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Alternate pooling for optimizing high-performance liquid chromatographic fractionation of complex peptide mixtures

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Abstract

Complete resolution of multi-component mixtures is achieved following a single RP-HPLC run. The resolution is generally limited both by peak broadening, which occurs due to an overloading of the column, and by the limited number of fractions which can be collected. In order to take full advantage of the separation capacity of the gradient, each fraction is often individually submitted to a second chromatographic run under the same conditions. Starting from a complex mixture from which a large number of compounds are expected, this conventional process implies multiple parallel chromatographic steps and rapidly generates a large number of samples resulting in considerable time-consuming manipulations and an increase of errors. To overcome these major drawbacks we propose an original strategy which relies upon the rational pooling of fractions issued from the initial chromatography in two groups, A and B. Since Group A and Group B contain the odd- and even-numbered fractions, respectively, each fraction is removed from its closest neighbors. Each group is then submitted to an additional fractionation under the same chromatographic conditions as used previously. The separation of toxins from *Dendroaspis viridis* snake venom was performed according to this strategy.

Keywords: Optimization; Toxins; Peptides

1. Introduction

High-performance liquid chromatography (HPLC) is the most versatile method for the separation and purification of polypeptides from both natural and synthetic origin [1–3]. This chromatographic technique affords rapid resolution of very complex mixtures including those comprising very similar polypeptides. Nevertheless, the approach to the purification of polypeptides must be tailored to the separation goals. Thus, purification of a single polypeptide from a complex mixture will require a different approach to that necessary for separating all components from a mixture. Separation methods employed for this purpose need to be highly resolutive, however this requirement can be restricted to the limited diversity of impurities closely related to the target compound and which must be eliminated during the purification. In some other cases, the aim is to separate all the components of a complex mixture, each one being of particular importance. For this type of separation, reversed-phase HPLC (RP-HPLC), using a gradient of organic modifier, is ideally suited since it allows the separation of a large

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variety of compounds from very hydrophilic to very hydrophobic during the same chromatographic procedure [2,4–6]. Classical examples of this type of separation include natural mixtures such as the venoms of poisonous animals which contain complex mixtures of toxins or, more recently, mixtures of peptides eluted from MHC class I and class II molecules [7–10]. This is also the case for peptide fragments obtained from protein cleavage in which the complexity will depend upon the nature and efficiency of the enzymatic or chemical agent used [11,12].

However, for most applications, fractionation of complex peptide mixtures is not only performed upon an analytical scale, since components of each fraction will be collected and submitted to various other methods of biological evaluation and/or structural determination such as amino-acid analysis, Edman degradation and/or mass spectrometry, or even NMR. The baseline separation of multi-component mixtures for preparative purposes is, in general, difficult to achieve using a single HPLC run. Even when the theoretical separation capacity of the chromatographic system suggests that it is adequate enough, practically, the resolution is limited both by peak broadening which occurs due to an overloading of the column and by the limited number of fractions which can be collected [13–17]. Not only can the overall resolution not exceed the total number of fractions but even the way the collection of a fraction is started or terminated can determine both the degree of purity and the yield of each component. It is obvious that, for highly complex mixtures, the probability of peak overlap increases with the number and diversity of components present [18]. With respect to these factors, only a limited part of the theoretical resolution capacity of the chromatographic system is generally used (this can be estimated by a comparison with the result for an analytical injection using a short time response for the detector). Thus it is not illogical to run a second chromatography under the same conditions with each of the fractions from the initial separation. Both the decrease in the quantity of neighboring contaminants and the possibility of performing a more accurate fraction collection lead, in general, to additional purification. However, this method has two major restrictions in being time-consuming, since each fraction must be processed individually, and leading to an exponential number of fractions which rapidly overwhelm storage capacities. Therefore, we report in this paper an alternative strategy, which to our knowledge has never been reported, and which allows optimization of the use of the resolving power of the preparative RP-HPLC system while avoiding the restrictions described above. This strategy, which is well adapted to the case of multi-component peptide mixtures, relies upon the rational pooling of fractions collected during the initial chromatography into two groups which are then ‘re-injected’ separately. The pooling is organized in such a way as to optimize further separation of the mixtures. In order to illustrate this method, we describe below its use for the fractionation of a venom containing a wide variety of peptide toxins.

