data (12 experimental runs – i.e. 3 x t_G – T models), semi-automated peak tracking, automated creation of the 3-dimensional resolution space (i.e. retention models), validation of the model using three extra experimental runs, exploration and evaluation of the Design Space for optimum, robust regions of the model to work in and confirmation of the desired modelled chromatogram within the Design Space to that obtained experimentally.

3.1 Peak tracking using PeakMatch®

The chromatograms are imported directly into the PeakMatch® software from the ChemStation data files as compatible AIA/AnDI files (*.cdf).

Fig. 1 highlights the usefulness of PeakMatch® in graphically displaying the chromatograms with strongly different selectivity's and their associated data (i.e. peak assignments, tR and peak areas), in an orderly manner. Each analyte should be tabulated in a single horizontal row to aid peak assignment. The numbers above the peaks correspond to peak areas, which are obtained directly from the integration software and are then reduced by a factor of 10^5 to

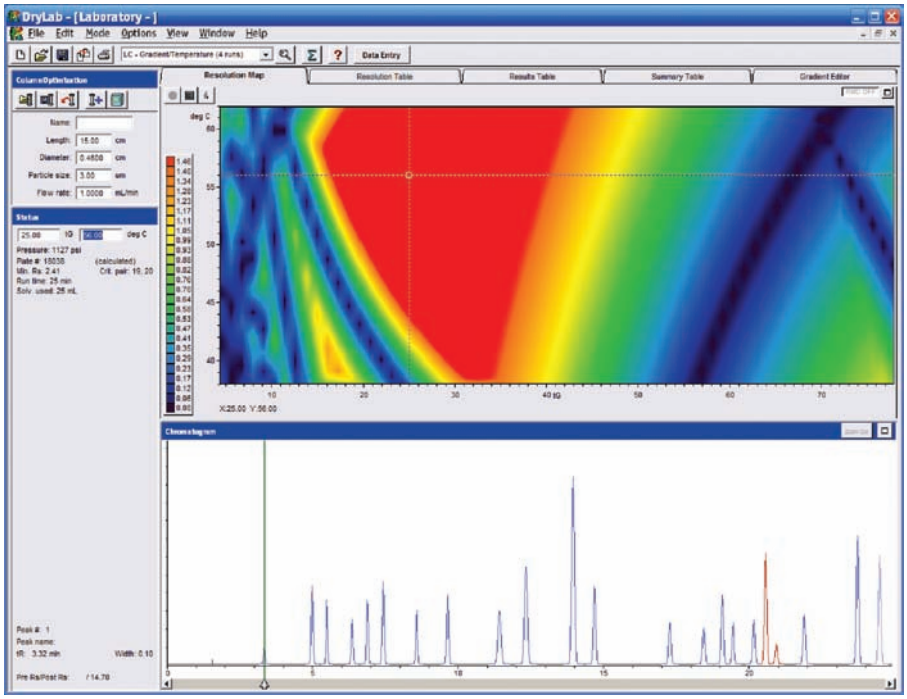

Figure 2. Screenshot in DryLab®2010 of the resolution map obtained using the chromatographic conditions stated in Fig. 1 – Binary gradients using as eluent B: B1 (MeOH) and t_G–T-model. Red regions in the resolution map represent regions of "baseline resolution" Rs, crit > 1.5, blue colour means co-elution of 2 or more peaks.

