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Computer-assisted optimization in the development of a high-performance liquid chromatographic method for the analysis of kava pyrones in *Piper methysticum* preparations

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Abstract

A computer simulation program was used to optimize the separation for six kava pyrones and two unidentified components obtaining the best resolution and the shortest run time. With DryLab it was possible to find the best separation conditions without running a large number of possible combination of variables in the laboratory. Additionally, due to the systematic progress in method development a new eluent was found with excellent properties, namely 2-propanol. With 2-propanol, the largest number of components could be revealed in the shortest analysis time. Starting with four initial experiments, the software allowed to optimize gradient time t_G and temperature T simultaneously. Changing other variables such as type of organic modifier, the eluent pH, the gradient form, and the flow-rate, the optimization resulted in resolution $R_s > 1.5$ for all kava pyrones and the two additional new bands. The HPLC method is used to analyze kava pyrones in *Piper methysticum* preparations.

Keywords: Optimization; *Piper methysticum*; Mobile phase composition; Plant materials; Pharmaceutical analysis; Method development; Computer simulation; Kava pyrones; Pyrones

1. Introduction

The characterisation of phytopharmaceuticals is difficult due to the large number of components present in plant extracts. Small changes in experimental conditions often results in confusing peak movements. Especially with charged species retention time can change with pH as much as a factor of 5 [1]. Potential corrections in the methods remain complicated as the understanding and for a complex mixture the control of peak movements is hard to accomplish without computing capabilities. In most cases gradient elution has to used to be able to see all components. The theory of gradient elution is clearly described by Snyder [2]; however, the chromatographer has problems to correlate his ideas with real experiments, if no software support is available. The use of computer programs is needed for the elaboration of the gradient profile and for the studies in actual critical resolution. The solution for several highly complex matrices has been published in the past using special software tools [3–10].

Piper methysticum, popularly known as kava, is an Oceanianic pepper plant widely used in the Pacific Islands. A drink prepared with the roots of this plant is used by the natives for its pharmacological properties including relaxation, local anaesthetic, and analgesic properties. As an integral part of traditional life, the symbolic use of kava has many cultural

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similarities like the tea ceremony in Japan. A multidisciplinary overview of the subject is given by Singh [11].

Extracts prepared from the roots have long been used in European phytomedicine as a sedative, tranquilizer, and muscle relaxant. Several European countries (e.g. Germany, UK, Switzerland and Austria) have approved kava preparations in the treatment of anxiety on the basis of detailed pharmacological data and clinical studies. A large number of compounds have been isolated from the plant—most of the compounds are six kava pyrones (Fig. 1). The kava pyrones are considered to be the active ingredients and used as a quality control measure in standardized phytotherapeutical and preparations.

A number of methods to determine the amount of kava pyrones have been published in recent years: Csupor [12] used a colorimetric method to determine the amounts of kavain, methysticin and yangonin. Young et al. [13] determined the amounts of kava pyrones using two-dimensional thin-layer chromatography on aluminium oxide. Duffield and co-workers [14,15] used gas chromatography with electron impact (EI) and methane chemical ionization mass spectrometry to investigate the components of *Piper methysticum*. Gracza and Ruff [16] published an HPLC method for the determination of kava pyrones by using normal-phase columns. A reversed-phase HPLC method developed to separate the kava pyrones by Boonen et al. [17] takes 120 min and is too time-consuming for routine analysis. In none of these papers was an attempt made to prove, that the final method presents ''the'' optimum, in terms as having the ''best'' values for individual working parameters. This can be shown however with the help of ''critical resolution maps'', shown in this paper.

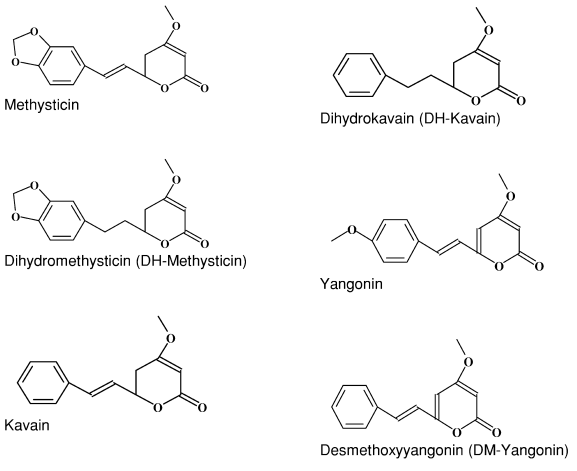

Fig. 1. Structures of the six kava pyrones in *Piper methysticum*.

Our continuing interest in plant drug analysis using HPLC led us to develop rapid, sensitive and accurate reversed-phase HPLC techniques. Previously, we described a new HPLC method for the determination of kava pyrones in kava extracts [18,19]. This paper is part of our on-going studies of the analysis of kava pyrones in *Piper methysticum*.

Optimization methods assist the chromatographer to obtain good separation with reduced time and effort needed, which in liquid chromatography involves the selection of experimental conditions for adequate separation and acceptable retention time. Finding the overall optimum conditions is therefore often a compromise between contradictory objectives. Especially in pharmaceutical laboratories obtaining a balance between resolution and analysis time is not always easy. An efficient optimization method should be employed during the method development process in order to deal with these problems. The optimization procedures can be manual, statistical or computer-aided.

Presently one software product in HPLC method development, DryLab, is widely used in the pharmaceutical and chemical industry. We used this program to optimize the separation of six kava pyrones obtaining the best resolution and the shortest run time. In the strategy of method development, changes in organic modifier content (% B), pH, temperature (T) and gradient run time (t_G), and the type of organic modifier should be tested to achieve a successful separation. Only four experimental runs for T vs. t_G and six runs for pH vs. t_G were needed. First, well-established eluents like acetonitrile and methanol were applied, later 2-propanol was also employed.

DryLab was expected to create, based on the input data, critical resolution maps of t_G vs. T and t_G vs. pH. With this systematic approach the maximum number of components present should be found and peak positions would be guided to a state of ''equal band spacing''. Correction in the gradient program, if

necessary, would speed up the elution by adjusting the flow-rate by carefully looking at potential changes in selectivity, which in gradient elution is eventually changing with changes in flow-rate. Finally the difference between starting and final % B should be reduced to a minimum, helping to shorten reequilibration time, and to cut down further the analysis time.