2. Experimental

2.1. Materials and reagents

Lyophilized crude venom from the snake *Dendroaspis viridis* was obtained from Latoxan (Rosans, France). Sephadex G-50 Fine media was from Pharmacia Biotech (Sweden) and C_{18} Nucleosil Silica Gel Spherical reversed phase (5 μm particle size, 300 Å pore size) from Marcherey–Nagel (Düren, Germany). The sources of reagents were: acetic acid glacial from Carlo Erba (Val de Reuil, France), trifluoroacetic acid (TFA) from Sigma (Saint Quentin Fallavier, France), and acetonitrile from Merck (Darmstadt, Germany). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Sample preparation

Crude venom (100 mg) was dissolved in 2.5 ml of 1% acetic acid and filtered through a 1 μm acrodisc (Gelman) before loading onto a Sephadex G-50 Fine column (1000 mm×25 mm I.D.). The column was equilibrated and eluted with 1% acetic acid at a flow-rate of 40 ml/h. The elution was monitored at 280 nm. The fractions were screened by plasma desorption mass spectrometry (PD-MS) on a Bio-Ion

20 ^{252}Cf fission fragment ionization time-of-flight (TOF) mass spectrometer (Uppsala, Sweden) for the presence of low-molecular-mass toxins (M_r 3000–8000). The fractions containing these polypeptides were pooled and lyophilized leading to 40 mg of a complex mixture of peptide toxins.

2.3. Preparative RP-HPLC

A preparative column with dimensions 500×10 mm I.D. was packed with Nucleosil C_{18} reversed phase (See *Materials and reagents*). Eluent A was an aqueous solution of 0.05% TFA in Milli-Q water, Eluent B was a 80% solution of acetonitrile in Milli-Q water containing 0.045% TFA. The elution gradient was 0 to 5% B over 15 min, 5 to 60% B over 90 min, and 60 to 100% B over 15 min. The flow-rate was kept at 2.5 ml/min, and the column was maintained at 50°C. The UV absorbance of the eluate was monitored at 215 and 280 nm. Fractions were collected manually according to UV absorbance. The HPLC apparatus (Beckman) consisted of a module 126 system Gold programmable solvent pump, and a module 168 system Gold diode array detector. System control and data acquisition were performed using the Gold Noveau date system.

2.4. Analytical RP-HPLC

The analytical column with dimensions 150×4.6 mm I.D. was packed with the same C_{18} Nucleosil reversed phase used for the preparative column. Eluent A was 0.05% TFA in Milli-Q water and eluent B was 80% acetonitrile containing 0.045% TFA in Milli-Q water, as during the preparative chromatography. The elution gradient was 0 to 60% of eluent B over 30 min. The flow-rate was kept at 1 ml/min and the column was maintained at 50°C. The UV absorbance was monitored at 215 nm. The HPLC apparatus (Shimadzu) consisted of a LC-10AS liquid chromatograph system pump, a SPD-10A UV–Vis detector and a SIL-10AXL autoinjector.

2.5. Capillary electrophoresis (CZE)

CZE was carried out on an Applied Biosystem HPCE 270A-HT capillary electrophoresis system (Foster City, CA, USA) in 20 m*M* sodium citrate buffer pH 2.5 at 30°C for 10 min, using a 50 cm×50 μm diameter capillary. Samples were injected under 5-in. Hg vacuum (1 in.=2.54 cm; 1 cm Hg=1333.22 Pa). Electrophoretic separation was monitored at 200 nm.

