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Fast development of a robust high-performance liquid chromatographic method for *Ginkgo biloba* based on computer simulation

I. MOLNAR*
Institut für angewandte Chromatographie, Berlin (Germany)
and
K. H. GOBER and B. CHRIST
A. Nattermann & Cie GmbH, Köln (Germany)

ABSTRACT

Rapid development of robust and reliable high-performance liquid chromatographic methods for routine quality control of *Ginkgo biloba* is possible with computer simulation. The goal is to reduce method development time and to increase transparency of the complex composition of plant extracts. With only two basic experiments and a peak tracking process based on the total area ratio compared to the individual peak-area ratios a robust method with more than 50 simulated experiments was completed in 8 h. The best method has been verified experimentally. The correlation between the best simulated run and the final experiment was satisfactory.

INTRODUCTION

Extracts of the leaves of *Ginkgo biloba*, a traditional pharmaceutical product in Asiatic medicine, are used in the treatment of vascular diseases. It contains flavonoids, biflavonoids, ginkgolides (diterpenes) and the sesquiterpene bilobalid [1].

Today quality control requires modern analytical techniques to monitor all compounds in *Ginkgo biloba*. For this purpose we tried to isolate preparative amounts of some major components in *Ginkgo biloba* mixtures. In the first step a methanolic extract was separated on a Lobar RP-18 column. About 2% of the total mass of the plant extract was taken as a sharp fraction, and investigated further in a second step.

Due to differences in the origin of *Ginkgo biloba* analytical runs often reveal differences in composition. This is a time-consuming problem and often delays production of the drug. Peak overlaps especially, which depend on the amount of the organic modifier, often require a readjustment in the method for the actual sample by "trial and error".

On the other hand DryLab software, a small expert system for rapid and sys-

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tematic high-performance liquid chromatographic (HPLC) method development is able to generate a large number of data from only two sets of experiments [2]. The goal of this work was to save time, using computer simulation techniques for the rapid development of a robust HPLC separation method for the isolation of standard components from preparative fractions of *Ginkgo biloba* extracts.

EXPERIMENTAL

Equipment

The HPLC system used was a Merck-Hitachi LiChrograph with an L-4000 UV–VIS detector (Merck, Darmstadt, Germany). The dwell volume of the instrument was 5.4 ml. Chromatograms were monitored at a wavelength of 330 nm. The injection device was a Rheodyne 7125 (Cotati, CA, USA).

The software DryLab I/plus (isocratic version) and DryLab G/plus (gradient version) (LC Resources, Lafayette, CA, USA; in Europe: Molnar, Berlin, Germany) are written for use with IBM compatible personal computers and were installed on a Victor 286 with a 20-MB hard disk (Victor, Langen, Germany). Graphical simulations of chromatograms were predicted in *ca.* 0.1 s and were printed with "Grafplus" and "Graflasr" software (Jewell Technologies, Seattle, WA, USA) on a Kyocera F-800 laser printer.

Chromatographic conditions

The system was operated at 30°C or in some cases at 25°C. The column in use was packed with Nucleosil C_{18} (Macherey & Nagel, Düren, Germany) of 5 μm particle size, and of dimensions 250 × 4 mm I.D. The flow-rate was 0.8 ml/min and the column pressure was 12 MPa. The aqueous eluent A was HPLC-grade water, adjusted to pH 3.5 with concentrated ortho-phosphoric acid. The organic modifier B was HPLC-grade acetonitrile (Merck). Phosphoric acid (Merck) was of analytical grade.

Standard samples of *Ginkgo biloba* leaves were supplied by S. B. Kwon (China). A methanolic extract of the drug was separated on a Lobar RP-18 column (250 × 32 mm I.D.) (Merck). One sharp fraction, containing about 2% of the total mass of the sample extract, was especially difficult to separate. This fraction was used for computer-supported rapid method development. The injection volume was 20 μl of a 1 mg/ml solution of the fraction in methanol.

