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Resolution of complex mixtures of non-flavonoid polyphenols by column-switching high-performance liquid chromatography using octadecylsilica and graphitized carbon columns[1]

E. Leira, A. Botana, R. Cela*

Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Química, Universidad de Santiago de Compostela, E-15706 Santiago de Compostela, Spain

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Abstract

Differences in the retention capacity and selectivity of C_{18} and graphitized carbon columns were used to resolve complex mixtures of non-flavonoid polyphenols by transferring fractions between columns arranged in a serial manner. Separation of mixture components was accomplished in a single switching operation by using mobile phases of the same composition but at different eluting strength in both separation steps. The elution conditions used in both columns were simplified by means of simulation software in order to obtain multiple fractions. The potential of this technique was realized by resolving a mixture of 38 very similar species that could not be separated with an isolated column.

Keywords: Column switching; Polyphenols; Computer simulation; Heart-cutting techniques

1. Introduction

High-performance liquid chromatographic (HPLC) procedures implemented in a single step are the normal choice for the analysis of a variety of simple and complex mixtures. However, especially complex samples cannot always be selectively resolved by altering the composition of the mobile phase. As a rule, the selectivity can be more readily changed by modifying the stationary phase, which, however, entails using two or more separation steps. A system consisting of several columns can be regarded as a multi-dimensional system when the essential mechanism via which the separation in each dimension takes place is different [1–3]. In addition, Giddings and Davis [4] suggest that, if two components are resolved in the first separation step, they should remain resolved throughout the process. Based on this criterion, most reported multi-column separations are one-dimensional as the separation mechanisms involved are not sufficiently different (they are not orthogonal). This is frequently the result of incompatibility or immiscibility between the mobile phases, the mobile phase for one column and the stationary phase for the other, or the precipitation of salts or some solute, etc. In these cases, automatic

*Corresponding author.

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fractions collection and re-injecting show clear advantages vs. on-line procedures, especially when fractions have to be manipulated to change solvents, buffers, etc. As a result, a large number of applications of multi-column techniques involve clean-up of complex samples for isolation and/or preconcentration of compound groups or families that are subsequently resolved by HPLC in a single step [5–7].

On the other hand, when selectivity differences between the two columns are large enough, the multi-column technique can be considered as a quasi-multi-dimensional technique. Many separations are currently being performed by reversed-phase HPLC; however, the more common columns, which use commercially available silica-bonded phases, do not ensure large enough selectivity differences to fulfil the above criterion. Previous work showed that the selectivity of bonded silica and commercially available graphitized carbon columns have significant differences in selectivity for a large number of non-flavonoid polyphenols [8]. Such differences can be used for the resolution of mixtures of this type of species by column switching.

Non-flavonoid polyphenols make up a large family of naturally occurring compounds in plants that are also present in natural and processed vegetable foods (preservation of which is favourably or adversely affected, depending on the particular compound [9,10]). Their chemical and spectral features are quite similar; also, because they occur in large numbers over wide concentration ranges, they make up extremely complex mixtures that are difficult to resolve, identify and quantify.

This paper reports the results obtained in the resolution of a mixture of 38 non-flavonoid polyphenols by switching between a C_{18} and a graphitized carbon column. The switching process (as regards both placement of the two columns, the groups used for heart cutting and the eluents employed) can be substantially simplified by computer simulation [11,13], which reduces experimental work. To this end, we used the software package PREOPT-W, developed by one of the authors [13]. The fact that both columns use the same type of mobile phase overcomes most of the problems posed by HPLC multi-dimensional techniques.

2. Experimental

2.1. Materials and reagents

We analysed a mixture comprising the 38 species listed in Table 1. As can be seen, many of them were chemically quite similar. All the species have been detected in various plants and processed foods and beverages of vegetable origin (wine, fruit juice, etc.) [9,10]. The substances studied were supplied by Fluka (Buchs, Switzerland), Aldrich (Alcobendas, Spain) and Merck (Darmstadt, Germany). The mobile phases used consisted of methanol 205 (Romil Chemicals, Louborough, UK) and water (Milli-Q, Millipore, Milford, MA, USA) in variable proportions; they were supplied with 1% acetic acid (Merck) in every case.

2.2. Apparatus

The experimental set-up used consisted of the following elements. (a) Model 600E and 501 pumps, both from Waters (Millipore- Waters, Milford, MA, USA); one can produce ternary gradients while the other can only effect isocratic elution. While the pump sequence in the experimental set-up could have been different, we used the gradient pump in the first separation step and the isocratic pump to resolve the cuts from the first column in the second step. Using gradients in the second step entailed recovering the initial conditions and reduced the number of cuts that could be processed in a single injection. (b) A Waters 700 autoinjector and two UV–Vis detectors, viz. an ERC-7211, variable-wavelength model from Erma (Tokyo, Japan) that was operated at 280 nm, and a Waters 996 diode-array spectrophotometer that was used over the wavelength range 200–450 nm. The two detectors used were interfaced to a Millenium 2010 data station (Waters) in order to monitor both signals simultaneously. The detectors were switched from one column to the other in order to confirm the identity of the species eluted through each column. (c) Column cuts were accomplished by using a Model C6W six-port valve from Valco Instruments (Schenden, Switzerland). (d) Two types of column were used, namely: a 50×4.6 mm I.D. Hypercarb column (Shandon Scientific,

