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Preliminary computer simulation for fine tuning of the high-performance liquid chromatography of some phenolic acids

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Abstract

Computer simulation (Drylab G) was used to optimize the reversed-phase high performance liquid chromatography of eleven phenolic acids. On the basis of the data for two preliminarily optimized solvent systems (I = acetonitrile–water–1% acetic acid, II = acetonitrile–tetrahydrofuran–1% acetic acid) obtained by computer simulation the subsequent simple step-by-step procedure allowed conditions for the separation of all solutes to be found.

1. Introduction

Generally it is easy to separate simple mixtures consisting of several solutes and to ensure that the capacity factors are in the optimum range of 1–10. However, for more complex mixtures the optimization of the chromatographic system can be complicated and the trial-and-error procedures are time consuming and lead to a greater consumption of solvents and materials. Computer-assisted methods, which have been extensively developed in recent years, can be applied to solve the problems more readily [1]. However, before starting with any optimization procedure, it is worthwhile studying the literature on the subject. It is also well known from the chromatographic literature [2] that adsorbents differ from batch to batch, which can result in selectivity changes, and it is often necessary to reoptimize the chromatographic system.

Examples of the chromatographic separations of phenolic acids are well documented [3–5]. The phenolic acids are relatively hydrophilic compounds and conventionally are separated by ion-pair or ion-suppression reversed-phase liquid chromatography. In this work, the latter mode was applied to obtain preliminary data for computer optimization. We used a typical procedure for optimization of the phenolic acid mixture by means of Drylab G software [6,7], which is based on the retention data of two preliminary runs and system variables. In our case the results for the two eluent systems investigated were only partially satisfactory. We could try other eluent systems or a longer, more efficient column, but the two experimental chromatograms obtained led us to the conclusion that the final chromatographic optimization could be easily performed by an additional simple step-by-step procedure.

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The method, which consists in the combination of two eluent systems, is presented below.

2. Experimental

An HP-1050 gradient liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a 20-μl sample injector (Rheodyne, Cotati, CA, USA) and a variable-wavelength UV detector (HP-1050) operated at 254 nm was used. The chromatograms were recorded with a Hewlett-Packard Model 3396 A reporting integrator. Stainless-steel columns (250 × 4.6 mm I.D. and 100 × 4.6 mm I.D.) were packed with 7-μm LiChrosorb RP-18 (Merck, Darmstadt, Germany) using laboratory-made apparatus with an Orlita pump. The first column had an efficiency of 6400 and the second 3600 theoretical plates, as determined using toluene as the test solute eluted with methanol–water (60:40) at a flow-rate of 1 ml/min. The dwell volume of the equipment was determined by running a blank gradient without the column.

In two gradient runs, acetonitrile (ACN), tetrahydrofuran (THF) and water (doubly distilled) were used as components of the eluent; all solvents contained 1% (v/v) of acetic acid to suppress the ionization of phenolic acids. The sample was a mixture of eleven components (see Table 1) dissolved in methanol–water (1:1).

Table 1
System variables, compounds and retention times

System variables			
Dwell volume (ml)		0.90	
Column length (cm)		25.0	
Column diameter (cm)		0.46	
Flow-rate (ml/min)		1.00	
Starting B (ACN) concentration (%)		0.0	
Final B (ACN) concentration (%)		50.0	
Gradient time, 1st run (min)		30.0	
Gradient time, 2nd run (min)		90.0	
Retention times			
Band No.	Compound	t_R (min)	
		Run 1	Run 2
1	Gallic acid	6.66	8.18
2	Protocatechuic acid	9.52	13.52
3	Chlorogenic acid	11.94	22.07
4	Vanillic acid	13.42	23.25
5	*trans*-Caffeic acid	13.64	24.43
6	Syringic acid	13.76	25.17
7	*cis*-Caffeic acid	14.42	26.13
8	*trans-p*-Coumaric acid	16.56	31.48
9	*cis-p*-Coumaric acid	17.17	32.97
10	*trans*-Ferulic acid	17.42	34.39
11	*cis*-Ferulic acid	18.05	36.60

3. Results and discussion

In accordance with the Drylab G instruction manual, preliminary data are needed for the optimization of resolution in reversed-phase liquid chromatography. The data consist of system variables and retention times of solutes for two typical linear gradient runs from 5% to 100% of modifier in water. For acetonitrile as modifier, too low retention times for some of the solutes were obtained for the 25-cm column. Especially gallic and protocatechuic acids show retentions near the dead time. This means that these bands are eluted under isocratic conditions and it is difficult to obtain precise data on retention times. Taking into account the aspects discussed above, we decided to perform two preliminary linear gradient runs starting from 0% ACN plus a constant acetic acid concentration (1%) for gradient times of 30 and 90 min. From a practical point of view it was of interest to test whether Drylab G can be applied to the prediction of retention and resolution in the system investigated (basing on retention data for a 0–50% gradient range).