2.6. Edman sequencing

Peptides were spotted on a glassfiber filter previously treated with Biobrene Plus. Sequences were performed on an Applied Biosystem, Procise 492 protein sequencer, using standard pulse liquid program. Phenylthiohydantoin (PTH) derivatives were separated and quantified by RP-HPLC over a Brownlee Spheri-5 PTH 5 μm, C_{18} column (220×2.1 mm I.D.) using the Model 140C Microgradient Delivery System and the Model 785A UV detector.

3. Results and discussion

The venomous glands of animals produce a complex mixture of toxins among which small peptidic toxins ranging in size from M_r 3000 to 8000 affect cellular receptors or ion channels with high selectivities and prove therefore to be of the highest interest for the characterization of these receptors [19,20]. Structural and pharmacological studies of these toxins are often hampered by the difficulties encountered in their purification which constitutes a critical step in these studies. RP-HPLC is by far the most widely used mode of HPLC at present. The ability of this technique to separate peptides of very closely related structure is ideally suited for the separation of peptidic toxins from complex samples. It performs separation in a practical and convenient manner. For these reasons, RP-HPLC using a gradient of organic modifier was selected from amongst the different modes of purification for the separation of the peptidic compounds from *Dendroaspis viridis* venom.

In order to select toxins in the range M_r 3000–8000, the venom was first submitted to gel filtration in order to discard compounds of low molecular mass, such as salts or organic polyamine components and large biomolecules corresponding to mucines or enzymes, thereby facilitating the following investigation of the polypeptidic mixture by RP-HPLC.

Gel filtration of the crude *Dendroaspis viridis* snake venom was performed on a Sephadex G-50 Fine column (1000×26 mm I.D.). TOF-PD-MS allowed the selection of a fraction containing polypeptides with a mass ranging from 3000 to 8000. After lyophilization, the mixture of peptidic toxins (40 mg) was dissolved in 0.05% TFA and loaded onto a preparative C_{18} Nucleosil column (500×10 mm I.D.). The elution gradient was 5 to 60% solvent B over 90 min and peaks were collected manually between two inflexion points. At the end of this first purification, 27 fractions were obtained (Fig. 1a). Each of these fractions was then controlled using two different criteria. Analytical RP-HPLC was performed with the same eluting buffers and a C_{18} Nucleosil column (250×4.6 mm I.D.) packed with the same stationary phase as preparative RP-HPLC. This method performed in non-overloading conditions was used to evaluate the resolving capacity of the chromatographic system under ideal conditions. Thus, the ability of this analytical system to separate several peaks in an individually collected fraction indicated that the separation capacity had not been fully exploited, due to both column overloading and/or inaccuracy in fraction collection. The second control was performed using an orthogonal method, capillary zone electrophoresis (CZE). In this case, it was possible to evaluate the homogeneity/heterogeneity of each fraction better, though no information was forthcoming concerning the possible