2. Experimental

2.1. Equipment

Chromatographic analysis of kava pyrones was performed using a Waters HPLC system (Waters, Eschborn, Germany) equipped with a 2690 separation module with in-line column heater and a 996 photodiode-array detector. Chromatograms were monitored at a wavelength of 240 nm. UV spectra were taken in the range of 210–440 nm. The dwell volume of the instrument was 0.5 ml. System control, data collection and data evaluation were performed using the Millennium^{32} client/server software 3.20 (Waters).

For computer-assisted optimization, we used the software DryLab 2000 version 3.1 (LC Resources, Lafayette, CA, USA; in Europe: Molnar, Berlin, Germany).

2.2. Chromatographic conditions

All solvents were HPLC-grade were purchased from Merck (Darmstadt, Germany). Water was purified by a Milli-Q water purification system (Millipore, Eschborn, Germany).

The Luna C_{18} column was packed with 5-μm particle size and had a dimension of 250×4.6 mm (Phenomenex, Aschaffenburg, Germany; number of theoretical plates was ca. 32 000). The flow-rate was 1.0 ml/min.

The method development started with unbuffered (pH 6.15) water (A)–acetonitrile (B) as the mobile phase with linear gradients from 10 to 90% B in 30 and 90 min at two different temperatures: 30 and 60 °C. In a second set of experiments, the mobile phase was exchanged against water (A)–methanol (B) or water (A)–2-propanol (B). The influence of the pH, measured in eluent A, was studied at three different values: At pH 4.35, 4.95 and 5.55 with 2-propanol as the organic modifier.

The correct peak tracking was first possible, after all three eluents were tested and the maximum number of peaks could be established.

3. Results and discussion

3.1. Method development strategy using DryLab

At the present time, the most succesful strategy by starting method development is to look at the influence of the temperature and of the elution force of organic eluents (t_G) in a simultaneous mode. Several successful attempts were made to use this approach in recent years for the separation of plant extracts, for peptides and proteins and other complex samples [20–22].

Therefore, we started with four gradient runs at two different temperatures. Initial experiments were carried out at 30 and 60 °C with linear gradients and with gradient times of 30 and 90 min. Always the same amount of sample was injected to keep peak areas constant. All experimental data, such as number, retention time and peak area of components, dwell volume, column information and gradient conditions were entered into DryLab (see Table 1 for acetonitrile, Table 2 for methanol, and Table 3 for 2-propanol).

Elution experiments were started with acetonitrile as the organic modifier. The experimental design consisted of four runs as shown in Fig. 2 as original chromatograms. The lower chromatograms were taken at 30 °C, the top ones at 60 °C. The two left chromatograms had $t_G = 30$ min run time, the other two on the right at $t_G = 90$ min run time.

3.2. Peak tracking

Integration data were exported in AnDI/AIA format to Excel and arranged in a table, in which one peak was located with all its data in one single horizontal line. The tables were produced after all the results with the three eluents acetonitrile (ACN), methanol (MeOH) and 2-propanol (i-PrOH) were available. Then we could see that we had 13 peaks,

Table 1
Experimental data collected with four initial gradient runs for acetonitrile

No.	Name	$T=30$ °C				$T=60$ °C			
		$t_G=30$ min		$t_G=90$ min		$t_G=30$ min		$t_G=90$ min	
		t_R	Area	t_R	Area	t_R	Area	t_R	Area
1	Methysticin	19.98	412	41.38	483	18.13	453	35.96	505
2	Dihydromethysticin	20.10	496	41.38	473	18.35	445	36.40	495
3	Unknown U1	20.54	55	42.46	53	18.82	47	37.99	55
4	Unknown U2	20.54	100	42.46	109	19.05	102	37.99	128
5	Kavain	20.77	821	42.95	875	19.05	805	37.99	885
6	Unknown NP1	21.33	33	44.49	32	19.42	15	39.46	15
7	Dihydrokavain	21.07	451	43.55	480	19.42	447	38.86	517
8	Unknown NP2	21.53	20	44.00	19	19.69	17	39.78	18
9	Unknown NP3	21.95	21	45.81	15	19.80	10	42.12	8
10	Yangonin	21.95	298	46.31	331	20.19	312	41.43	353
11	Demethoxyyangonin	22.09	141	46.31	134	20.33	133	41.43	144
12	Unknown NP4	22.29	24	46.31	20	20.63	28	42.36	12
13	Unknown NP5	22.70	15	47.07	12	21.10	20	42.69	7

Gradient: 10–90% B; dwell volume: 0.5 ml; column: 25×0.46 cm I.D.; d_p: 5 μm; flow-rate: 1 ml/min.

which should be measured and represented in each table. The table was marked and inserted into the input data table for DryLab. Consequently the input runs were generated by DryLab for the purpose of controlling the correctness of the peak tracking process. With the help of the plate, a number small adjustments of the resolution of the DryLab model could be accomplished, as shown in Figs. 2 and 3 for acetonitrile.

In Figs. 2 and 3 we can observe an unexpected chromatographic behaviour: the faster runs with steeper gradients have a better resolution of the first peak pair, methysticin and dihydromethysticin and the last peak pair, yangonin and demethoxyyangonin.

The excellent correlation between measured and by DryLab-predicted retention times shows the correctness of the peak tracking process. There are, however, several double peaks in all runs.

The two-dimensional critical resolution map (t_G vs. temperature) for acetonitrile is shown Fig. 4. The

Table 2
Experimental data collected with four initial gradient runs for methanol

No.	Name	$T=30$ °C				$T=60$ °C			
		$t_G=30$ min		$t_G=90$ min		$t_G=30$ min		$t_G=90$ min	
		t_R	Area	t_R	Area	t_R	Area	t_R	Area
1	Methysticin	24.88	455	56.69	485	22.18	492	47.74	482
2	Dihydromethysticin	25.28	444	57.48	476	22.57	530	48.60	463
3	Unknown U1	25.28	55	57.48	60	22.84	49	49.25	53
4	Unknown U2	25.28	111	57.92	115	22.84	116	49.77	121
5	Unknown Kavain	25.28	791	57.48	844	22.84	805	49.25	845
6	Unknown NP1	26.86	21	60.43	22	23.83	20	51.61	18
7	Dihydrokavain	25.91	460	58.94	498	23.47	496	50.82	490
8	Unknown NP2	26.53	36	61.09	27	24.27	25	53.19	36
9	Unknown NP3	26.86	21	61.09	25	24.01	20	52.89	17
10	Yangonin	27.50	319	64.29	341	24.89	340	55.54	328
11	Demethoxyyangonin	27.31	123	63.35	136	24.89	150	54.98	134
12	Unknown NP4	28.30	20	66.80	20	25.7	20	58.20	20
13	Unknown NP5	28.60	20	67.00	15	26.4	20	58.90	20

Gradient: 10–90% B; dwell volume: 0.5 ml; column: 25×0.46 cm I.D.; d_p: 5 μm; flow-rate: 1 ml/min.