In Fig. 1 the resolution map is presented for the system using a 25-cm column with a real plate number $N = 6400$. It can be seen that good resolution can be obtained for a very long gradient time (90 min). The most efficient resolution was obtained by simulation of chromatogram using another gradient range. The final gradient range was found to be from 0 to 23.1% ACN during 46.2 min. The simulated chromatogram for this gradient is shown in Fig. 2 and the real chromatogram in Fig. 3. Good agreement of

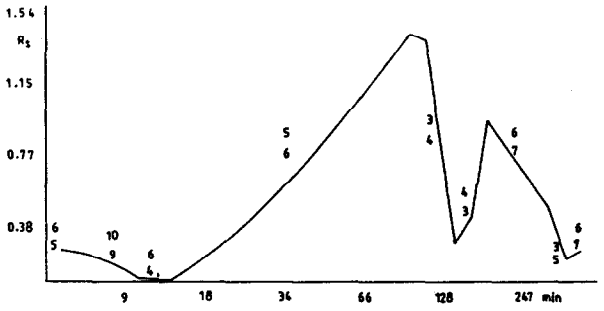

Fig. 1. Relative resolution map for phenolic acids. 0–50% acetonitrile–water gradient; compounds as in Table 1.

the simulated and real retention data is observed; compare also the data in Table 2.

Only the last band, *cis*-ferulic acid, shows a large deviation, 2.4%, from simulated value. Otherwise the resolution, especially of peaks 3–7, shows larger discrepancies which are probably due to the tailing of the peaks and/or the column being poorly packed. Instead of 6400 theoretical plates the efficiency of the column should be about 10 000.

In the subsequent experiments a new 10-cm column was packed with better efficiency per unit length, $N = 3600$, and the composition of the eluent was varied. THF as the second modifier with a low constant concentration, 2%, was introduced into the mobile phase. As reported earlier [8–10], a low concentration of another modifier with stronger hydrophobic properties than the first can lead to retention and selectivity changes, especially when the solutes interact specifically with the modifier. Modifiers such as

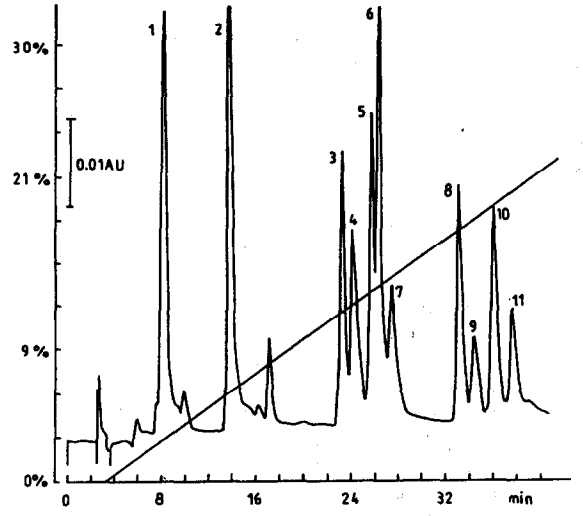

Fig. 3. Real chromatogram of phenolic acids. Conditions as in Fig. 2.

THF can be strongly extracted to the C_{18} stationary phase and then its interaction with proton-donor solutes (phenolic acids) in the stationary phase can introduce a marked influence on retention.