Table 1
Listing of the 38 non-flavonoid species studied; key numbers match the chromatogram and spectrum labels in the figures, and heart-cut groups the labels in Fig.6

Key number	Compound	Heart-cut Group
1	3-Hydroxybenzoic acid	III
2	4-Hydroxybenzoic acid	II
3	2,4-Dihydroxybenzoic acid (β-resorcylic acid)	II
4	2,5-Dihydroxibenzoic acid (gentisic acid)	II
5	2,6-Dihydroxybenzoic acid (γ-resorcylic acid)	I
6	3,4-Dihydroxybenzoic acid (protocatechuic acid)	I
7	3,5-Dihydroxybenzoic acid (α-resorcylic acid)	I
8	3,4,5-Trihydroxybenzoic acid (gallic acid)	
9	4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	III
10	3-Hydroxy-4-methoxybenzoic acid (isovanillic acid)	III
11	4-Hydroxy-3,5-dimethoxybenzoic acid (syringic acid)	IV
12	2,4-Dimethoxybenzoic acid	
13	2,6-Dimethoxybenzoic acid	IV
14	3,4-Dimethoxybenzoic acid (veratric acid)	V
15	3,5-Dimethoxybenzoic acid	VII
16	2-Hydroxycinnamic acid (*o*-coumaric acid)	VI
17	3-Hydroxycinnamic acid (*m*-coumaric acid)	V
18	4-Hydroxycinnamic acid (*p*-coumaric acid)	
19	3,4-Dihydroxycinnamic acid (caffeic acid)	III
20	4-Hydroxy-3-methoxycinnamic acid (ferulic acid)	V
21	3,5-Dimethoxy-4-hydroxycinnamic acid (sinapic acid)	V
22	3,4,5-Trimethoxycinnamic acid	VII
23	2-Hydroxybenzaldehyde (salicyl aldehyde)	V
24	3-Hydroxybenzaldehyde	III
25	4-Hydroxybenzaldehyde	III
26	2,5-Dihydroxybenzaldehyde	III
27	3,4-Dihydroxybenzaldehyde (protocatechialdehyde)	II
28	3,5-Dimethoxy-4-hydroxybenzaldehyde	
29	2-Hydroxy-3-methoxybenzaldehyde (*o*-vanillin)	V
30	4-Hydroxy-3-methoxybenzaldehyde (vanillin)	IV
31	3-Hydroxy-4-methoxybenzaldehyde (isovanillin)	IV
32	2,4-Dimethoxybenzaldehyde	
33	3,4-Dimethoxybenzaldehyde (veratraldehyde)	V
34	3,5-Dimethoxybenzaldehyde	VII
35	3-Methoxybenzaldehyde (*m*-anisaldehyde)	VI
36	4-Methoxybenzaldehyde (*p*-anisaldehyde)	VI
37	3,4,5-Trimethoxybenzaldehyde	VI
38	Chlorogenic acid	II

Runcorn, UK) consisting of porous graphitized carbon and featuring a mean particle size of 7 μm and 5000 theoretical plates; and a 150×3.9 mm I.D. Novapak C_{18} column (Waters) of 4 μm mean particle size and 8000 theoretical plates. Both columns were kept at the same temperature (35°C) by means of a Waters TCM-HCM oven in order to avoid the influence of this experimental variable on the elution times for the polyphenols studied [14].

Fig. 1 depicts the operational scheme used. The injected sample was passed through column 1 and eluted by using the mobile phase supplied by the first pump. During the process, the valve was switched off and the column effluent monitored by means of detector 1 (usually the variable-wavelength detector). Whenever a fraction was to be transferred to the second column, the valve was switched on (we did it manually even though both valve positions could

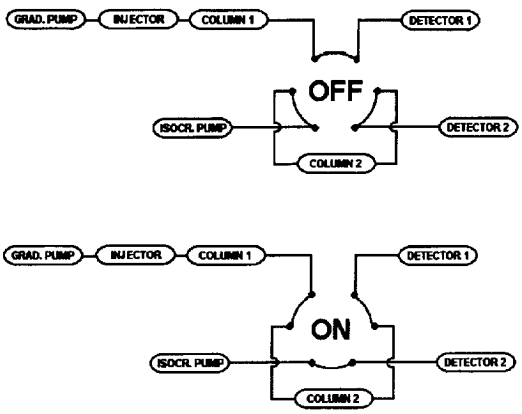

Fig. 1. Experimental set-up used.

readily have been changed pneumatically or electrically and cuts could have been simply and reproducibly timed through programmable external options included in the software of the data station used). In this way, it was not necessary to stop the elution of the first column as elution of the second column was started, by means of the mobile phase propelled by the second pump, in order to record the chromatogram from detector 2.

3. Results and discussion

3.1. Column sequence

As a rule, the sequence in which the columns are placed in a column-switching system has a marked effect on the results obtained and the scope of the ensuing procedure. The final choice is very often dictated by the specific separation objectives. When several cuts are to be performed in the same chromatogram, the column with the higher retention capacity should be placed first. In this way, the fractions transferred to the second column are completely eluted before a new cut is performed. Otherwise, several injections are usually needed for each analysis of a complex mixture. For the same reason, the first column should be eluted with a weaker phase than the second. In addition, this operating mode has the advantage that the transferred fraction is concentrated at the head of the second column, so the chromatographic bands are only moderately degraded.