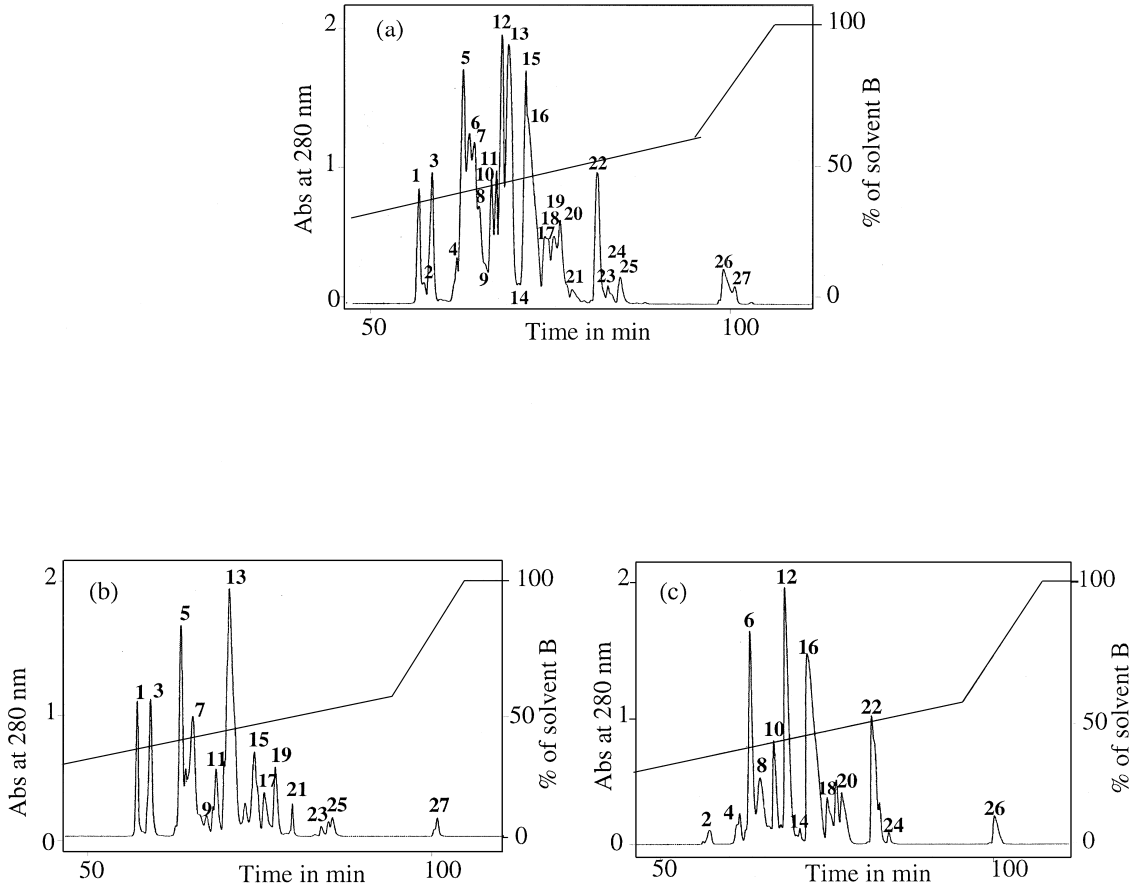

Fig. 1. (a) Initial RP-HPLC of the peptidic fraction from *Dendroaspis viridis* snake venom; (b) and (c) alternate second RP-HPLC of pool A containing odd fractions and pool B containing even fractions, respectively. Experimental conditions: column, 500×10 mm I.D., packed with C_{18} Nucleosil reversed phase, 5 μm, 300 Å; flow-rate, 2.5 ml/min; gradient elution, 5 to 60% B in 75 min; detection, UV at 215 and 280 nm; temperature, 50°C.

separation of these components using the initial system.

Due to the complexity of the initial peptide mixture, to the overloading of the column and to the difficulty of collecting fractions with accuracy, only five (1, 2, 16, 23 and 26) of the 27 fractions collected were found to be homogeneous according to both analytical RP-HPLC and CZE criteria. When examined by analytical RP-HPLC, most of the remaining fractions displayed several component indicating that the actual resolving capacity of the chromatographic system (stationary phase and gradient) had been underexploited (Fig. 2). This observation implies that further purification could still be obtained by re-injecting each fraction under the same chromatographic conditions. In order to accelerate and simplify the chromatographic steps to be repeated, we have adopted an original pooling strategy before the re-injection. Fractions 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27 were pooled leading to 'pool A' while fractions 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26 were pooled leading to 'pool B'. Thus, as illustrated in Fig. 3 (left) the pooling is organized in such a way that when a peak is present in pool A, both the peak eluting immediately before and after it in the gradient will be added to 'pool B'. The incidence of overloading on peak overlapping is greatly decreased due to the fact that each major peak in a pool is now contaminated by only traces of its most closely eluting congeners. This not only decreases contamination by peak overlapping but also allows more accurate fraction collection consistent with the decrease in the number of peaks in the chromatogram [Fig. 3 (right)]. Pools