The optimization of the system with ACN–water–THF was performed as described above based on two linear gradient runs from 0% to 50% of ACN with constant THF (2%) and acetic acid (1%) concentrations. Table 3 reports the values of the system variables and retention times of the solutes for two runs of 20 and 60 min. The sequence of the solutes is different in comparison with the data in Table 1. Additionally, in Table 3 there is some sequence variation

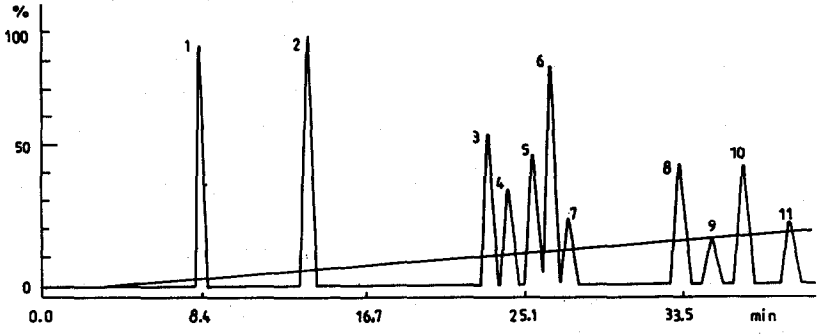

Fig. 2. Simulated chromatogram of phenolic acids by gradient elution with 0–23.1% acetonitrile–water during 46.2 min. Column length, 25 cm; compounds as in Table 1.

Table 2
Retention times for a linear gradient from 0.0 to 23.1% ACN in 46.2 min for simulated and experimental chromatograms using a 25-cm C_{18} column

Band No.	Compound	t_R (min)	
		Simulated	Experimental
1	Gallic acid	8.30	8.23
2	Protocatechuic acid	13.90	13.95
3	Chlorogenic acid	23.39	23.65
4	Vanillic acid	24.41	24.53
5	*trans*-Caffeic acid	25.75	26.05
6	Syringic acid	26.60	26.68
7	*cis*-Caffeic acid	27.57	27.91
8	*trans-p*-Coumaric acid	33.39	33.56
9	*cis-p*-Coumaric acid	35.01	34.93
10	*trans*-Ferulic acid	36.64	36.57
11	*cis*-Ferulic acid	39.12	38.17

Table 3
System variables, compounds and retention times

System variables	
Dwell volume (ml)	0.90
Column length (cm)	10.0
Column diameter (cm)	0.46
Flow-rate (ml/min)	1.0
Starting B (ACN) concentration (%)	0.00 + 2% THF
Final B (ACN) concentration (%)	50.00 + 2% THF
Gradient time, 1st run (min)	20.0
Gradient time, 2nd run (min)	60.0

Retention times

Band No.	Compound	t_R (min)	
		Run 1	Run 2
1	Gallic acid	3.27	3.41
2	Protocatechuic acid	5.11	6.59
3	Chlorogenic acid	6.58	10.32
6	Syringic acid	7.15	10.41
4	Vanillic acid	7.41	10.24
5	*trans*-Caffeic acid	7.88	12.76
7	*cis*-Caffeic acid	8.14	13.12
8	*trans-p*-Coumaric acid	9.63	17.18
9	*cis-p*-Coumaric acid	9.87	17.18
10	*trans*-Ferulic acid	9.88	17.73
11	*cis*-Ferulic acid	10.23	18.58

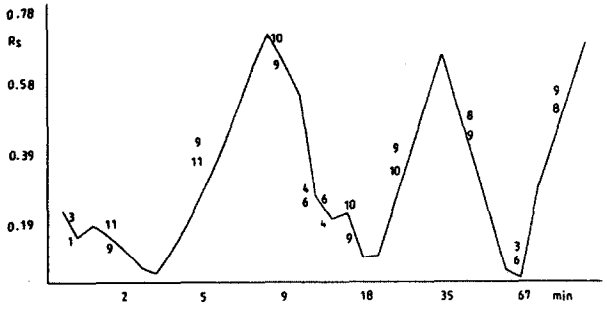

Fig. 4. Relative resolution map for phenolic acids. 0–50% acetonitrile–water gradient with constant concentration of THF (2%); compounds as in Table 1.

between the two runs, it is contrast to the data in Table 1 where the sequence of the solutes is the same in the two runs. Fig. 4 shows the relative resolution map for the system. The image is different to that in Fig. 1. No satisfactory resolution of the complete set of the solutes can be obtained in the gradient time range shown in Fig. 4.

It is interesting that generally in both Figs. 1 and 4 different pairs of bands show minimal resolution. This suggests that mixing of the two modifiers (ACN and THF) is another possibility for improving the separation. However, in our computer optimization procedure we obtained a new gradient range from 7 to 30% ACN (with constant THF and acetic acid concentrations) during 31 min.