Table 3
Experimental data collected with four initial gradient runs for 2-propanol

No.	Name	$T=30$ °C				$T=60$ °C			
		$t_G=30$ min		$t_G=90$ min		$t_G=30$ min		$t_G=90$ min	
		t_R	Area	t_R	Area	t_R	Area	t_R	Area
1	Methysticin	13.92	495	26.52	520	11.73	506	19.61	540
2	Dihydromethysticin	14.13	513	26.91	513	12.01	492	20.26	519
3	Unknown U1	14.13	61	27.20	61	12.15	59	20.84	61
4	Unknown U2	14.13	129	27.20	129	12.36	125	21.21	129
5	Kavain	15.09	886	29.24	915	12.99	846	22.63	921
6	Unknown NP1	15.09	19	29.70	20	12.99	18	23.34	20
7	Dihydrokavain	15.47	484	30.06	531	13.42	480	23.67	547
8	Unknown NP2	15.47	31	30.79	24	13.42	30	24.38	35
9	Unknown NP3	15.73	20	31.09	23	13.68	20	24.90	21
10	Yangonin	15.73	381	31.71	370	13.68	375	25.20	379
11	Demethoxyyangonin	16.52	151	33.34	155	14.41	155	26.70	162
12	Unknown NP4	17.06	30	34.01	25	15.08	30	27.73	26
13	Unknown NP5	17.21	12	34.32	14	15.36	13	28.28	21

Gradient: 10–90% B; dwell volume: 0.5 ml; column: 25×0.46 cm I.D.; d_p: 5 μm; flow-rate: 1 ml/min.

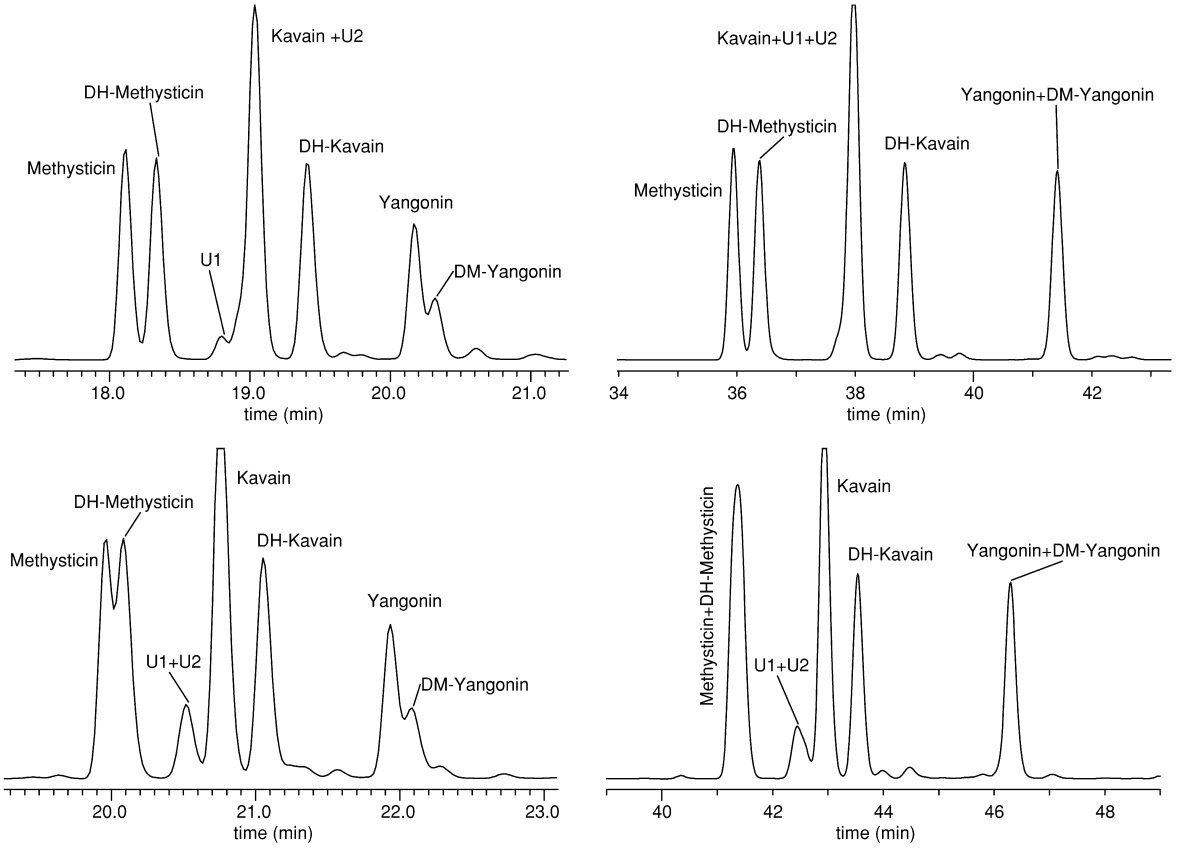

Fig. 2. Four original chromatograms with different t_G/T values using acetonitrile as the organic modifier. Lower left: 30 min/30 °C; lower right: 90 min/30 °C; upper left: 30 min/60 °C; upper right: 90 min/60 °C. For other conditions, see Section 2.