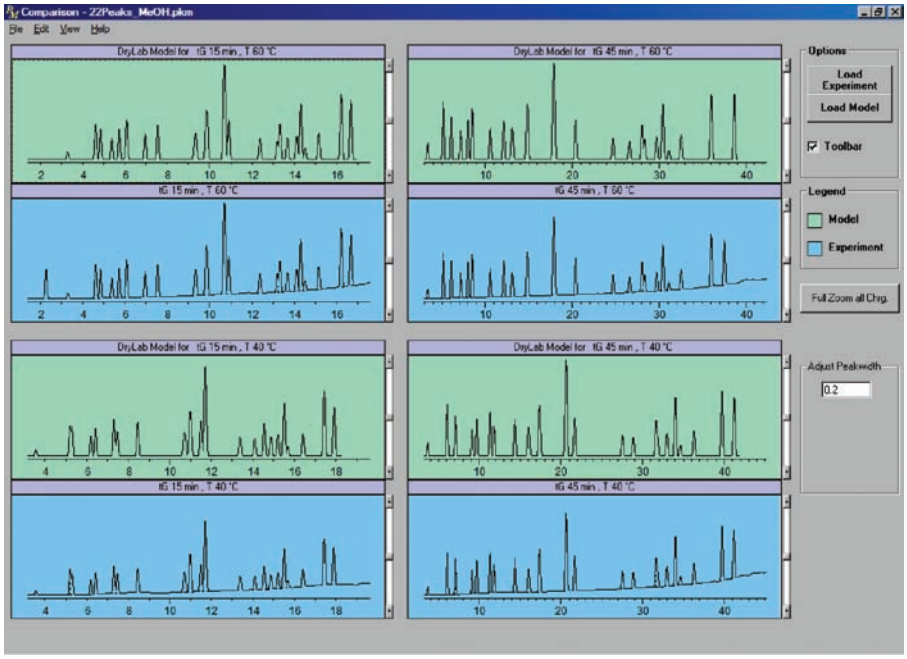

Figure 3. Comparison of the experimental (in blue) and modelled chromatograms (in green) in PeakMatch® obtained using chromatographic conditions stated in Fig. 1 – (Binary gradient using MeOH-t_G-T plane) for the control of the model.

generate numbers of 2-4 digits. The legends associated with each chromatogram permit easy identification of the parameters that have been changed for each chromatogram (i.e. composition of eluent B – ratio of MeOH:AN, the temperature and gradient time). The low standard deviation (i.e. 0.7%) of the sums of the peak areas per runs. Fig.1., green table at lower right corner shows the precision of the experiments in the tabulated data to be excellent. This observation proves that peak areas which correspond to sample masses can be used in an easy and precise way for peak assignment / tracking. Decomposition or large extinction changes on altering the chromatographic conditions due to the dilution of the sample in the column are very seldom. UV or mass spectra are often helpful in peak assignment / tracking, but their use requires more expertise and cost. The UV-spectra of related substances and impurities are often too similar for discriminatory and identification purposes and up to 30-40% of analytes may not be ionised and hence not detected by mass spectroscopy. However, the only prerequisite for successful peak tracking using peak area is, that the same sample mixture and volume must be injected and that the injector must be working reproducible.

Critical peak pairs are clearly seen in some of the input runs seen in Fig. 1, for example in the lower left chromatogram (t_G: 15 min and T: 40°C) benzylamine t_R: 5.16 min, peak area 258) and procainamide peaks (t_R: 5.25 min, peak area 200) only exhibit partial separation. Phenol (t_R: 11.46 min, peak area 317), one of the neutral components) which elutes before quinoxaline (t_R: 11.67 min, peak area 940) when chromatographed with a shallower gradient (i.e. lower right chromatogram, t_G: 45min, T: 40°C) now elutes (phenol, t_R: 21.57 min, peak area 317) after quinoxaline (t_R: 20.53 min, peak area 918). Interestingly, increasing the operating temperature from 40°C (bottom left chromatogram) to 60°C (top left chromatogram) induces the same movement of phenol with respect to quinoxaline as on increasing the gradient time. It is quite evident from Fig.1 that some peak pairs are better separated at the higher temperatures. These observations are based on the different tendency of peak movements and are definitely not the result of decomposition.

The critical peak pair was not constant between the input chromatograms, indicating the presence of several *"critical moving peaks"* in the complex mixture.

3.2 Generation of the t_G –T models

Once a matched peak table (identified by a green colour) had been obtained, all the data were automatically transferred into DryLab®2010, where the resolution map of the MeOH-plane of the cube was generated and optimal conditions could then be ascertained.

From the 4 basic input experiments, a t_G –T model could be created, showing a resolution map in which approximately 10,000 virtual chromatograms can be represented with extremely high precision (Fig. 2).

The accuracy of the DryLab®2010 software to model the original input experiments is shown in Fig. 3. The original experiments are in blue and the DryLab®2010 models are in green. The comparison shows that all four input runs are precisely calculated by DryLab®2010 and hence validates the model (i.e., the model does what is expected). Fig. 3 highlights that these four input experiments of the same sample generate very different selectivities.

The above process is then repeated using the ternary mobile phase composition of (B1:B2)(MeOH:AN)(50:50 V/V) as eluent B as shown in Fig. 4.