Figs. 5 and 6 show the simulated and experimental chromatograms, respectively, of the mixture investigated. The retention times of the phenolic acids are about 0.7 min (mean value) lower in the real than in the simulated chromatogram (Table 4) but the resolutions are similar. In both Figs. 5 and 6 peaks 8–10 are only partially separated, in contrast to the chromatograms in Figs. 2 and 3, where the peaks are well separated. The opposite situation occurs for the peaks with lower retention. Bands 3–7 are poorly separated in Fig. 2 but show, in principle, acceptable resolution in Fig. 6. This means that bands 3–7 are well resolved with an eluent containing THF but bands 8–11 are better separated without THF in the mobile phase (compare also Fig. 7, where the chromatogram for the

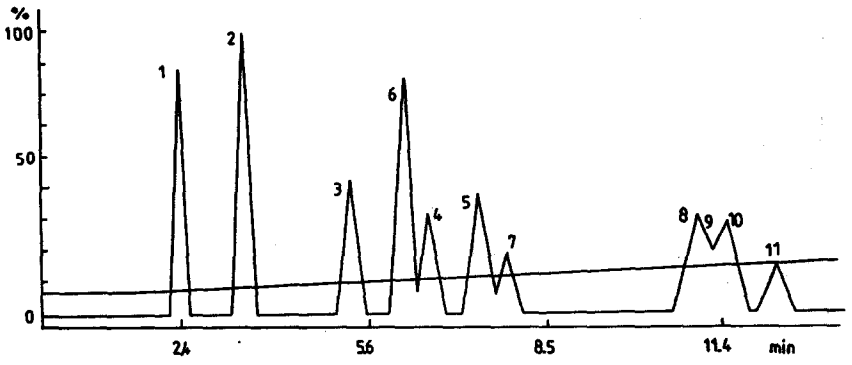

Fig. 5. Simulated chromatogram of phenolic acids by gradient elution with 7–30% ACN during 31 min with a constant concentration of THF (2%). Column length, 10 cm; compounds as in Table 1.

10-cm column, $N = 3600$, and an ACN gradient range of 7–30% is simulated based on the system variables in Table 1, without THF in the eluent).

The above observation can also be supplemented by the variation of the retention sequence within the band 3–7 set. Bands 3 (*cis*-chlorogenic acid) and 7 (*cis*-caffeic acid) are the first and the last band, respectively, in the set in both chromatograms. However, syringic acid shows a stronger retention than vanillic acid and *trans*-caffeic acid in the ACN system. In the ACH–THF (2%) system the retention of syringic acid is weaker than that of vanillic acid and *trans*-caffeic acid. Also, *trans*-caffeic acid shows an increase in retention relative to *cis*-caffeic acid in the ACN–THF system in comparison with the ACN system. The retention variations suggest that the separation of bands 3–7 can possibly be achieved by fine tuning of the THF and ACN gradient simultaneously, e.g., by applying a procedure analogous to that described previously [11,12].

Instead of looking for another system with a different selectivity characteristic or changing the

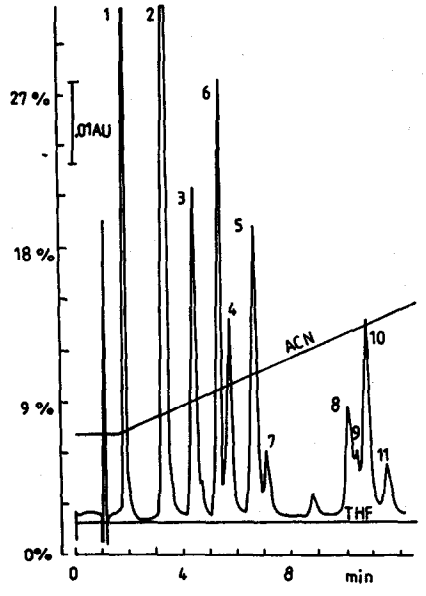

Fig. 6. Real chromatogram of phenolic acids. Conditions as in Fig. 5.