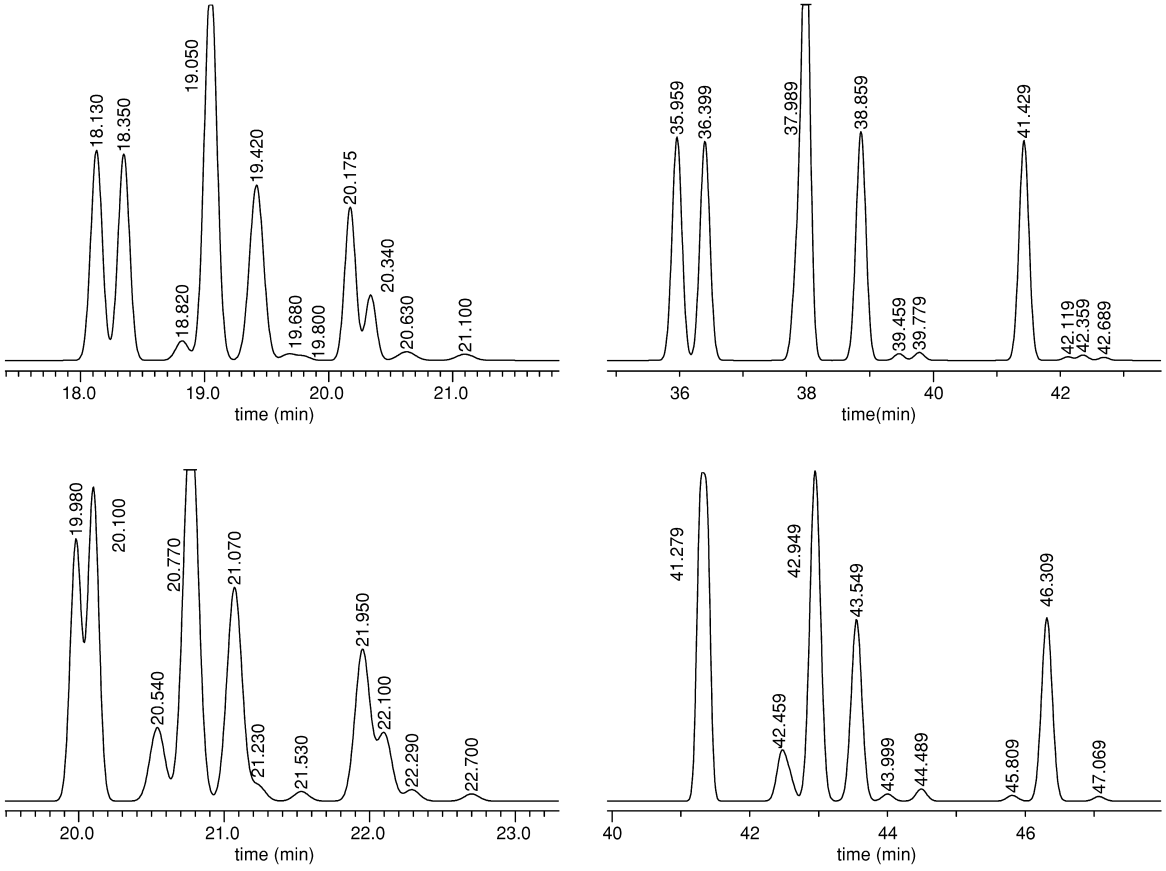

Fig. 3. Four chromatograms developed by the computer software DryLab with t_G/T values as shown in Fig. 2, using acetonitrile as the organic modifier.

white circles are representing the position of the four basic experiments. As the colour blue means ''coelution'', we can see from the critical resolution map, that all four basic runs must have had at least one or more coeluting band pairs. The maximum critical resolution is 0.54, which is insufficient for routine analysis. It becomes clear, that there is no reason to make any further trial and error-type of experiments as there is no choice to separate all peaks of interest with this set of conditions.

Therefore, in the following experiments acetonitrile was replaced by methanol (MeOH) (Fig. 5). Here, U1 is overlapping with kavain in all four basic experiments; we had to treat these bands as one peak otherwise the resolution map would be all blue (critical resolution, $R_{s,crit}=0$). Methanol is, therefore, not useful as an organic modifier and any further optimization with methanol would be meaningless.

3.3. Use of 2-propanol as eluent B

By using 2-propanol (i-PrOH) as eluent B, Fig. 6 shows the original chromatograms of the four input runs. Peaks are moving quite strongly, especially additional peaks U1 and U2, which are at 30 °C and 30 min completely hidden under the first two peaks, but at 60 °C and 90 min they are completely separated.

2-Propanol is a well known eluent in the separation of biopolymers and in normal-phase chromatography. It is comparable with tetrahydrofuran in terms of isoeluotropic power but it is more conveni-

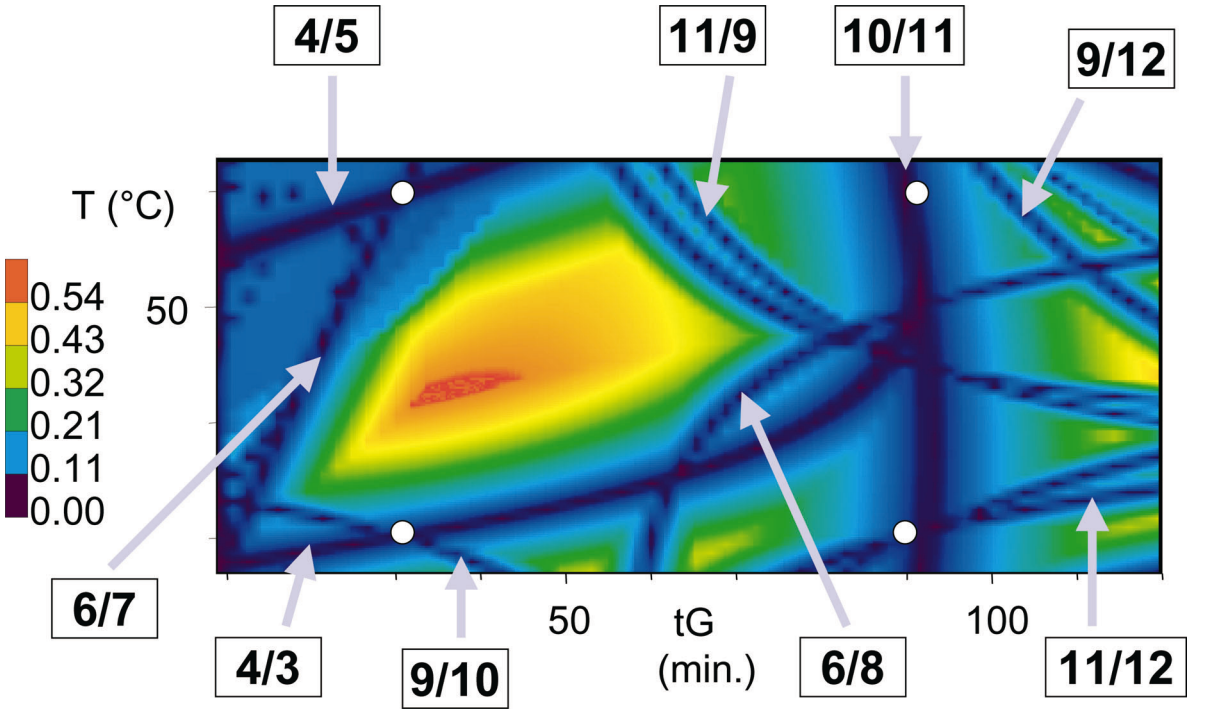

Fig. 4. Plot of the critical resolution vs. temperature and gradient time. $R_s = 0$, i.e. dark blue indicates that peaks overlap. Eluent B was acetonitrile. The region with the highest critical resolution is between 40 and 50 °C and t_G 30 and 60 min. The analysis time is about 25 min. However, this low critical resolution is not acceptable. The many blue lines in the map indicate numerous critical peak pairs.