Comparison of Figs. 1 and 4 highlights the differing elution orders (i.e. differing chromatographic selectivity) that are

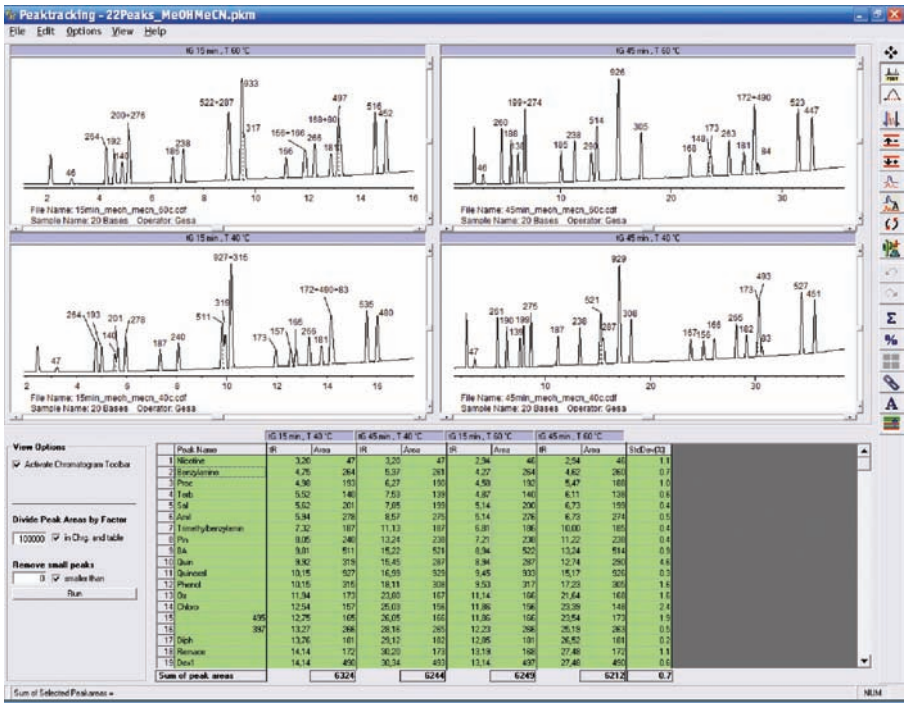

Figure 4. Collection of the experimental chromatograms and tabulated peak assignments, retention times and areas in PeakMatch® obtained using the chromatographic conditions stated in Fig. 1 with the exception that eluent B was (B1:B2)(50:50 V/V) which corresponds to a MeOH:AN ratio of (1:1 V/V).

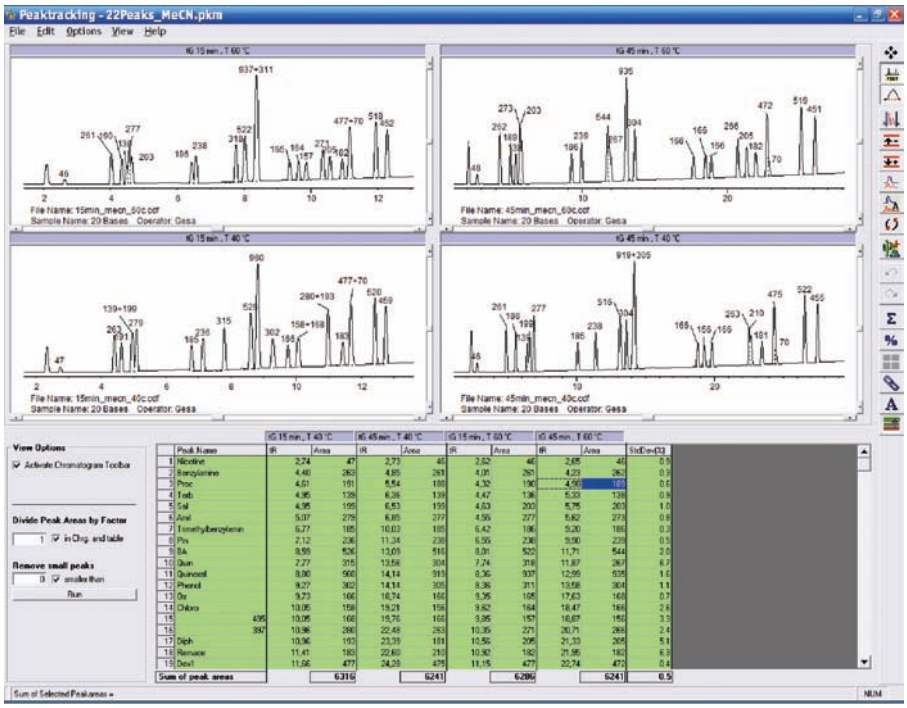

Figure 5. Collection of the experimental chromatograms and tabulated peak assignments, retention times and areas in PeakMatch® obtained using the chromatographic conditions stated in Fig. 1 with the exception that eluent B is now B2: (AN: Water)(65:35 V/V) – Binary gradient using AN-t_G-T-plane.

achievable when (Water:MeOH:AN) ternary mobile phase compositions are compared to that of a binary composition (Water:MeOH). Peak areas represent the mass of the sample (concentration x elution volume = sample mass). As long as the flow rate is constant, it can be expected that the peak areas will be additive in overlapping peaks. In DryLab®2010, the area of peaks in overlapping bands can be calculated, such as the largest peak (corresponding to co-elution of quinoxaline and phenol) in the lower left at tR: 10.15 min, originally with a peak area of 1242, is now subdivided into peak areas of 927+315. Other areas of greater co-elution can be observed (peak area 745), such as the peak in the same run at 14.14 min, corresponding to three peaks (remacemide, doxepin isomers 1 and 2) with the areas of 172+490+83. Each experiment in a t_G – T plane has three other chromatograms, which can greatly assist in understanding peak movements.