Table 4
Retention times for a linear gradient from 7.0 to 30.0% ACN and a constant THF concentration (2%) in 31 min for simulated and experimental chromatograms using a 10-cm C_{18} column

Band No.	Compound	t_R (min)	
		Simulated	Experimental
1	Gallic acid	2.43	1.81
2	Protocatechuic acid	3.37	3.32
3	Chlorogenic acid	5.09	4.42
6	Syringic acid	6.07	5.35
4	Vanillic acid	6.51	5.71
5	*trans*-Caffeic acid	7.32	6.61
7	*cis*-Caffeic acid	7.76	7.06
8	*trans*-*p*-Coumaric acid	10.95	10.07
9	*cis*-*p*-Coumaric acid	11.22	10.36
10	*trans*-Ferulic acid	11.48	10.76
11	*cis*-Ferulic acid	12.27	11.53

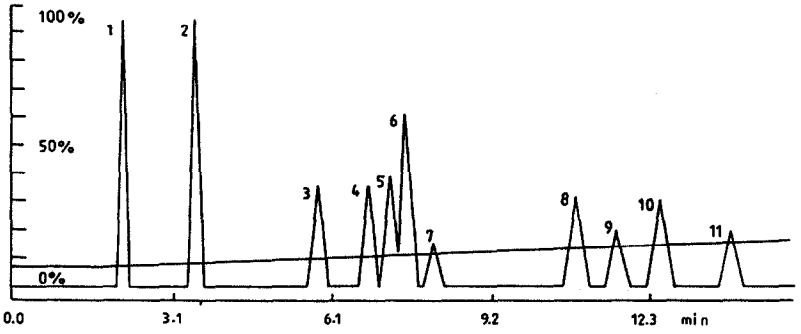

Fig. 7. Simulated chromatogram of phenolic acids by gradient elution with 7–30% ACN and a 10-cm column based on the data in Table 1.

operational parameters, e.g., column length, we decided to perform the next optimization procedure step by step based on the data for the chromatograms presented in Figs. 3, 6 and 7. We started using the same gradient of ACN as in Fig. 6 and the THF concentration was kept at the same level, 2%, during 0–6 min then decreased linearly to 0% from 6 to 12 min. The real chromatogram is shown in Fig. 8 and is very similar to that in Fig. 6. This means that the decrease in THF concentration in the last stage of the chromatographic process does not influence the retention of bands 8–11. The decrease should be greater to reflect the retention change of bands 8–11. The retention of these solutes is probably strongly influenced by extraction of THF into the stationary phase. Hence the expulsion of THF from the stationary phase in the later stages of the chromatographic process can be achieved by a higher ACN concentration.

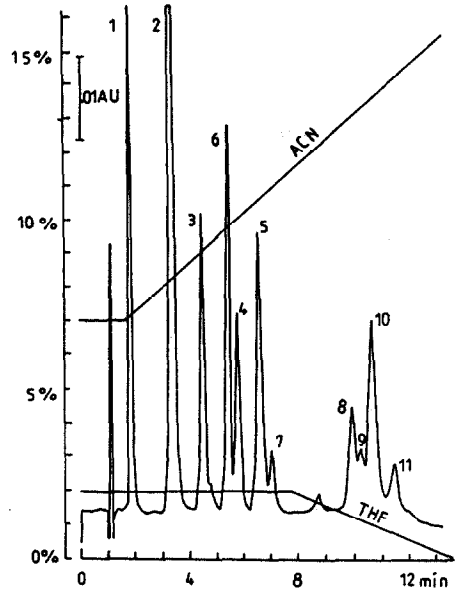

Fig. 8. Separation of phenolic acids by gradient elution with 7–30% ACN during 31 min, 2% THF during 0–6 min and 2–0% THF during 6–12 min. Compounds as in Table 1.

We then decided to increase the gradient slope of ACN and to decrease the starting time of the THF gradient from the start of the chromatogram. The next chromatogram was obtained using a gradient of THF concentration from 2 to 0% during the initial 6 min. The gradient of ACN concentration was divided into two segments: (1) during the first 6 min the slope of the gradient was the same as in Figs. 6 and 8 (i.e., 7–11.6% ACN), and (2) during the next 6 min, i.e., from 6 to 12 min, the gradient was from 11.6 to 30% ACN (Fig. 9). The second segment with a higher slope of the ACN gradient was introduced mainly, as suggested above, to elute THF rapidly from the stationary phase.