Method development with DryLab

In HPLC, computer-supported method development is becoming increasingly important [3–10]. DryLab is able to predict robust methods and conditions of equal band spacing, which are necessary for high-speed separations for small as well as for large molecules, such as ribosomal proteins [11,12]. It can generate new chromatograms under user-selected chromatographic conditions. For this purpose the software has to be supplied with two sets of experimental data.

All experimental parameters, such as dwell volume, column parameters, elution conditions, the number of components, their retention times and the peak areas of the reference run were entered into the program [3,4] (Tables I–III).

Method of peak tracking

Method development with DryLab was started using a previously developed method in isocratic mode with 27% B (Fig. 1). The separation, however, was not satisfactory, especially between the two last major peaks, which showed a strong overlap.

Next, two gradients were made from 5 to 100% B in 15 and 45 min, respectively. Both runs showed closely eluting peaks. Here we could only work with some of the major peaks when using DryLab I/plus and found an optimum at 29% B, a result which was within 2% of the routine method used previously. For the very close elution (Fig. 2) DryLab I/plus suggested less-steep gradients.

Consequently, two more gradients were run, each from 20 to 70% B, with a gradient run time (t_G) of 20 and 60 min, respectively (Fig. 3). Data from the integrator are listed in Table I.

Peak range

We first determined the range of interest in both chromatograms. In Fig. 3a, the

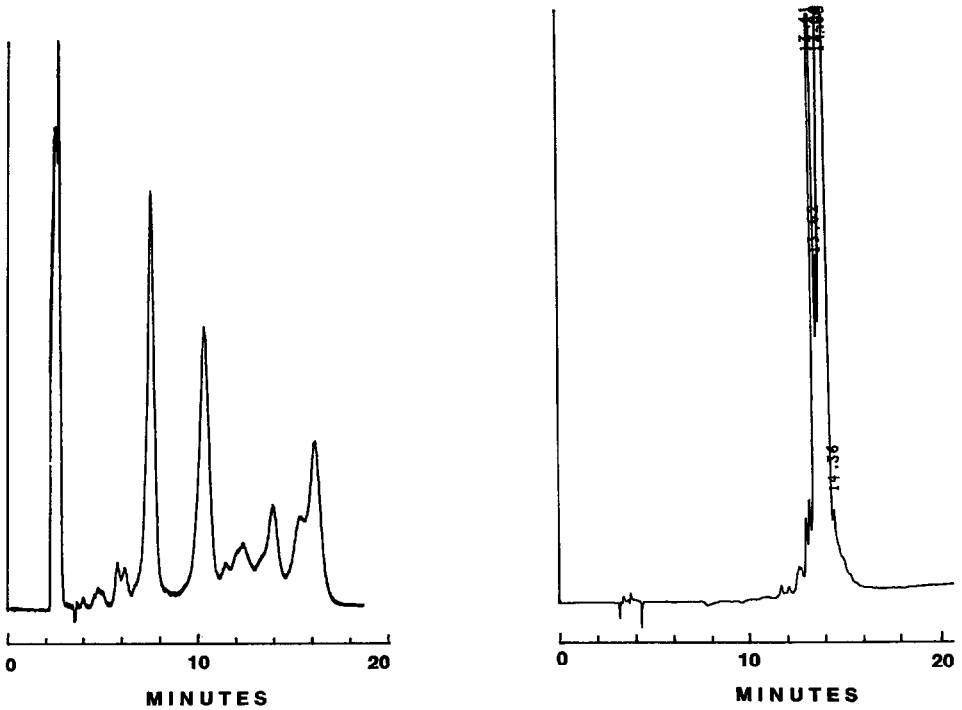

Fig. 1. Separation of a *Ginkgo biloba* extract. Elution conditions: column, Nucleosil C_{18}, 250 × 4 mm I.D., particle size, 7 μm; isocratic separation using 27% acetonitrile; temperature, 30°C. Other conditions as in Experimental.

Fig. 2. Separation of a *Ginkgo biloba* extract. Elution conditions: column, Nucleosil C_{18}, 250 × 4 mm I.D., particle size, 7 μm; gradient from 5 to 100% B in 15 min; temperature, 30°C. Other conditions as in Experimental.