An improved understanding of peak movements as a function of the influence of physico-chemical parameters (or factors) such us gradient time t_G, temperature, pH, ternary composition, additive concentration or changing instrumentation (with differences in dwell volume), differences in flow rate, in column length and diameter, should permit the chromatographer to control his/her methods much more precisely and hence to reduce run failures and generate higher quality data.

The final t_G –T plane using AN, an eluent, which is often selected initially, due to its lower viscosity, UV cut-off and associated better peak shape than MeOH, is shown in Fig. 5. The price of AN still remains high after the worldwide shortages some months ago (ca. 5-10 times higher than HPLC grade MeOH) therefore many laboratories now favour MeOH over AN. It is therefore expedient to investigate if MeOH offers any selectivity advantages over AN in method development strategies.

As expected, the elution times are somewhat shorter with AN than with MeOH. However we observe much more cases of co-elution with AN than with MeOH in eluent B.

3.3 Generation of the 3-dimensional resolution model (the Cube)

After the three t_G –T models have been created, the DryLab®2010 software calculates the 3-dimensional resolution model (the Cube), representing the simultaneous influence of three parameters (%MeOH in AN, t_G and T) on the chromatographic selectivity and critical resolution of the separation. The advantage of the 3-D resolution volume is the fact, that the 8 corner points and 4 intermediate points of the space are all measured and form a "cube" as a true "Design Space", allowing us to predict > ca. 10^6 separations within the cube with an unparalleled precision of the retention times and chromatographic separation selectivity.

Fig. 6 illustrates the graphical representation of the three-dimensional resolution cube (top right side) of a Design Space (DS) with 3 factors: Gradient time (t_G) (x-axis), temperature (T) (y-axis) and ternary composition (z-axis) (%MeOH in AN). The front page of the cube is shown on the top left and corresponds to the the t_G–T- plane with MeOH as eluent B (comparable to Fig. 2). The robustness of the method can be easily visualized as a geometrical body within the resolution space, in which the critical resolution does not fall

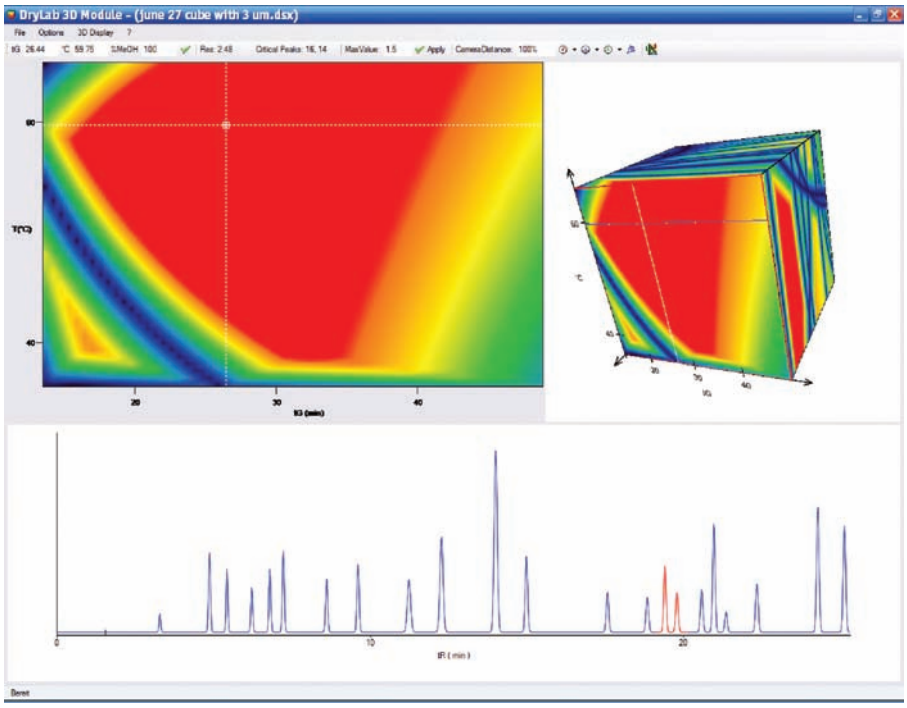

Figure 6. 3-D Resolution space showing the t_G – T (MeOH) plane. Red coloration within the resolution space indicates operating regions with $R_{s,crit}$ > 1.5, whereas blue coloration indicates operating regions associated with co-elution or poor resolution.