However, the difference in retention capacity for polyphenols between C_{18} and graphitized carbon columns is enormous [8,15]; eluting mixture components from the Hypercarb column require a mobile phase with a high content of organic modifier. In this situation, if the column with the higher retention capacity (the graphitized carbon column) is placed first, then the fractions transferred to the second (the C_{18} column) are carried in a mobile phase of a high eluting strength that allows them to travel very rapidly through the second column; as a result, elution is too fast and hardly improves on the resolution obtained with the first column. It is therefore preferable to place the C_{18} column first and transfer its effluent to the Hypercarb column. Under these conditions, the transferred mobile phase has a lesser effect on elution from the second column.

3.2. Cut length

Fig. 2 shows the optimal isocratic separation theoretically possible for the mixture using the C_{18} column. It entailed using a mobile phase consisting of 10% methanol and 90% water. Under these conditions, an overall 28 peaks out of the 38 theoretically possible were acceptably resolved, even though some peak couples were completely overlapped, as can be seen in the resolution map of Fig. 3. However, the time needed to complete the separation (about 200 min) was quite unacceptable. Therefore, we chose to elute the mixtures in gradients through the first column. In this way, we maximized the separation capacity of the first column and minimized the elution time and the number of cuts required to resolve the mixture. Thus, the transferred mobile phase for those components that were rapidly eluted from the first column was fairly weak, so the compressive effect of head bands at the second column (with a much higher retention capacity) was exerted as described above. By passing a mobile phase with a much higher eluting strength through the second column, complete resolution of the transferred fraction would be possible before a new transfer. On the other hand, the mobile phase in which the fractions of strongly retained components were transferred would have a much higher eluting

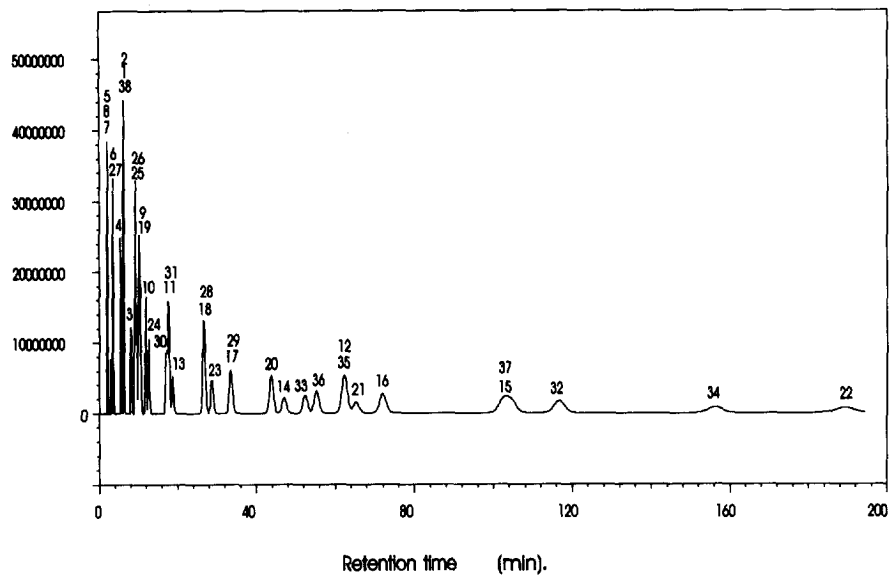

Fig. 2. Optimal isocratic elution of the 38 species studied from the Novapak C_{18} column. Mobile phase, methanol–water (10:90) containing 1% acetic acid. Peak labels match the key numbers in Table 1.

strength. Transferred bands would hardly concentrate at the head of the second column; rather, they would continue to be eluted (at a lower linear speed, however, owing to the higher retention capacity of the second column). This should be taken into account in programming the separation through the first column, which poses an additional difficulty. The gradient used for elution of the first column need not always be the optimal in terms of the overall resolution of the mixture components in the column.