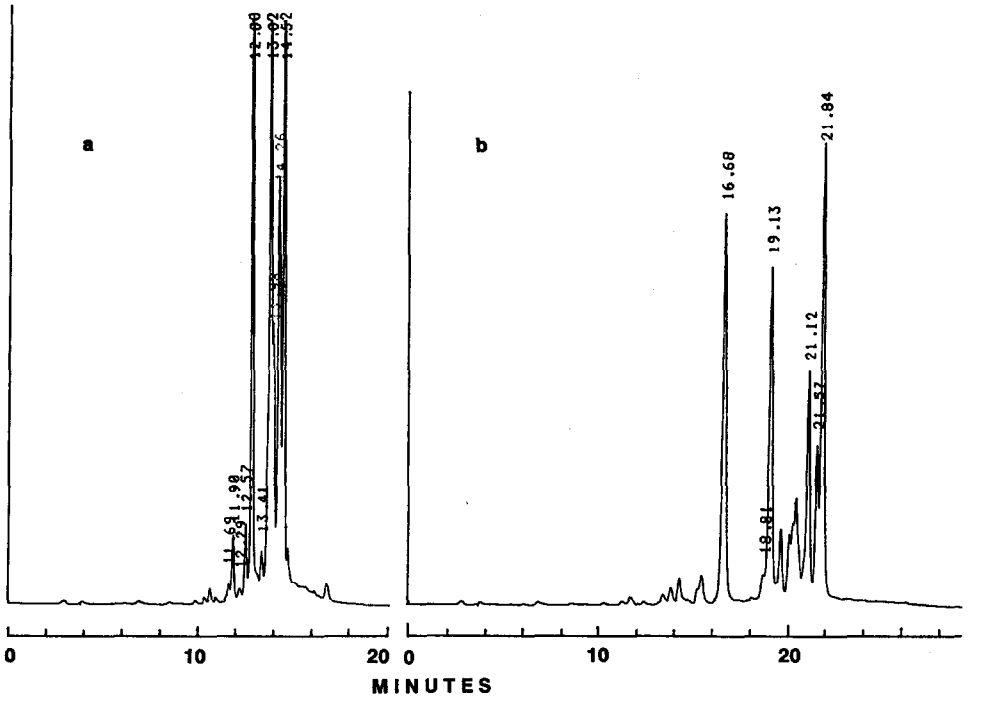

Fig. 3. Separation of a *Ginkgo biloba* extract. Elution conditions: column, Nucleosil C_{18}, 250 × 4 mm I.D., particle size, 7 μm; (a) gradient from 20 to 70% B in 20 min; (b) gradient from 20 to 70% B in 60 min; temperature, 30°C. Other conditions as in Experimental.

20-min run shows the range 8.56–16.80 min, and in Fig. 3b, the 60-min run range is 8.74–21.84 min. We observed a larger number of peaks in the 60-min run. By direct comparison we have the problem that in terms of the peak order the large peaks do not match.

Correspondence between components

The correspondence between components of the two runs had to be correctly established. The two chromatograms with their integration reports were compared as follows.

Determination of the reference run. By definition, the reference run contains the larger number of components. Using DryLab G/plus for gradient elution, the reference run is normally the one with the smaller slope, or larger gradient run time, t_G.

Trial run. We called the other chromatogram with the smaller number of peaks the trial run.

Peak assignment

This process is also called peak tracking [13,14], as our goal was to find each peak in both chromatograms. As the position of a component was changing, often unpredictably, with changing elution conditions, peak tracking was necessary for the establishment of the identity of the components.