	Predicted		Actual		
# Peak name	tR[min]	W (½ ht)	tR[min]	% error	W (½ ht)
1 Nicotine	3.572	0.07	3.576	0.11	0.06
2 Benzylamine	5.702	0.10	5.698	-0.07	0.07
3 Procainamid	6.269	0.08	6.272	0.05	0.06
4 Terbutaline	7.811	0.09	7.805	-0.08	0.06
5 Salbutamol	8.215	0.09	8.214	-0.01	0.06
6 Amilorid	9.555	0.10	9.546	-0.09	0.07
7 Trimethylbenzyalamine	9.875	0.10	9.857	-0.18	0.07
8 Pindolol	11.697	0.10	11.676	-0.18	0.07
9 Phenol	13.893	0.17	13.898	0.04	0.12
10 Benzylalcohol	14.721	0.16	14.702	-0.13	0.11
12 Quinoxaline	16.700	0.14	16.664	-0.22	0.11
11 Quinine	17.017	0.12	16.979	-0.22	0.09
13 Oxprenolol	20.877	0.12	20.870	-0.03	0.09
14 Chloropheniramine	21.970	0.13	22.003	0.15	0.10
15 ARC-68397	23.482	0.11	23.471	-0.05	0.09
16 ARD-12495	23.788	0.12	23.799	0.05	0.09
17 Diphenhydramine	24.528	0.12	24.541	0.05	0.09
20 Remacemide	26.818	0.12	26.835	0.06	0.09
21 Desipramine	29.010	0.12	29.025	0.05	0.09
22 Nortriptyline	29.960	0.12	29.958	-0.01	0.09
				Mean	
				error in %	**0.09**

Table 2. Comparison of the predicted and experimental retention times, peak width and resolution using a binary mobile phase composition of MeOH, t_G: 30 min and T: 40°C. Average precision of predictions > 99.9%.

below a baseline separation of $R_{s,crit}$ > 1.5. By setting the $R_{s,crit}$ > 1.5 in the resolution map, robust regions within the Design Space can be easily identified. The resolution map shown in Fig. 6 highlights that the separation ($R_{s,crit}$ > 1.5) has an extended robust region (shown in red) when MeOH is used in eluent B

3.4 Validation of the 3-dimensional resolution model (the Cube)

The validity of the accuracy of the 3-D cubic retention model was determined by comparing the predicted and experimental retention times from the three validation runs in the 3-D design space, as shown in Fig.7. The

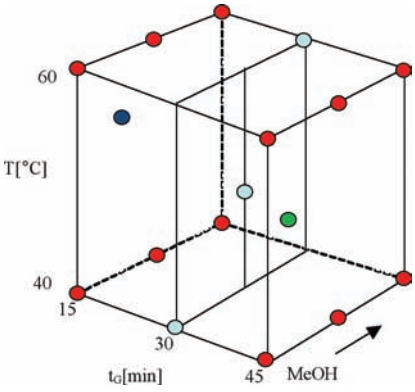

Figure 7. Experimental design for the 3-D retention model and its control. Red circles represent the twelve input experiments for the 3-D model, the light blue circles the validation experiments and the dark blue (t_G = 22 min, T = 55°C and MeOH in eluent B) and green (t_G = 45 min, T = 50°C and MeOH:AN [80:20 V/V] in eluent B) circles optimum conditions for comparison (see Fig. 11 and 10 respectively).

accuracy of the predicted retention times was excellent for the three validation experiments of the model, i.e., better than 99% accurate (deviations are in average less than 0.2%) as can be seen for the binary mobile phase composition using MeOH and a t_G of 30 min and a T of 40°C (see Table 2 for a typical result from one of the validation exercises). The average difference between predicted and experimental retention times is approximately 6 sec and the largest deviation is 14 sec.

Retention modelling with 99.9% accuracy in t_R has the additional advantage that it allows the chromatographer a much higher degree of flexibility, in that the effect of changing operating parameters inside of the cube model can be quickly evaluated in order to test the robustness and improve the chromatography without the need for costly and time consuming method revalidation activities.

Fig. 8 illustrates the corresponding separation plane with 100% eluent B2 (organic modifier is AN). The separation throughout the plane is poor, indicating that no combination of the operating parameters (t_G and T) can afford acceptable resolution. Evaluation of slices of the t_G-T planes throughout the cubic model visually shows that baseline resolution for the 22 compounds is only possible with an eluent B, which is rich in MeOH (i.e. > 80% B1) (see Fig.6). Hence, it is quickly established that it would be a waste of time and money to attempt to use AN in eluent B in this separation.