The chromatogram shown in Fig. 9 demonstrates a good resolution of peaks 8–11, which now have decreased retention relative to the chromatogram in Fig. 8. However, peaks 6 and 4 overlap. The overlap of these peaks is probably caused by a too low concentration of THF in the

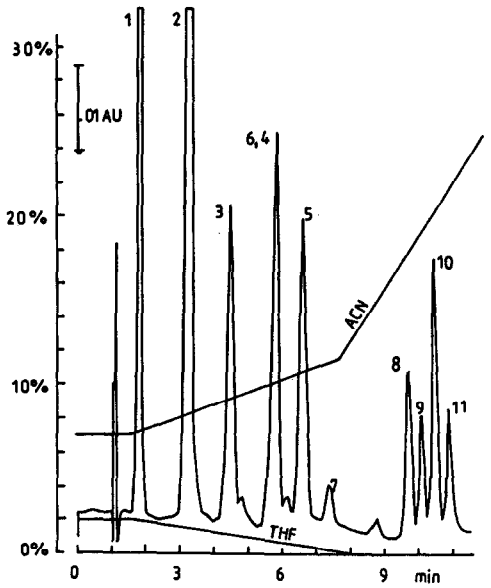

Fig. 9. Separation of phenolic acids by gradient elution with 7–11.6% ACN and 2–0% THF during 0–6 min and 11.6–30% ACN during 6–12 min. Compounds as in Table 1.

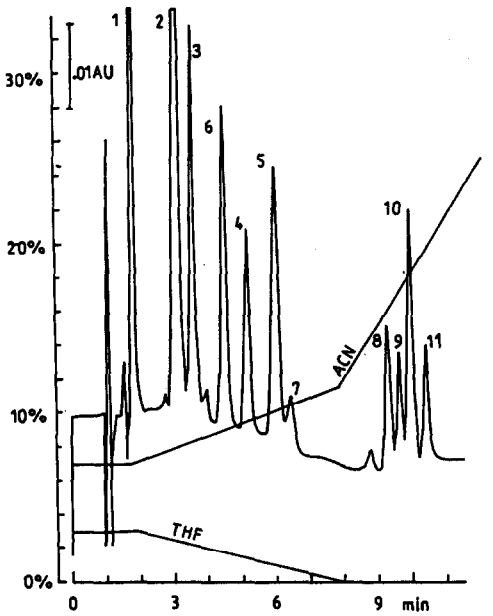

Fig. 10. Separation of phenolic acids by gradient elution with 7–11.6% ACN and 3–0% THF during 0–6 min and 11.6–30% ACN during 6–12 min. Compounds as in Table 1.

initial stage of elution relative to the conditions in Fig. 8. With a greater THF concentration, syringic acid shows a weaker retention than vanillic acid and the band of *trans*-caffeic acid is closer to that of *cis*-caffeic acid (Fig. 6). Therefore, for the next chromatogram a higher starting THF concentration (3%) was applied, which caused a poorer resolution of peaks 5 and 7 (Fig. 10). In the last stage of the fine tuning of the system, an intermediate starting concentration of THF was chosen, i.e., 2.5%. Fig. 11 shows the chromatogram with satisfactory resolution of all the bands under the final gradient conditions as follows: 0–6 min, gradient from 7% to 11.6% ACN and from 2.5 to 0% THF; 6–12 min, gradient from 11.6 to 30% ACN. In the whole gradient range the acetic acid concentration was constant at 1%.

The procedure described above for optimization of the chromatographic system is not typical in chromatographic practice but shows that it is a worthwhile effort to compare various chromatograms obtained after computer optimization. This can sometimes help to devise a more

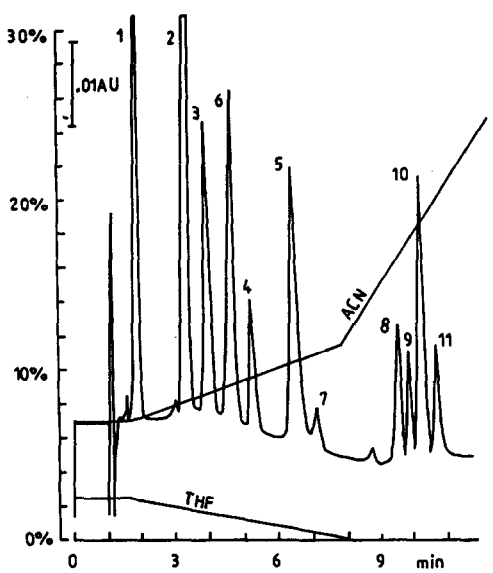

Fig. 11. Separation of phenolic acids by gradient elution with 7–11.6% ACN and 2.5–0% THF during 0–6 min and 11.6–30% ACN during 6–12 min. Compounds as in Table 1.

complicated chromatographic system permitting improved resolution of the solutes.

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