TABLE I

INTEGRATION REPORT FOR THE 20- AND 60-min RUNS

Peak No.	Reference run (60 min)			Trial run (20 min)			$R_{i,1}$[c]
	t_R (min)	Estimated resolution values[a]	Peak area[b]	t_R (min)	Estimated resolution values[a]	Peak area[b]	
01	8.74	bl	7	8.56	bl	7	1.00
02	10.42	bl	8	9.94	bl	9	1.13
03	11.37	bl	12	10.42	bl	14	1.17
04	11.84	bl	30	10.72	bl	33	1.10
05	12.52	bl	10	11.01	bl	15	1.50
06	13.56	bl	38	11.69	0.5	51	1.34
07	13.97	1.0	52	11.90	1.1	137	2.63
08	14.40	bl	96	12.29	0.9	44	0.46
09	15.57	bl	122	12.57	0.9	156	1.28
10	16.68	bl	1112	12.88	0.8	1199	1.08
11	18.24	bl	25	13.41	0.7	127	5.08
12	18.81	0.5	79	13.82	0.5	1806	22.86
13	19.13	1.0	931	13.98	0.9	80	0.09
14	19.70	1.4	220	14.26	0.8	1015	4.61
15	20.16	0.4	149	14.52	0.6	1629	10.93
16	20.34	0.3	143	14.76	0.6	17	0.12
17	20.48	1.2	364	15.13	0.5	11	0.03
18	21.12	1.3	658	16.17	bl	4	0.01
19	21.57	0.8	352	16.80	bl	330	0.94
20	21.84	bl[d]	1207				
21	23.09	bl[d]	6				
22	26.25		3				
Total			5624			6668	1.19

[a] Resolution values are only roughly estimated.
[b] Peak-area values are given in thousands of integration units.
[c] $R_{i,1} = A_{T,i}/A_{R,i}$.
[d] bl = Baseline-separated.

RESULTS AND DISCUSSION

Peak tracking

There are 22 peaks in the 60-min run, but only 19 peaks in the 20-min run. The sum of the peak areas is, as expected with similar sample injection amounts, comparable in size. The ratio of the total peak areas is 6684:5624 = 1.19. In ideal injection and integration conditions both total peak areas should be about equal [6].

When properly assigned, individual peak-area ratios should have the same value as the total peak-area ratio. As we can observe in our case, not all peak-area ratios correspond to this number, which turned out to be 1.19. There are some strong deviations, such as at reference peak No. 7 and also at the peak group 11–19.

Resolution values can be helpful if the chromatogram is not at hand. We can see that for peaks which are baseline-separated (bl), the peak-area ratio is close to the total area ratio. Such peaks are the so-called "well behaving" ones. In overlapping

ABLE II

EAK TRACKING PROCESS

ıtegration report, uncorrected						Peak tracking after corrections					
eak lo.	60 min		20 min		R_i	Peak No.	60 min		20 min		R_i
	t_R	Area	t_R	Area			t_R	Area	t_R	Area	
1	8.74	7.00	8.56	7.00	1.00	01	8.74	7.00	8.56	7.00	1.00
2	10.42	8.00	9.94	9.00	1.13	02	10.42	8.00	9.94	9.00	1.13
3	11.37	12.00	10.42	14.00	1.17	03	11.37	12.00	10.42	14.00	1.17
4	11.84	30.00	10.72	33.00	1.10	04	11.84	30.00	10.72	33.00	1.10
5	12.52	10.00	11.01	15.00	1.50	05	12.52	10.00	11.01	15.00	1.50
6	13.56	38.00	11.69	51.00	1.34	06	13.56	38.00	11.69	51.00	1.34
7	13.97	52.00	11.90	137.00	2.63	07	13.97	52.00	11.90	137.00	1.22[a]
8	14.40	96.00	12.29	44.00	0.46	08	14.40	96.00	12.29	44.00	1.22[a]
9	15.57	122.00	12.57	156.00	1.28	09	15.57	122.00	12.57	156.00	1.28
0	16.68	1112.00	12.88	1199.00	1.08	10	16.68	1112.00	12.88	1199.00	1.08
1	18.24	25.00	13.41	127.00	5.08	11	18.24	25.00	13.41	30.53	1.22
2	18.81	79.00	13.82	1806.00	22.86	12	18.81	79.00	13.41	96.47	1.22
3	19.13	931.00	13.98	80.00	0.09	13	19.13	931.00	13.82	1806.00	1.31[b]
4	19.70	220.00	14.26	1015.00	4.61	14	19.70	220.00	13.98	80.00	1.31[b]
5	20.16	149.00	14.52	1629.00	10.93	15	20.16	149.00			1.31[b]
6	20.34	143.00	14.76	17.00	0.12	16	20.34	143.00			1.31[b]
7	20.48	364.00	15.13	11.00	0.03	17	20.48	364.00			0.99[c]
8	21.12	658.00	16.17	4.00	0.01	18	21.12	658.00	14.26	1015.00	0.99[c]
9	21.57	352.00	16.80	330.00	0.94	19	21.57	352.00			1.04[d]
0	21.84	1207.00				20	21.84	1207.00	14.52	1629.00	1.04[d]
1	23.09	6.00				21	23.09	6.00	14.76	17.00	−[e]
2	26.25	3.00				22	26.25	3.00	15.13	11.00	−[e]
3						23		0.01	16.17	4.00	−[e]
4						24		0.01	16.80	330.00	
um		5624.00		6684.00	1.19			5624.00		6684.00	1.19
lean (1–24)					3.02						1.19
.D. (1–24)					5.46						0.14