3.5 Robustness of the 3-dimensional resolution model (the Cube)

Fig. 9 illustrates that there are other additional robust regions within the design space for the separation for this complex separation, for example the use of the operating a ternary mobile phase composition containing (B1:B2)(80:20 V/V).

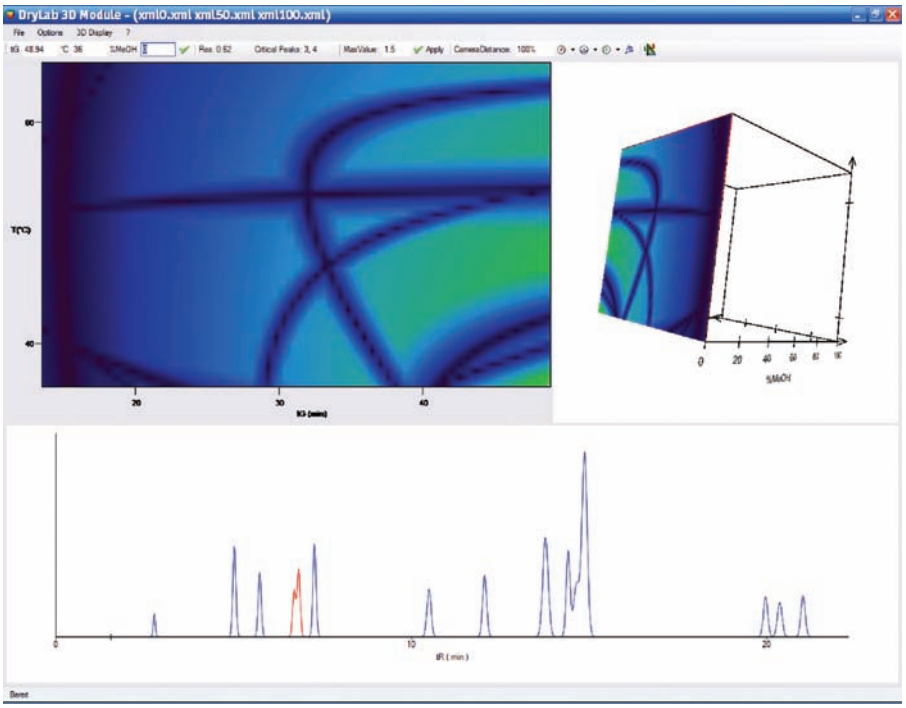

Figure 8. 3-D Resolution space showing the t_G-T-AN- plane with eluent B: B2. Colour code as shown in Fig. 6.

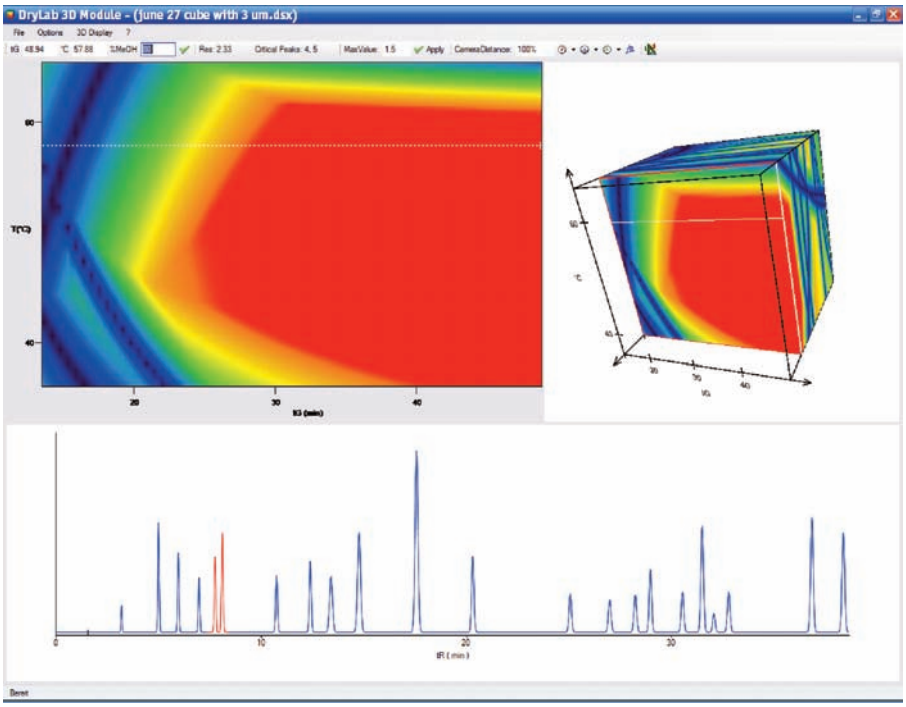

Figure 9. 3-D Resolution space showing the t_G-T-plane for (B1:B2)(80:20 V/V) as eluent B. Colour code as shown in Fig. 6.