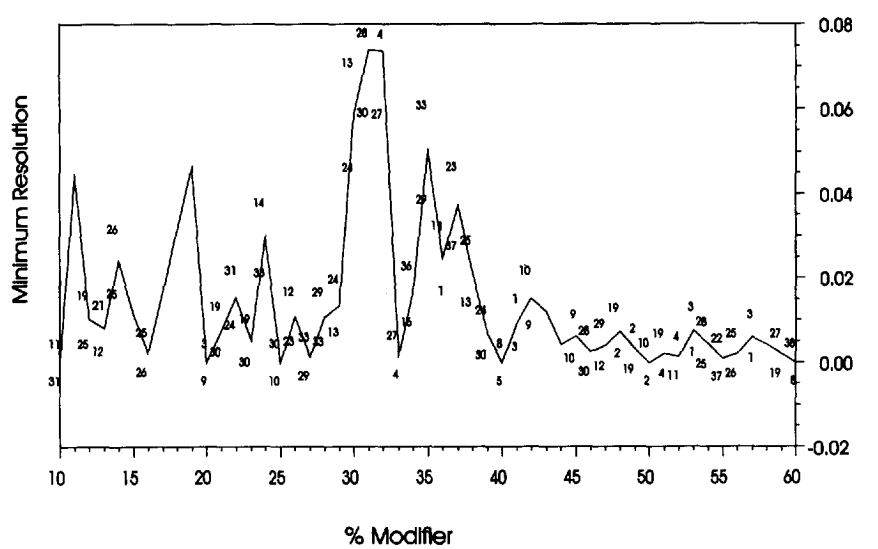

Fig. 3. Resolution map for the mixture of 38 species studied in terms of the amount of methanol in the mobile phase. Labels indicate the peak couple resulting in the minimum resolution in each case.

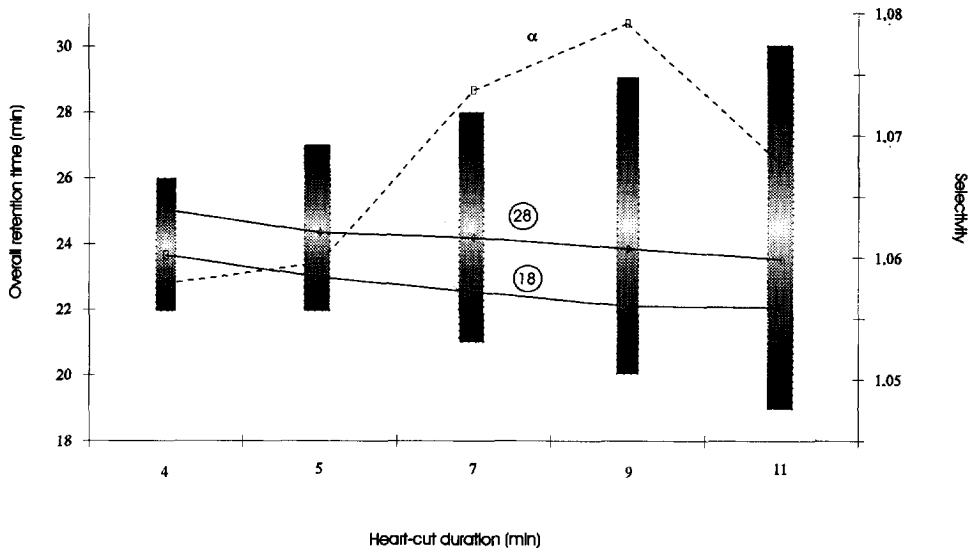

Fig. 4. Variation of the overall retention and selectivity factor for two species transferred in the same cut as a function of the cut duration and its start. Vertical bars indicate the start and end of each cut.

Rather, a specific gradient must frequently be designed in order to ensure that efficient cuts are transferred from the first to the second column.

In intermediate gradient regions, fraction transfers can give rise to different results depending on the volume of the fraction that is transferred to the second column. In these fractions, the mobile phase has an intermediate eluting strength, so the transferred fraction will travel a relatively short but long enough distance through the second column to alter the expected retention times for this column (in the absence of coupling). This effect is illustrated in the graphs of Fig. 4, which shows the overall retention times for two species (4-hydroxycinnamic acid and syringaldehyde) transferred in a fraction from the first column to the second, in cuts started at different times (denoted in the graphs by vertical bars indicating the cut start and end), with the transfer line open for increasing times (also indicated in the graphs by both the length of the vertical bars and the labels of the horizontal axis). The gradient used was the same in every case (curve 10 on the Waters 600E programmer, in 60 min, from 10% to 60% methanol, at a fixed flow-rate of 1.0 ml/min). Under these conditions, the two species studied in this experiment had a retention time on the C_{18} column of 21.9±0.2 and 25.6±0.2 min, respectively. As can be seen, the overall retention times decreased with increasing total cut time since the two chromatographic bands travelled through the second column during the time that the transfer took place, propelled by the mobile phase from the first column, which gained in strength with time as the gradient increased simultaneously with the transfer. The relation was not linear, however, because the two cuts were started at a different time. Nor was the selectivity constant because the speed at which the two species travelled through the second column depended on the nature of its stationary phase and on interactions with the solutes. The net conclusion is that both factors (the overall transferred volume and the time at which the cut is started) must be considered. Obviously, if the first column is eluted isocratically, then the latter factor will not be influenced. Timing cuts is critical in order to ensure reproducibility in results, therefore the use of an automated system is highly recommended.

In practice, it is fairly easy to calculate the overall retention times for the different species, provided the flow-rate and composition of the mobile phase are kept constant and the retention data for the species are isocratic on both columns, the dead volumes of both columns and the delay time for the particular chromatographic system are known. However, when the first separation step is carried out in the gradient

mode, these calculations are somewhat more complicated, so it pays to use some simulation model for the retention peaks in both the isocratic and the gradient mode. We used the model PREOPT [11–13], which allows the simulation of any type of gradient (linear, curved or composite) with a precision of 1–5% even when retention varies non-linearly with the amount of modifier in the mobile phase, as is the case with elution from graphitized carbon columns [8].