Bad integration causes R_i of 2.63 and 0.46. The mean value of both peak areas in both runs gives a reasonable R_i of 1.22 for both peak pairs No. 7 and 8, a value which is fairly close to the total ratio of $R_i = 1.19$.

Peaks 12 and 13 of the 20-min run correspond obviously with peaks 13, 14, 15 and 16 of the 60-min run. The group ratio is 1.31, which is fairly close to 1.19.

Peaks 17 and 18 of the 60-min run join to one peak in the 20-min run. The new ratio after correction is 0.99, which is fine.

Similar case to note *c*. The corrected ratio is 1.04.

These peaks were neglected.

groups, however, there are some "not well behaving" bands, especially in the trial (faster), but in many cases also in the reference run.

Taking a look at both chromatograms, we can distinguish five large peaks, having a distinct size ratio. On the basis of their shape and peak area we tried to match them. One of these peaks is reference peak No. 10. It has a peak-area ratio of 1.08, which is close to the statistically robust total ratio of 1.19. Other large peaks in the 20-min run are overlapping with other peaks and therefore have larger peak areas,

TABLE III

DRYLAB G, PART I INPUT VALUES

System variables: dwell volume, 5.40 ml; column length, 25.00 cm; column diameter, 0.40 cm; flow-rate, 0.80 ml/min; starting %B, 20.0; final %B, 70.0; gradient time run 1, 20.0 min; gradient time run 2, 60.0 min. Default N value for R_s calculations, 10 000. Number of bands = 13.

Band No.	Retention time (min)		Area
	Run 1	Run 2	
1	12.57	15.57	122.00
2	12.88	16.68	1112.00
3	13.41	18.24	25.00
4	13.41	18.81	79.00
5	13.82	19.13	931.00
6	13.82	19.70	220.00
7	13.82	20.16	149.00
8	13.98	20.34	143.00
9	14.26	20.48	364.00
10	14.26	21.12	658.00
11	14.52	21.57	352.00
12	14.52	21.84	1207.00
13	14.76	23.09	6.00

as expected. We have to deconvolute such peaks and this is shown in Table II. For example, it is obvious that peaks 13, 14, 15 and 16 of the reference run correspond with peak Nos. 12 and 13 of the trial run. Dividing the sum of the trial peaks 12 + 13 (1808 + 80 = 1888) by the sum of reference peaks 13–16 (931 + 220 + 149 + 143 = 1443), we have a ratio for all peaks of 1.31.