3.6 Selection and verification of optimum robust chromatographic conditions

Given the fact that there was excellent correlation between the three predicted and experimental validation runs, it was expected that the accuracy of the simulated chromatograms within the resolution cube would also be good. This was confirmed by comparison of the predicted and experimental chromatograms using the chromatographic conditions: t_G : 45 min, T: 50 °C and a ternary eluent composition: (B1:B2 80:20 V/V) (i.e. the dark blue circle represented in Fig. 7).

The experimental chromatogram was obtained using a different batch of stationary phase material, differing buffer batch and a different mobile phase preparation and the chromatography performed on a different day which highlighted that, even with a worst case scenario, excellent correlation between experimental and modelled data is obtained and that the chosen chromatographic conditions are robust (see Fig 10).

Evaluation of the t_G –T-ternary resolution model (80% MeOH plane – see Fig. 9) permits the chromatographer to rapidly establish that the robustness (i.e. Rs,crit > 1.5 for the sample mixture without the doxepin isomers) of the chosen chromatographic conditions can be maintained using the following chromatographic operating parameter ranges of t_G : 45 ± 1 [min], T: 50 ± 1 [°C], ternary eluent composition: 80 ± 1 [%B1:B2], flow rate: 1.0 ± 0.1 mL/min, dwell volume: 1.18 ± 0.05 mL, Bstart: 5±1%B. A comparison of the predicted and experimental retention times for this chromatographic condition within the 3-D resolution map is shown in Fig. 10. The correlation was observed to be excellent, the average difference between predicted and experimental retention times is approximately 0.4 min and the largest difference is 0.7 min.

The t_G –T-ternary resolution model also allows the chromatographer to rapidly establish that the use of a binary eluent containing MeOH is also feasible for the separation of the sample mixture (without the doxepin isomers) which, for economic reasons and ease of method transfer, would be very attractive. Examination of the t_G –T-ternary resolution model (Fig. 6, eluent B: 100% B1)("MeOH plane") indicates that the binary LC method using MeOH would be sufficiently robust (i.e. Rs, crit, >1.6) if operated within the following operating parameters – t_G : 22 ± 1 [min], T: 55 ± 1 [°C], ternary eluent composition: 100% B1 (MeOH modifier), flow rate: 1.0 ± 0.1 mL/min, dwell volume: 1.18 ± 0.05 mL, Bstart: 5±1%B. Fig. 11 once again highlights that excellent correlation was observed, the average difference between predicted and experimental retention times is approximately 0.1 min and the largest difference is 0.2 min.

Summary

A method for the separation of all 20 basic drug molecules and two neutral components was rapidly achieved and was proven to be robust with respect to the three critical operating parameters – gradient time, temperature and ternary mobile phase composition. The methodology presented in this work is in accordance with Quality by Design (QbD) principles and results in the definition and visualization of the Design Space and the identification of robust working regions for the chromatographic conditions.

The HPLC modelling software has been shown to increase efficiency and productivity in routine method development, optimization and method transfer. The new 3-D modelling technology locates the global optimum of highly influential chromatographic operating parameters with respect to separation, analysis time and robustness.

The graphical presentation of the critical parameters with an optimization Design Space greatly assists in assessing the robustness of the chromatographic separation.