ent in handling and less strongly smelling. Unlike *n*-butanol, it is completely miscible with water. Viscosity is an issue, but only at room temperature. Another point is the good HPLC quality of 2-propanol (Merck). The investigation of a ternary eluent acetonitrile vs. 2-propanol revealed the excellent properties of 2-propanol against acetonitrile. With ACN, we only could see four bands plus a shoulder. With i-PrOH, we saw the six major bands plus two clearly separated additional components. This was the reason for choosing i-PrOH in the final method.

The corresponding resolution map is shown in Fig. 7. The critical peaks at low t_G are Nos. 7+10, 9+10 and 7+8 as well. At $t_G > 40$ min the critical peak pair is 2+3. Baseline resolution is therefore possible at higher temperatures of 50 °C and with t_G of 90–100 min (5 to >95% 2-propanol). This behaviour is contradictory to the expectation that resolution would decrease at higher temperature. Here, the opposite is true.

3.4. Influence of the pH of the aqueous eluent A

As a further option, the pH value of the aqueous eluent was varied between 4.35, 4.95, and 5.55 at t_G 30 and 90 min. The resolution map, based on six experiments (Fig. 8), shows clearly that pH changes (in vertical direction) over the range of pH 4.3–5.7 result in no change in critical resolution as expected because the sample compounds are neutral. Critical peaks are 5+6 and 2+3. Further experiments in this pH range would not supply any new information.

3.5. Is isocratic work possible?

For this option, with the help of DryLab, a two-dimensional isocratic % B vs. temperature model was calculated. The critical resolution map for the isocratic elution (Fig. 9) shows, that there is only a small range at 23% and 54 °C, where a resolution (R_s 1.39) can be expected. However, the analysis time

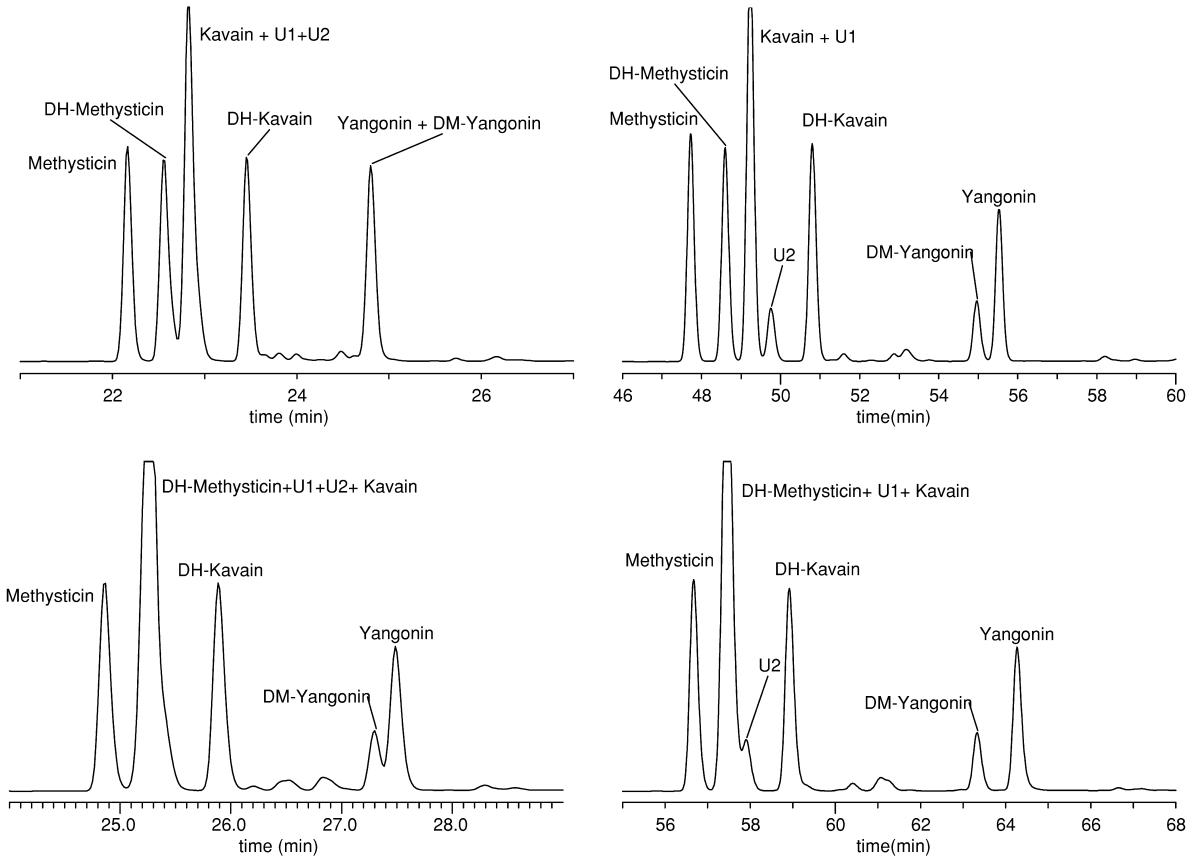

Fig. 5. Four original chromatograms with different t_G/T values using methanol as the organic modifier. t_G and T values as in Fig. 2. For other conditions, see Section 2.

would be over 30 min. As the pressure drop at 1.0 ml/min is already 2825 p.s.i., only a small reduction of the analysis time can be expected from a higher flow-rate (1 p.s.i. = 6894.76 Pa).