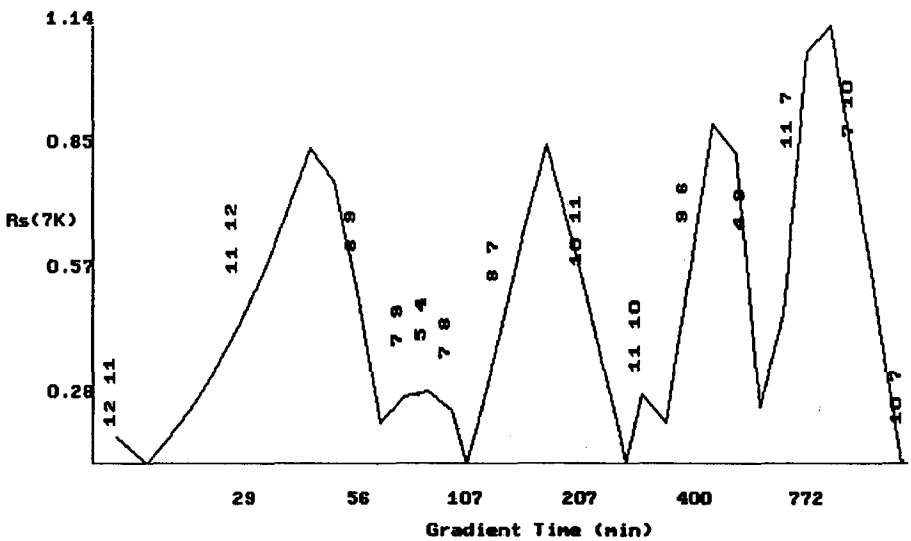

Fig. 4. Relative resolution map for the *Ginkgo biloba* sample by Dry Lab G/plus (gradient from 20 to 70% B). The highest resolution can be observed at 50 min gradient time. Other conditions as in Experimental.