3.3. Design of the elution programs and resolution of mixtures

A column switching system is the obvious choice when the mixture or components of interest cannot be properly resolved with a single column, whether isocratically or by gradient elution. Fig. 5 shows the optimum chromatogram obtained for a mixture of the 38 polyphenols studied (peak labels match the number codes given in Table 1) with the C_{18} column using a linear gradient from 10% to 60% methanol in 60 min. Under these conditions, the mixture was resolved into 27 peaks, of which some were extensively overlapped, within 40 min. The graphitized carbon column provided much poorer separation: the 38 species provided 21 peaks for a gradient from 60% to 100% methanol. After the need to perform cuts between the columns in order to resolve the mixture was identified, we took into account that resolving the peaks transferred to the second column in each cut would rely on retention differences for the same species on such a column. Because the number of cuts that could be performed in each run was limited (ideally, each must be completely eluted from the second column before the next cut), separation through the first column should logically be adapted to the needs of the second. Thus, the optimal gradient for the first column (i.e. the one resolving the largest number of species in the shortest possible time, as in the example of Fig. 5) may not be the most favourable for transferring a few eluate fractions to the second. However, establishing the new conditions may involve intensive preliminary experimental work, success of which is not always granted. Using a simulation package such as PREOPT-W in this situation allows reliable decisions to be made in this respect in a few minutes without the need for additional experimentation. PREOPT-W constructs a retention model for the species in the mixture from isocratic retention data alone. The model can and is normally used to evaluate the elution potential of each column (see graphs of Figs. 2 and 5). If, as in our case, the mixture cannot be fully resolved under such conditions, the same retention model can be used to design any other type

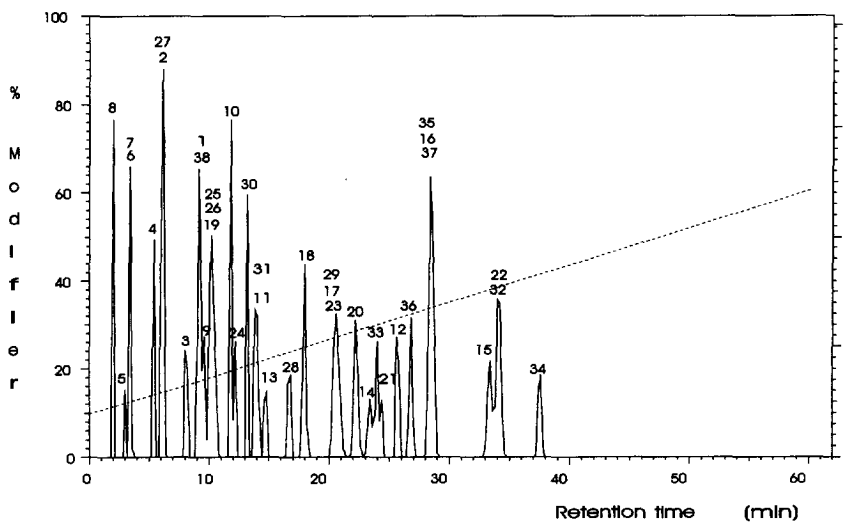

Fig. 5. Optimal gradient program for the separation of the 38 species studied based on the results provided by the simulation program PREOPT-W. The dashed line denotes the gradient program.

of gradient (without the need for additional experimental data) on the basis of two criteria, namely: (a) the species with marked retention differences on the second column should be coeluted from the first; and (b) those with similar retention properties on the second column should not be coeluted in the same transferred fraction. Obviously, both conditions cannot always be met, depending on the particular mixture; however, the ability to simulate this situation facilitates decision-making and increases the likelihood of accomplishing satisfactory separations in a column switching system. The model also obviously allows one to select the sequence in which the columns are arranged in the experimental set-up based on the above considerations. In addition, if the purpose is to quantify a given species that cannot be resolved on the first column, the same simulation mechanism can be used to design its gradient; in this way, the species is transferred in a cut that can be satisfactorily resolved on the second.

Even though we also analysed various mixtures of a lower complexity, we only report the results for the full polyphenol mixture (38 species). The chromatogram of Fig. 6 illustrates the elution of the 38 species from the first column (C_{18}) by use of the step gradient described in Table 2. The gradient was designed by means of PREOPT-W in order to fully resolve the mixture, irrespective of the number of cuts needed. Those groups that can be transferred to the second column are labelled on the graph (the species included in each group are listed in Table 1). As can be seen, the gradient was designed in such a way that peaks grouped in chromatographic regions separated by baseline stretches, which facilitated cutting and eased timing, particularly when cuts were made manually. Those peaks not included in any transfer group were eluted in pure form from the first column under these gradient conditions, so they lent themselves readily to direct quantitation.