3.6. Optimum separation of U1 and U2 from dihydromethysticin and kavain

In further work, the investigation focussed on the two new compounds U1 and U2 with the goal of developing robust conditions for their analysis and preparative chromatography. Using a special feature of DryLab to calculate maps of the critical resolution either for one peak or for a group of peaks, we could see that for the new unknowns U1 and U2 there is a region at 55–60 °C and in the t_G range of 70–90 min. So far the condition t_G: 90 min and T: 60 °C can be considered as fairly robust.

3.7. Which organic eluent is the best?

The best organic eluent, as can be seen in Fig. 10 and in a different cutout in Fig. 11, is 2-propanol. The retention times of the kava pyrones are the shortest and the separation is the best possible.

3.8. Gradient shape and flow-rate

Further evaluation of the model kava in the gradient mode included a change in gradient shape and different flow-rates. To shorten the retention times, the gradient shape was changed from 20 to 30% 2-propanol in 16 min. The flow-rate was

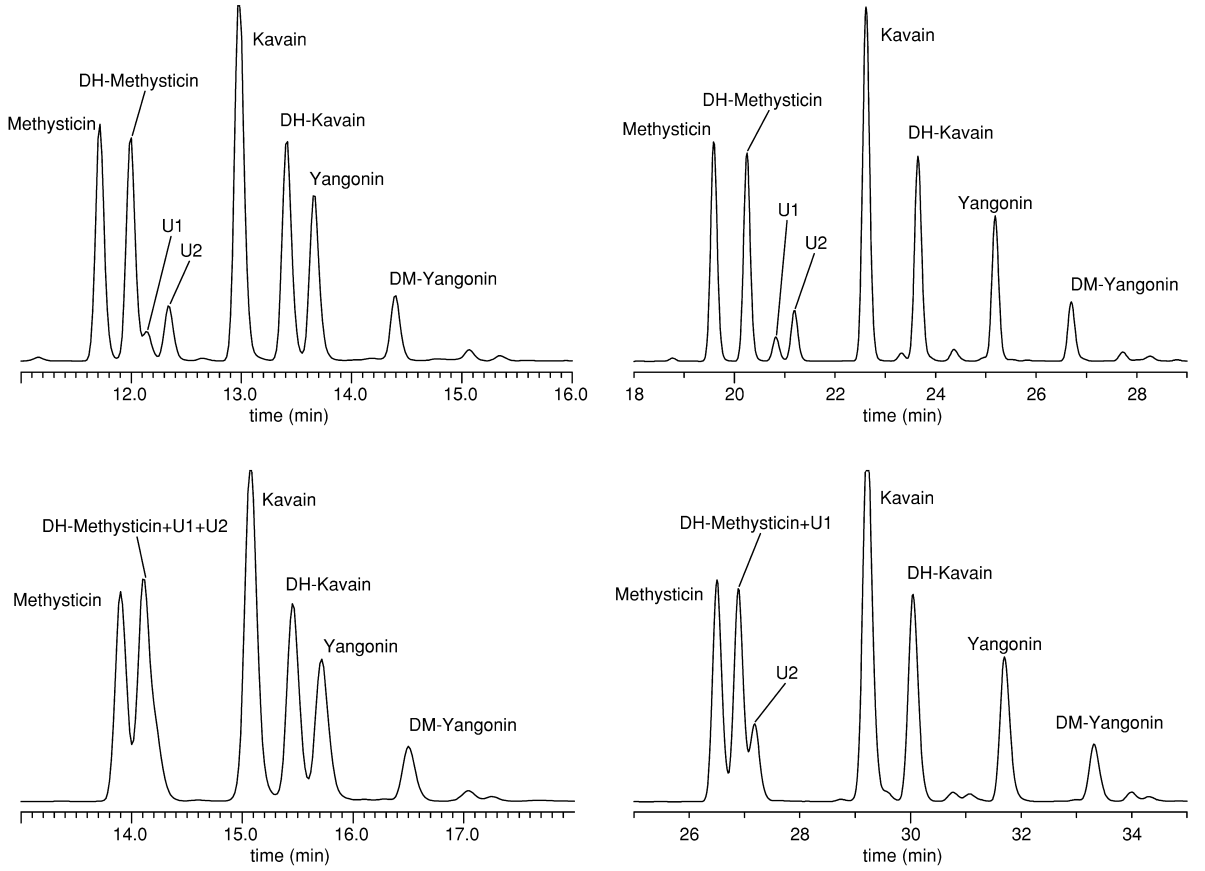

Fig. 6. Four original chromatograms with different t_G/T values by using 2-propanol as the organic modifier. t_G and T values as in Fig. 2. For other conditions, see Section 2.

increased corresponding to DryLab prediction from 1.0 to 1.2 ml/min and finally to 1.4 ml/min, without producing pressure problems. We favor the separation with a flow-rate of 1.4 ml/min because of the shorter analysis time. Therefore, a resolution of 1.4 for the peaks U1 (No. 3) and U2 (No. 4) seems to be sufficient for this minor peak pair. The chromatograms are shown in Fig. 12.

4. Conclusions

It has been demonstrated that 2-propanol is the best eluent for the separation of the kava pyrones. Acetonitrile and methanol deliver hidden components and long analysis times. In methanol, two bands can not be separated at all. Computer-supported optimization by DryLab made it easier to find optimum conditions or to avoid work without useful information. DryLab was used mainly to produce resolution maps and virtual chromatograms. Trying to vary the pH value by using 2-propanol as eluent B, the resolution map demonstrated impressively that there are no further improvements possible. To prove whether an isocratic or a gradient run gives better results, we used experimental gradient runs and made a transformation with DryLab in an isocratic method. The isocratic method did not, however, show better resolution values. We, therefore, could conclude that we have a fairly rugged region with 2-propanol in the gradient mode at $T = 50–60$ °C and $t_G = 70–200$ min. Further reduction of analysis time was possible by adapting a linear gradient and increasing the flow-rate from 1.0 to 1.4 ml/min. The