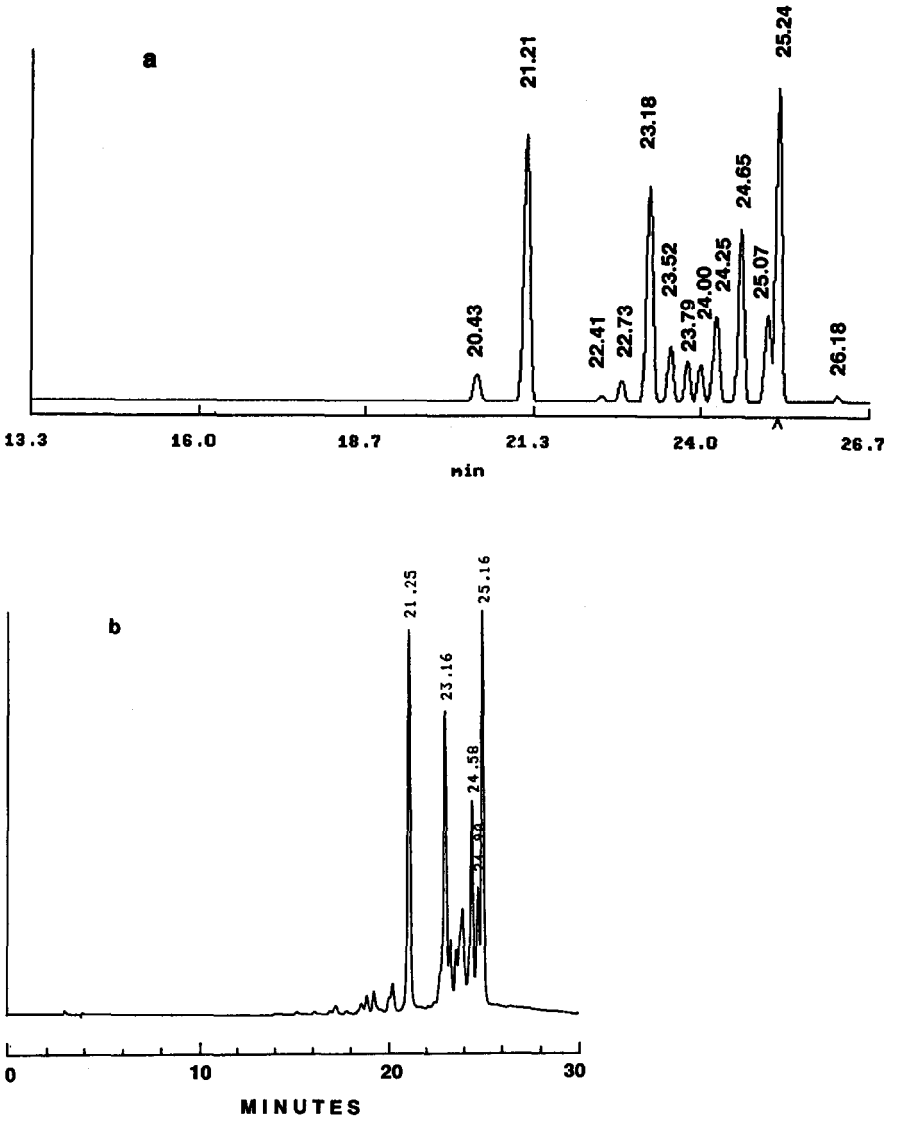

Fig. 5. Optimum gradient conditions from 15 to 40% B in 25 min by (a) simulation by DryLab G/plus and (b) the corresponding experiment. Other conditions as in Experimental.

One possible measure of the peak match is the mean value and the standard deviation of the individual peak-area ratios compared to the ratio of the sums of all peak areas [6]. As we can see in Table II, the correction of the peak identities brings the mean peak-area ratio very close to the ratio of the sums of all peak areas. As long as the uncorrected mean peak-area ratio is 3.02 with a standard deviation of 5.46, after peak tracking we have a mean peak-area ratio of 1.19, the same value as the total area ratio, 1.19. The standard deviation is now decreased to 0.14 (Table II).

TABLE IV

COMPARISON OF PREDICTED AND EXPERIMENTALLY FOUND RETENTION TIMES OF *GINKGO BILOBA* SAMPLE IN GRADIENT ELUTION FROM 15 TO 40% ACETONITRILE

Other conditions as in Fig. 1a and b.

No.	Retention time (min)		
	Predicted	Experimental	Difference
1	20.43	20.41	0.02
2	21.21	21.25	0.04
3	22.41	22.30	0.11
4	22.73	22.61	0.12
5	23.18	23.16	0.02
6	23.52	23.45	0.07
7	23.79	23.71	0.08
8	24.00	24.06	0.06
9	24.25	24.06	0.19
10	24.65	24.58	0.07
11	25.07	24.90	0.17
12	25.24	25.16	0.08
13	26.18	26.65	0.47

Average deviation < 0.4%

It has to be mentioned at this point that the quality of integration is essential for a reliable peak tracking process. The chromatographer has to be in the position to be able to change any wrong integration and to repeat integration with new data as often as necessary. This is an easy job using integration software programs such as Nelson, BarSpec's Chrom-A-Set, Maxima and many others, which are now commercially available. Stand-alone type integrators should set a mark at the start and at the end of integration, and they also should be in position to re-integrate the same data set under different baseline settings.

Simulation of chromatograms with DryLab G/plus

Following the peak assignment process, all necessary data were entered into DryLab G/plus (Table III). In accordance with the fact that the HPLC system had a low-pressure gradient mixer, an estimated dwell volume of 5.4 ml was taken.

The range of the components has been reduced to the thirteen most important peaks. For the simulation of gradient runs we started with a consideration of the relative resolution map (RRM) (Fig. 4). According to the RRM, an optimum is at a run of 50 min duration, going from 20 to 70% B. This run, however, takes rather a long time.

Another gradient is from 15 to 40% B in 25 min; this run is complete after 25 min (Fig. 5).