Table 2
Elution program for the mixture of 38 polyphenols that gave the chromatogram of Fig. 6, in the format used by Waters 600E programmer

Time (min)	Methanol (%)	Curve No.
0	15	*
4	20	11
7	30	11
12	35	11
15	45	11
19	60	11
25	60	11
35	15	6

Fig. 7 shows the chromatograms obtained from the second, graphitized column, using the 7 cuts designed from the chromatogram of Fig. 6. Fig. 7a shows the separation of α-resorcylic, γ-resorcylic

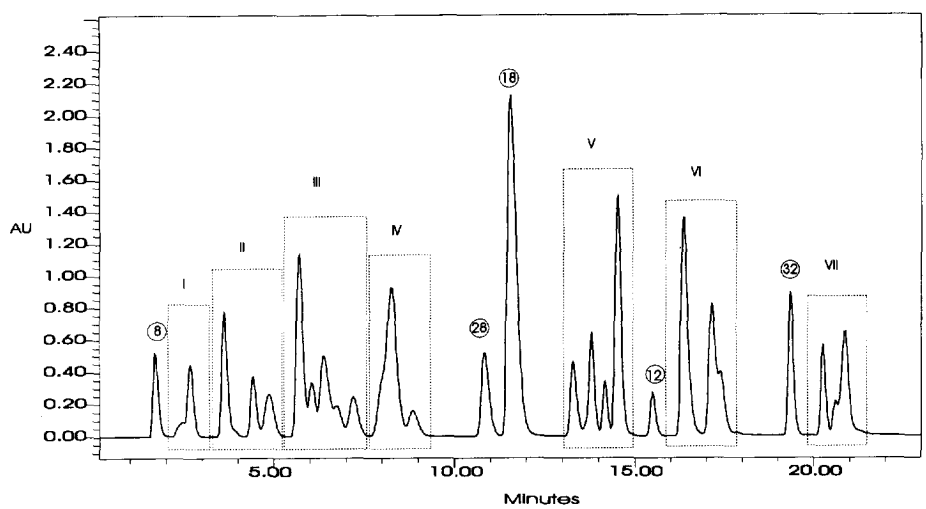

Fig. 6. Elution of the 38 species studied through the Novapak C_{18} column by use of the gradient described in Table 2. The dotted insets show possible cuts and match the results in Fig. 7.

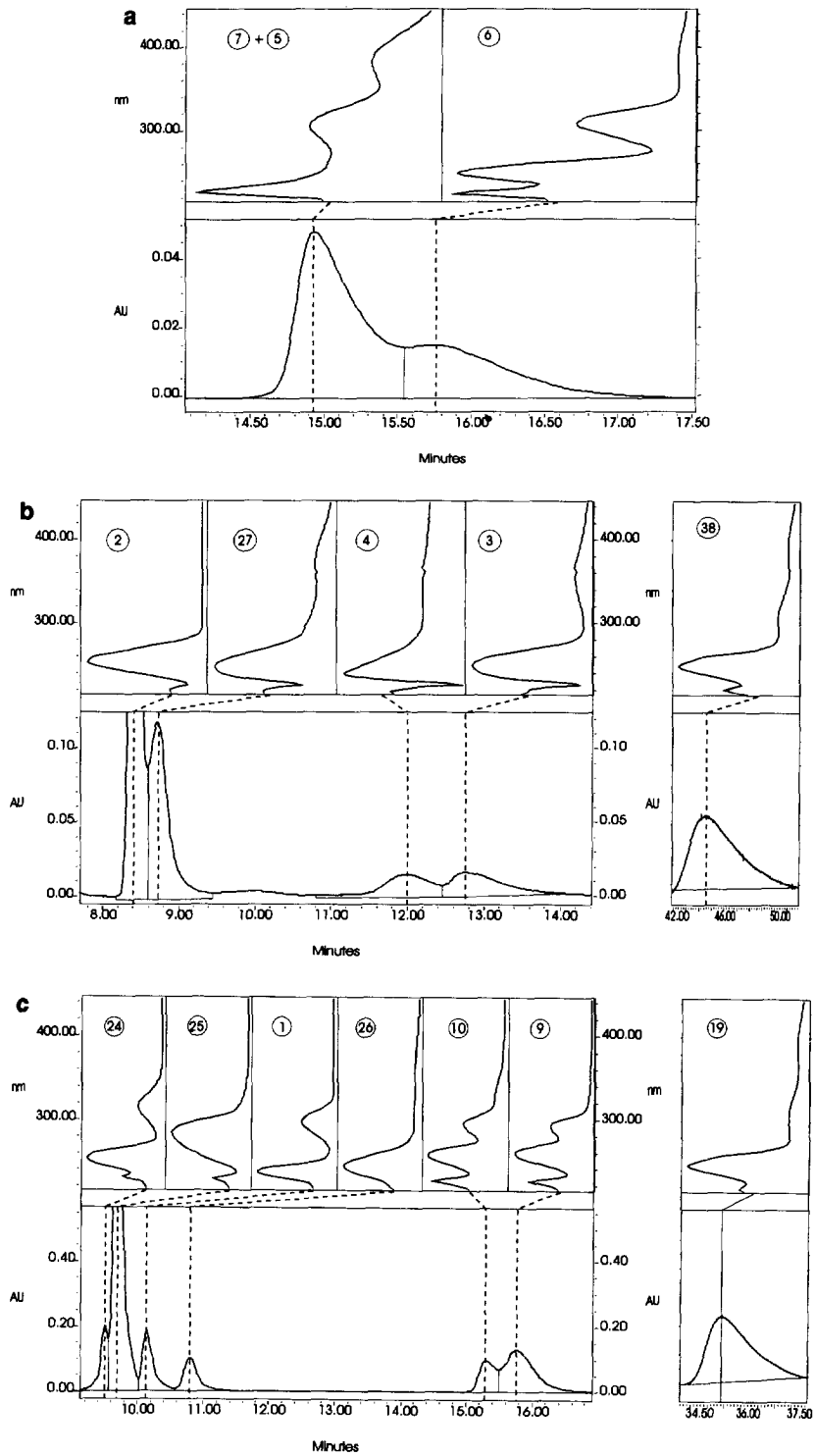

Fig. 7. (*Continued on page 76*).