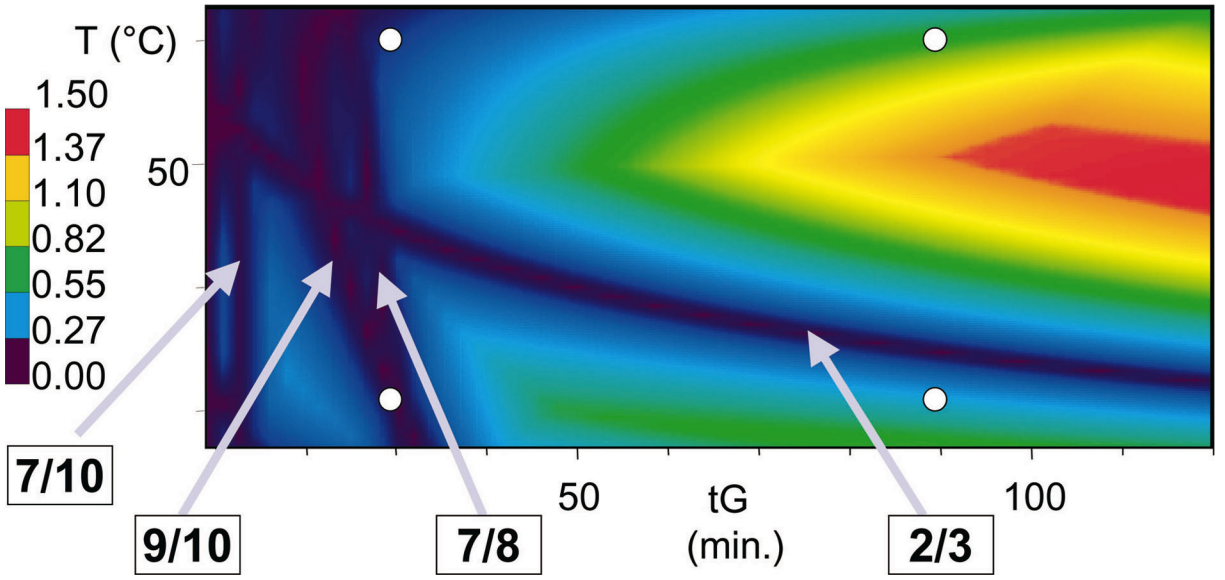

Fig. 7. Plot of critical resolution against temperature and gradient time. Eluent B was 2-propanol. There is a fairly rugged region at $T = 50–60$ °C and $t_G = 100–200$ min. The analysis time is about 25 min ($T = 50$ °C; $t_G = 70$ min). Using 2-propanol, we obtain an acceptable baseline separation.

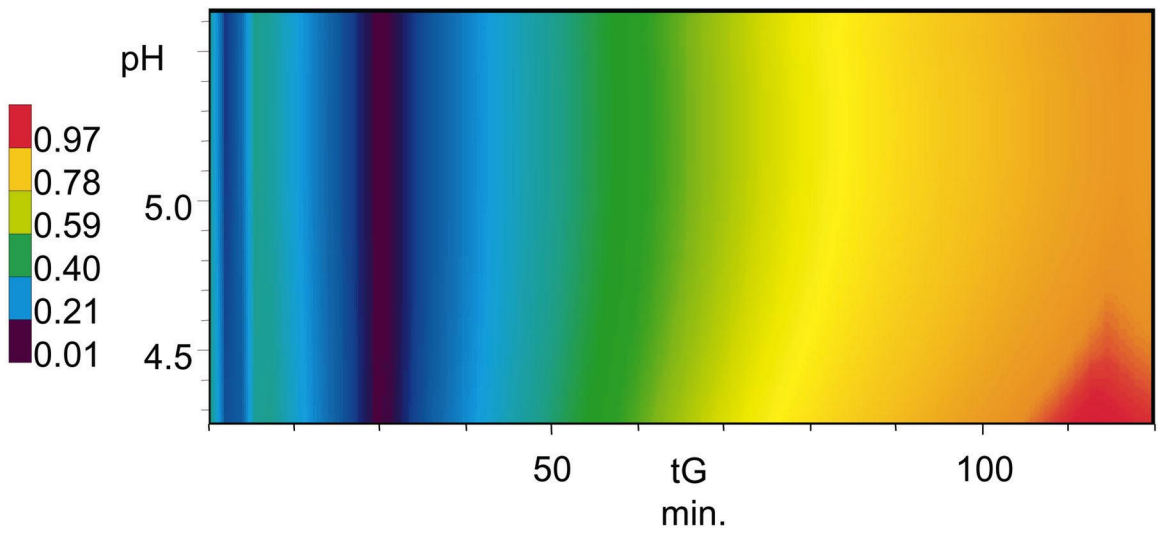

Fig. 8. Plot of the critical resolution against pH and gradient run time (t_G) showing the critical resolution. We can see that the pH has no influence on the critical resolution.

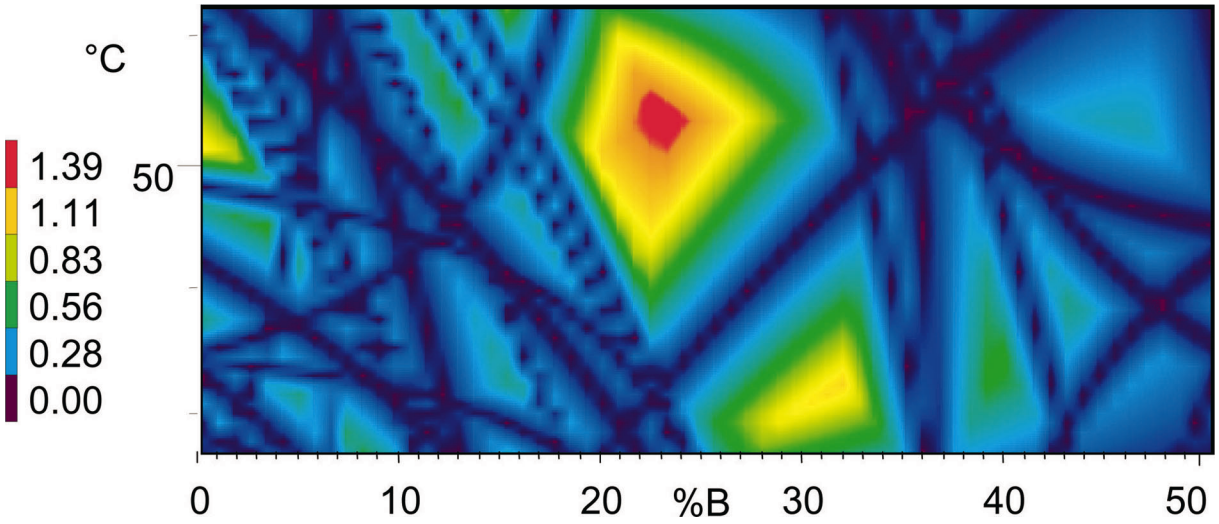

Fig. 9. Isocratic resolution map from gradient input data. Around 30% B there are areas with fairly rugged regions. The largest rugged area is at 18–25% B 2-propanol and temperature from 45 to 60 °C, where the analysis time is about 25 min. The plot indicates, however, the difficulty in finding the correct temperature and % B without peak overlaps in complex plant extracts.