Reliability of the predictions

The precision of the predictions of DryLab G/plus with two other sets of samples (five benzoic acid esters and thirty ribosomal proteins) showed a coefficient of

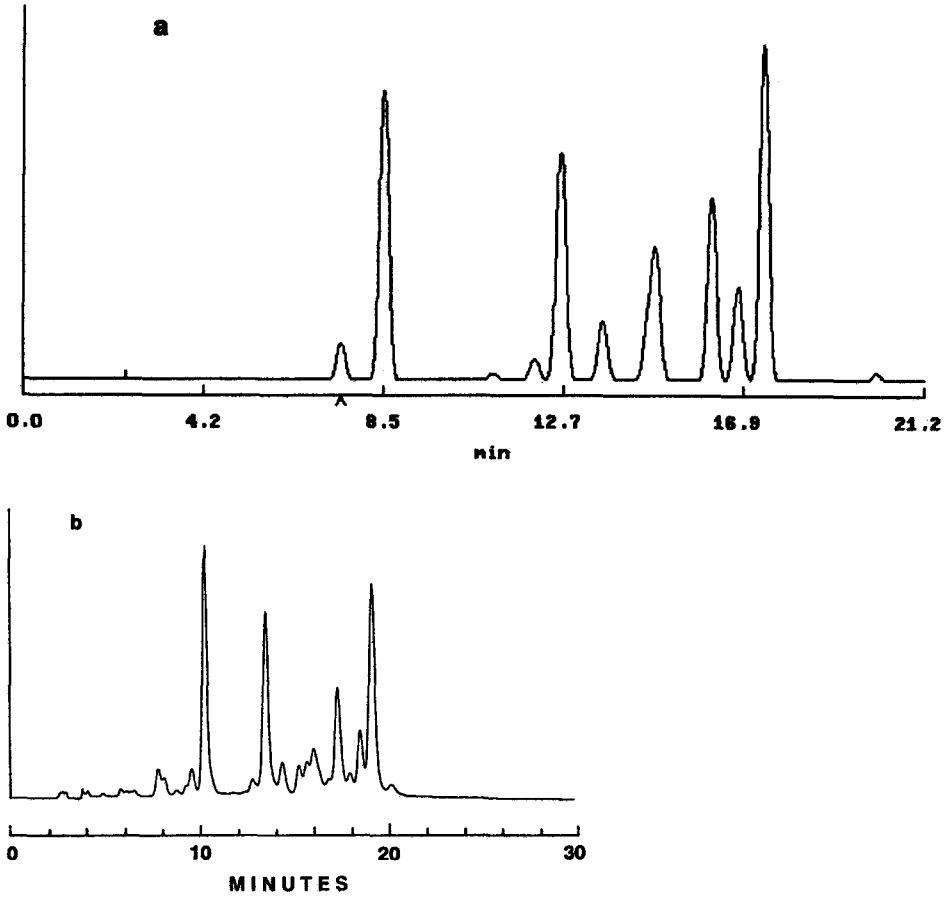

Fig. 6. Optimum gradient conditions from 25 to 30% B in 10 min by (a) simulation by DryLab G/plus (30°C) and (b) the corresponding experiment, at 25°C. Other conditions as in Experimental.

variation of less than 1% [6,11]. We expected, therefore, similar precision in the case of the *Ginkgo biloba* sample between predicted and experimentally verified values. The retention data shown in Fig. 5a were tested for correlation with the experimental values (Fig. 5b). As we can see in Table IV the reliability of the prediction is satisfactory: the average deviation between prediction and experiment is less than 0.4%.

After trying several gradients, we decided on a final run of higher peak resolution, as shown in Fig. 6a. The conditions were experimentally verified, resulting in the run shown in Fig. 6b. The correlation is also sufficient, although for better resolution the temperature in Fig. 6b was reduced from 30°C to 25°C, making the experimental retention times longer than predicted.

CONCLUSIONS

We have shown the application of computer-aided HPLC method optimization in the separation of a natural product mixture. After two initial gradient elution runs,

peaks were matched between runs using an area-ratio matching technique. Good integration software is necessary to obtain the correct peak areas. The matched peaks were input into the optimization software and the best run conditions were found using computer-based simulation tools. The predicted optimum compared well with an experimental run under the predicted conditions, with an average deviation in retention times of less than 1%. The total development time of about 1 day saved considerable time over traditional trial-and-error optimization techniques.

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