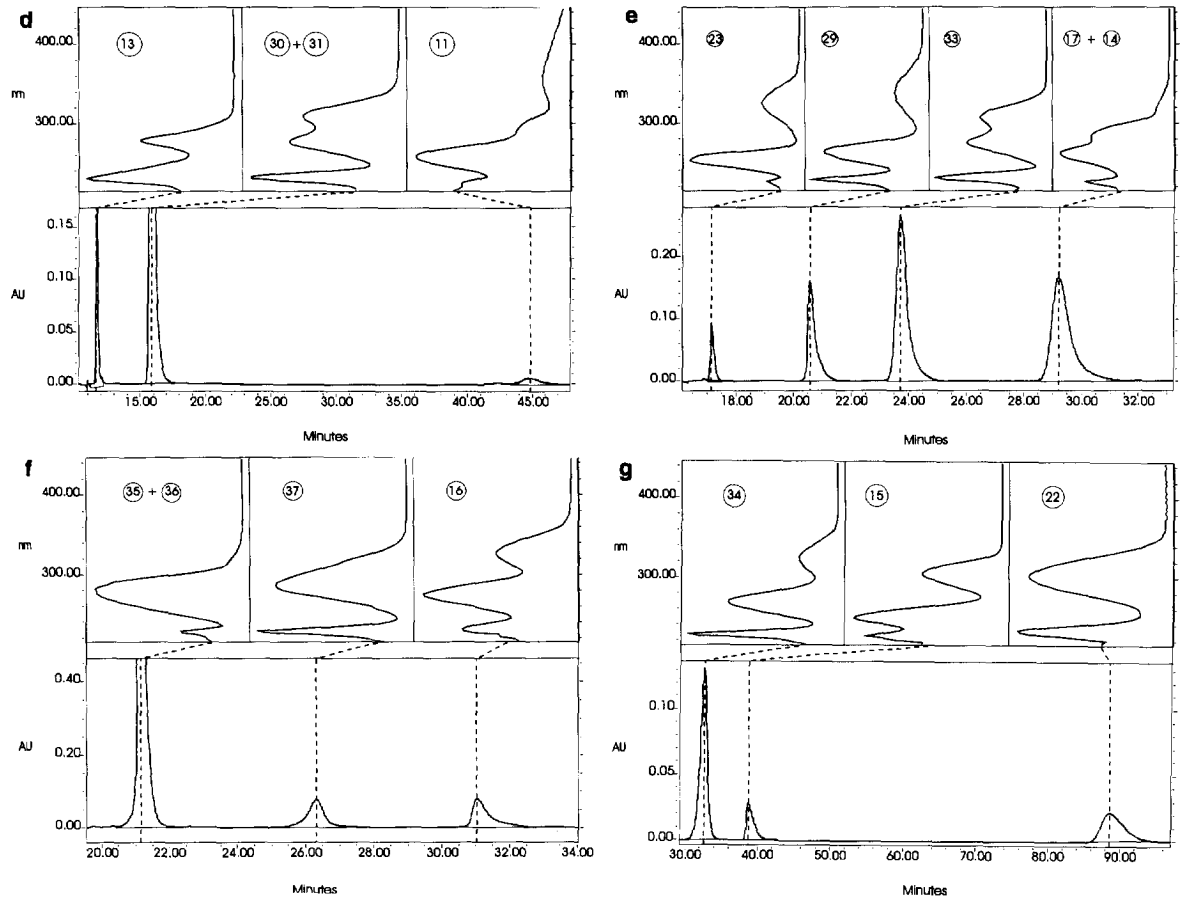

Fig. 7. Elution of the species making up each of the seven cut groups through the Hypercarb column. The insets at the top show the spectrum for each species at the peak maximum.

and protocatechuic acid. No significantly improved resolution was achieved because the two columns were very similarly selective for the three species. The chromatogram of Fig. 7b corresponds to Group II and allowed the resolution of the five peaks that made up this transfer group by using a methanol–water (90:10) mobile phase for elution from the Hypercarb column. Fig. 7c shows the separation of the seven species making up Group III. Improved resolution could be achieved by using methanol–water (60:40) as the mobile phase for the second column; however, caffeic acid was strongly retained as a result. The chromatogram of Fig. 7d shows the separation of Group IV, consisting of five species, of which vanillin and isovanillin were fully overlapped. Both can be separated by means of a methanol–water (60:40) mobile phase, which, however, results in strong retention of syringic acid. Fig. 7e illustrates the separation of Group V, comprising seven species, of which three (salicylaldehyde, *o*-vanillin and veratraldehyde) were well resolved on transfer to the second column; two other species (*m*-coumaric and veratric acid) were fully overlapped and could not be resolved by decreasing the eluting strength for the second separation step; and the other two species (synapic and ferulic acid) were retained virtually irreversibly on the graphitized column, so they could not be quantified. Irreversible retention of these and other polyphenols on graphitized carbon phase was previously reported [8]. The chromatogram of Fig. 7f shows the separation of Group VI, made up of four species, of which *m*- and *p*-anisaldehyde coeluted

from the second column. Nevertheless, both species can be resolved on a C_{18} column (see Fig. 4); therefore, if both species are to be quantified, cuts must be designed in such a way as to avoid the two of them being transferred to the second column in the same fraction. Finally, the chromatogram of Fig. 7g shows the separation of the species in Group VII.