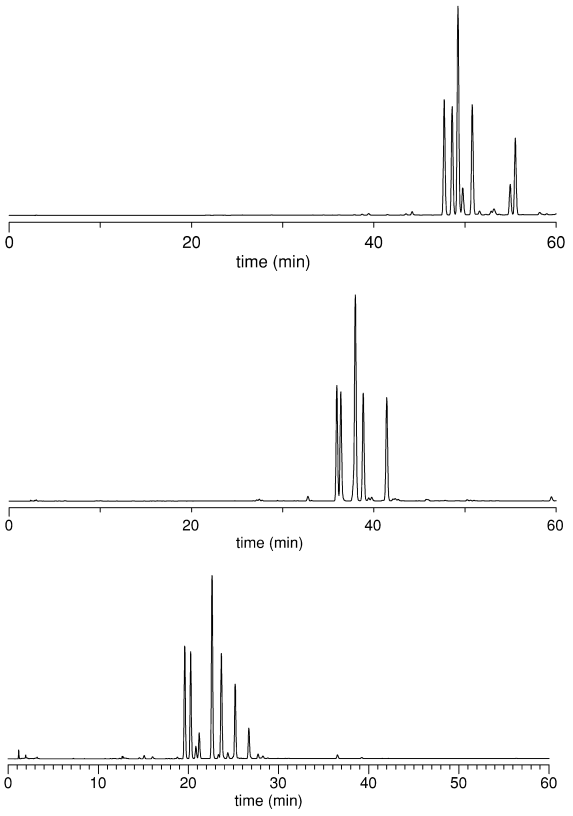

Fig. 10. Comparison of the three organic modifier. t_G: 90 min, T: 60 °C. Top: methanol; middle: acetonitrile; bottom: 2-propanol. For other conditions, see Section 2.

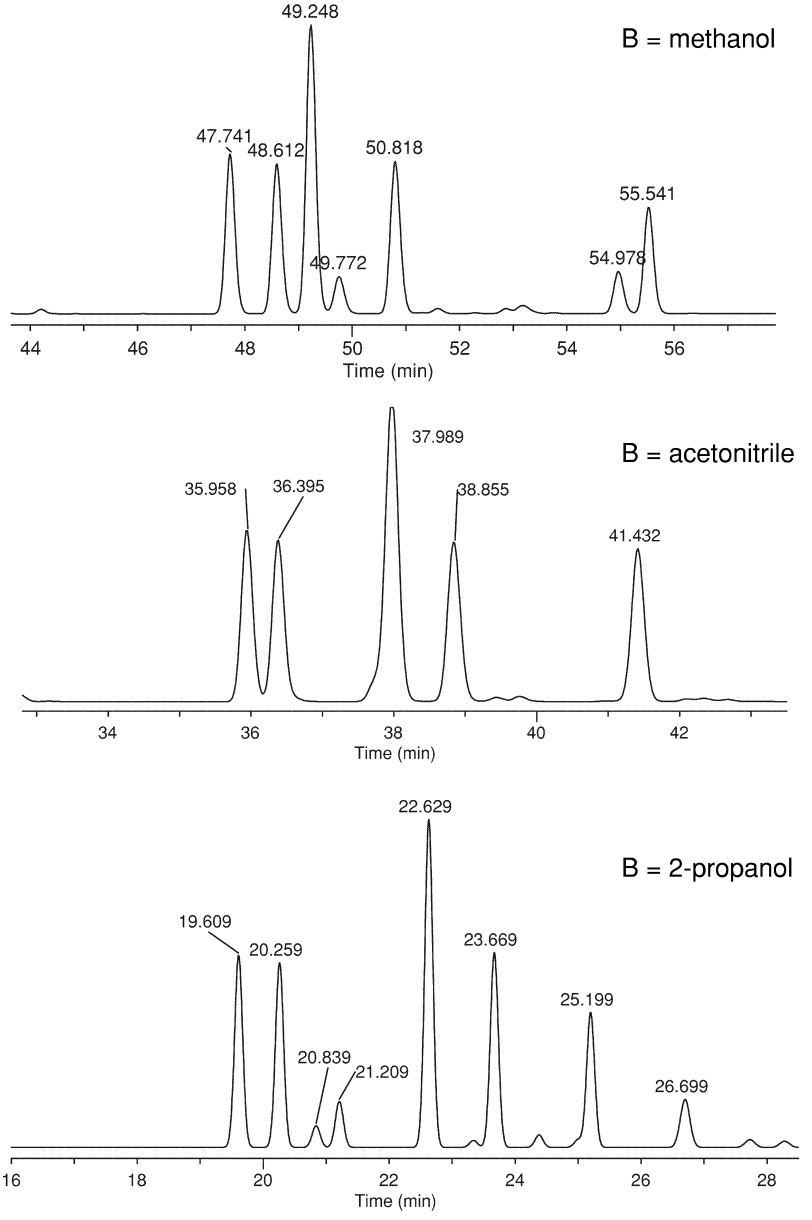

Fig. 11. Same as Fig. 12, but with a different cutout to show the selectivity differences.

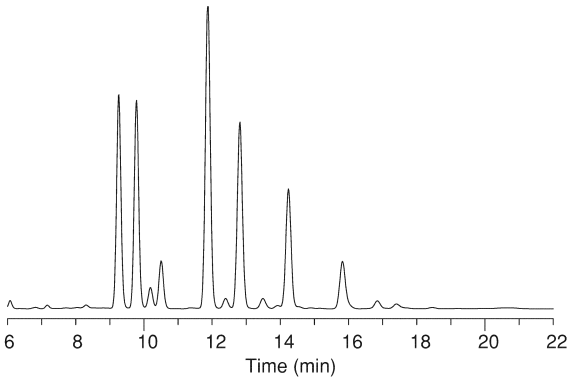

Fig. 12. Optimization of speed of analysis using different flow-rates. Other conditions T: 60 °C, linear gradient from 20 to 30% 2-propanol in 16 min.

analysis time is about 15 min for eight major components at 1.4 ml/min.

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