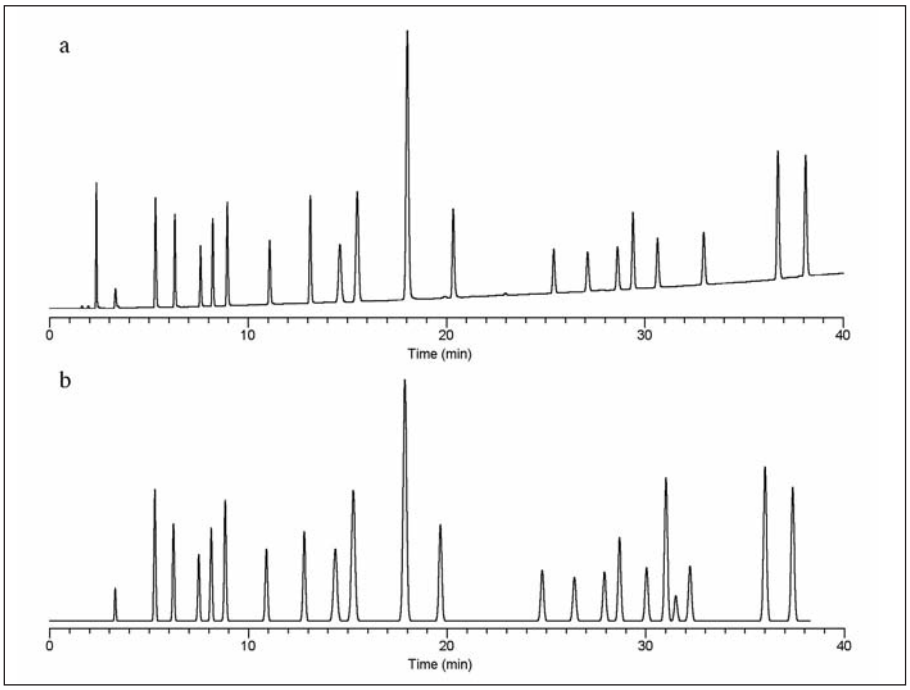

Figure 10. Experimental (a) and predicted (b) chromatograms obtained using the following chromatographic conditions - t_G: 45 min, T: 50°C and eluent B: (80:20)(MeOH:AN V/V) (green circle depicted in Fig.7). Doxepin isomers are shown in the predicted chromatogram (Rt = 31.014 and 31.509 min). The peak at 2.25 min in Fig 10a corresponds to the maleate counterion.

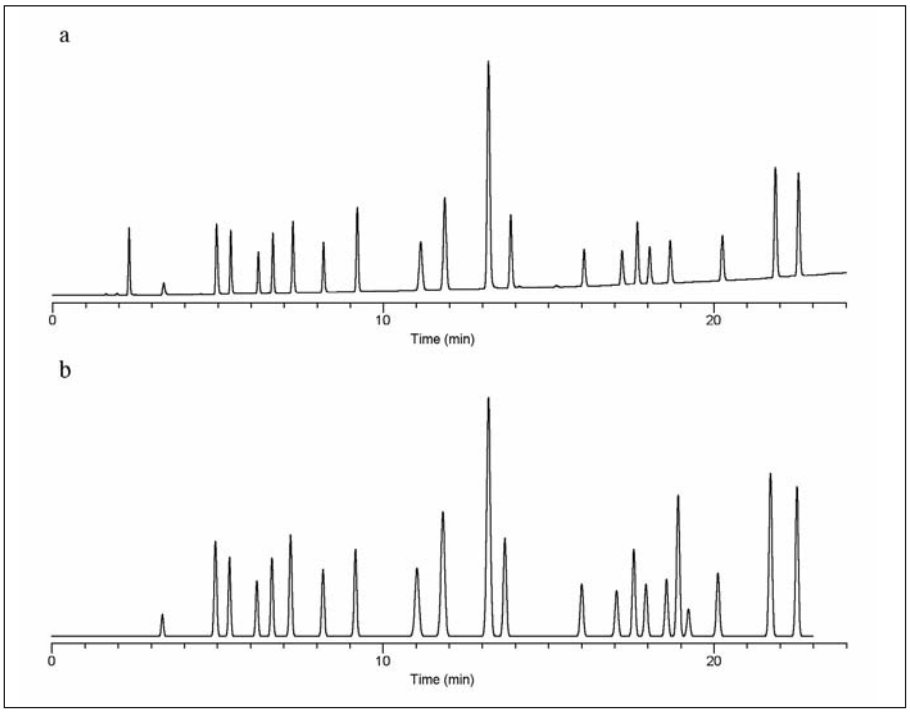

Figure 11. Experimental (a) and predicted (b) chromatograms obtained using the following chromatographic conditions - t_G: 22 min, T: 55°C and eluent B: 100% MeOH (dark blue circle depicted in Fig.7). Doxepin isomers are shown in the predicted chromatogram (Rt = 18.913 and 19.223 min). The peak at 2.25 min in Fig 11a corresponds to the maleate counterion.

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Data Mining Software Adopted as a Teaching Tool at University of Melbourne

SpectralWorks AnalyzerPro® mass spectrometry data mining software has been adopted as a teaching tool for Masters students studying metabolomics and proteomics at the University of Melbourne, Australia. "Data processing and analysis is a key element in this field", said Scott Campbell, Vice President (R&D) of SpectralWorks, "and we are very pleased to support this teaching initiative."

The AnalyzerPro® software is used in the practical metabolomics workshop of the Masters of Biotechnology course.

For more information email johnm@spectralworks.com