As an example of application to a real sample Fig. 8 shows the obtained chromatogram for a white wine extract. Superimposed to this chromatogram appear those corresponding to several cuts made. Since selectivity of the extraction procedure (counter current continuous solvent extraction with a 2:1 ether–pentane mixture) is somewhat poor, it is clear that this extract contains not only polyphenolic species so direct quantization of the species of interest could not be reliably carried out in the C_{18} chromatogram. In cuts, many peaks appear showing excellent results in peak-purity tests. For example, cut number 2 which corresponds to only one peak in the C_{18} column decomposes in nine peaks in the second dimension. Protocatechuic acid being present in this cut can now be clearly identified and quantified. Similarly, cut number 1 allowed the accurate quantization of gallic acid, overlapped with other four unknowns in this cut. Cut number 3 appears to be composed by eight species, allowing when developed in the second dimension the quantitation of caffeoiltartaric and feruiltartaric acids. Cut number 4, which appears as two peaks in the C_{18} chromatogram decomposes in five peaks in the Hypercarb chromatogram. Free caffeic acid can now be reliably quantified in this chromatogram. Similarly, in cut number 6, 4-hydroxycinnamic acid and syringaldehyde could be detected.

The conclusion is that most of the species in highly complex mixtures of polyphenols can be resolved by a careful choice of the gradient used to elute the first column and the eluting strength of the mobile phase for the second, in addition to careful selection of cuts. All these can be dramatically simplified by using a simulation model such as that of PREOPT-W to design both the elution programs for the two columns and their cuts. In any case, a

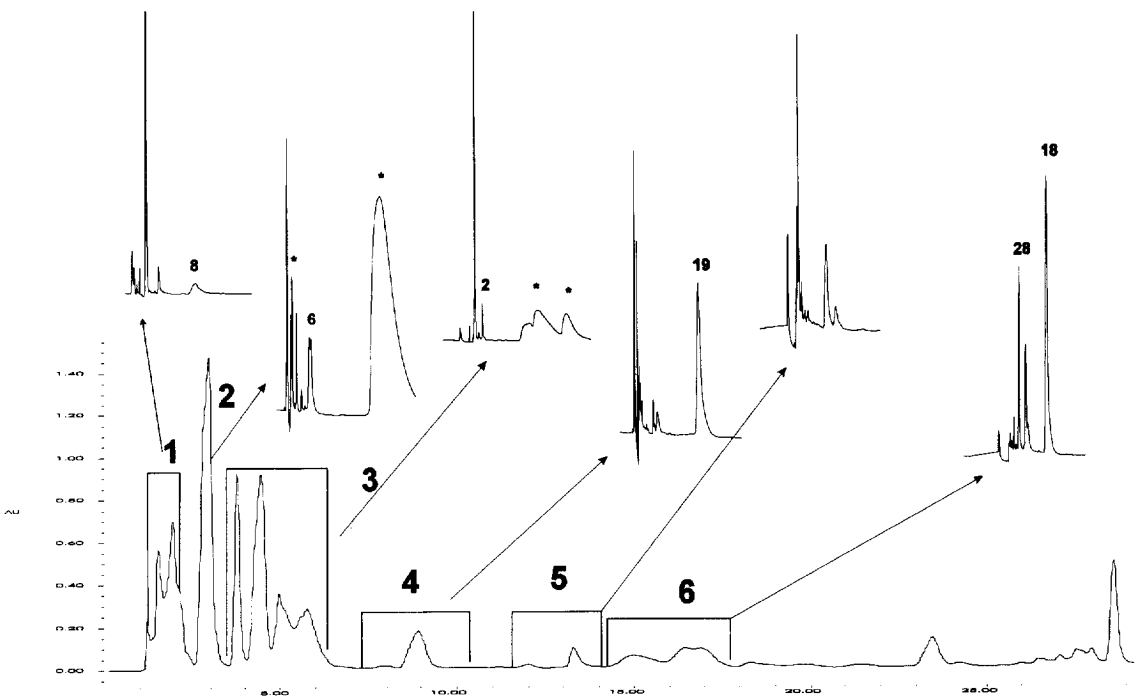

Fig. 8. Chromatogram of a white wine extract obtained in the first dimension showing six cuts through the Hypercarb column. Numbers in chromatogram cuts correspond to the key of compounds in Table 1; asterisks indicate caffeic and ferulic acid derivatives.

mixture such as that studied in this work will require two or more injections if every species in the mixture is to be accurately resolved and quantified.

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