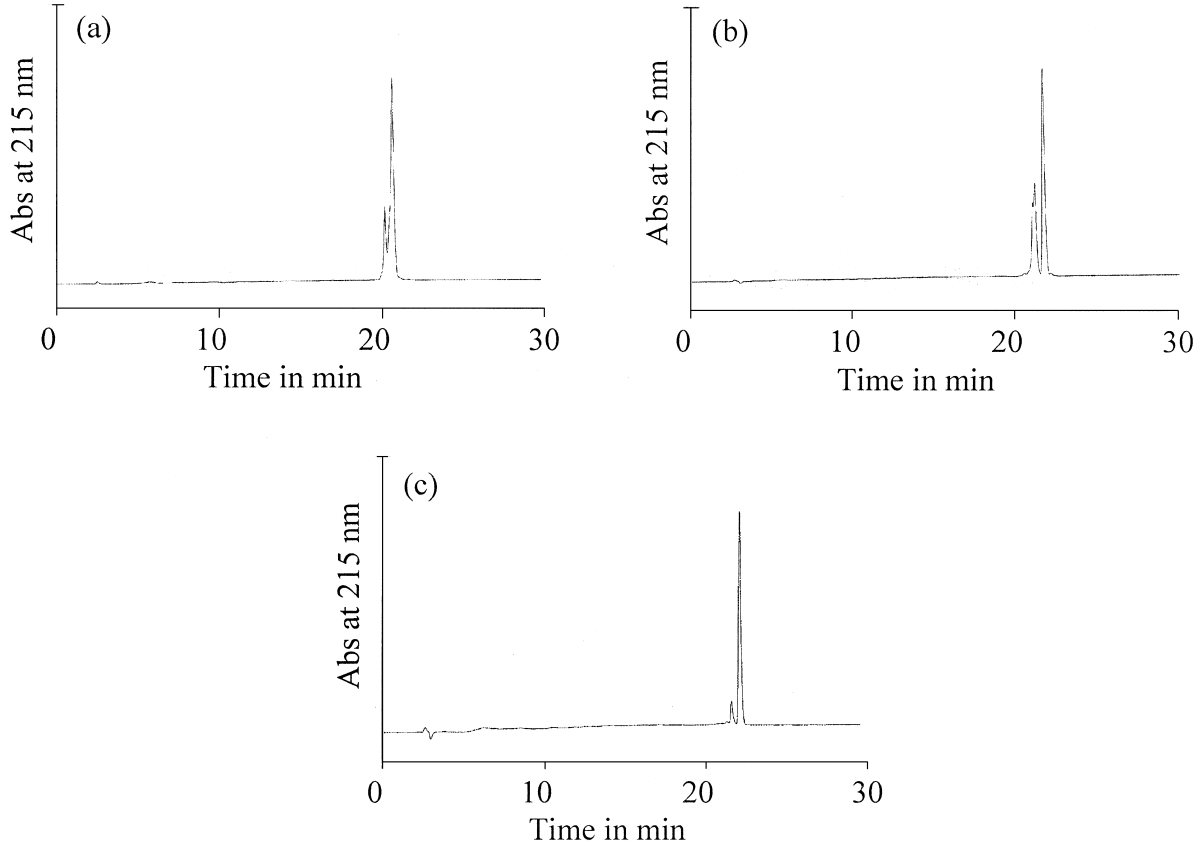

Fig. 2. Analytical RP-HPLC profiles following initial chromatography of fractions 19 (a), 20 (b) and 21 (c). Experimental conditions: column, 150×4.6 mm I.D., packed with the same reversed phase used for the preparative column; flow-rate, 1.0 ml/min; elution gradient, 0 to 60% B in 30 min; detection, UV at 215 nm; temperature, 50°C.

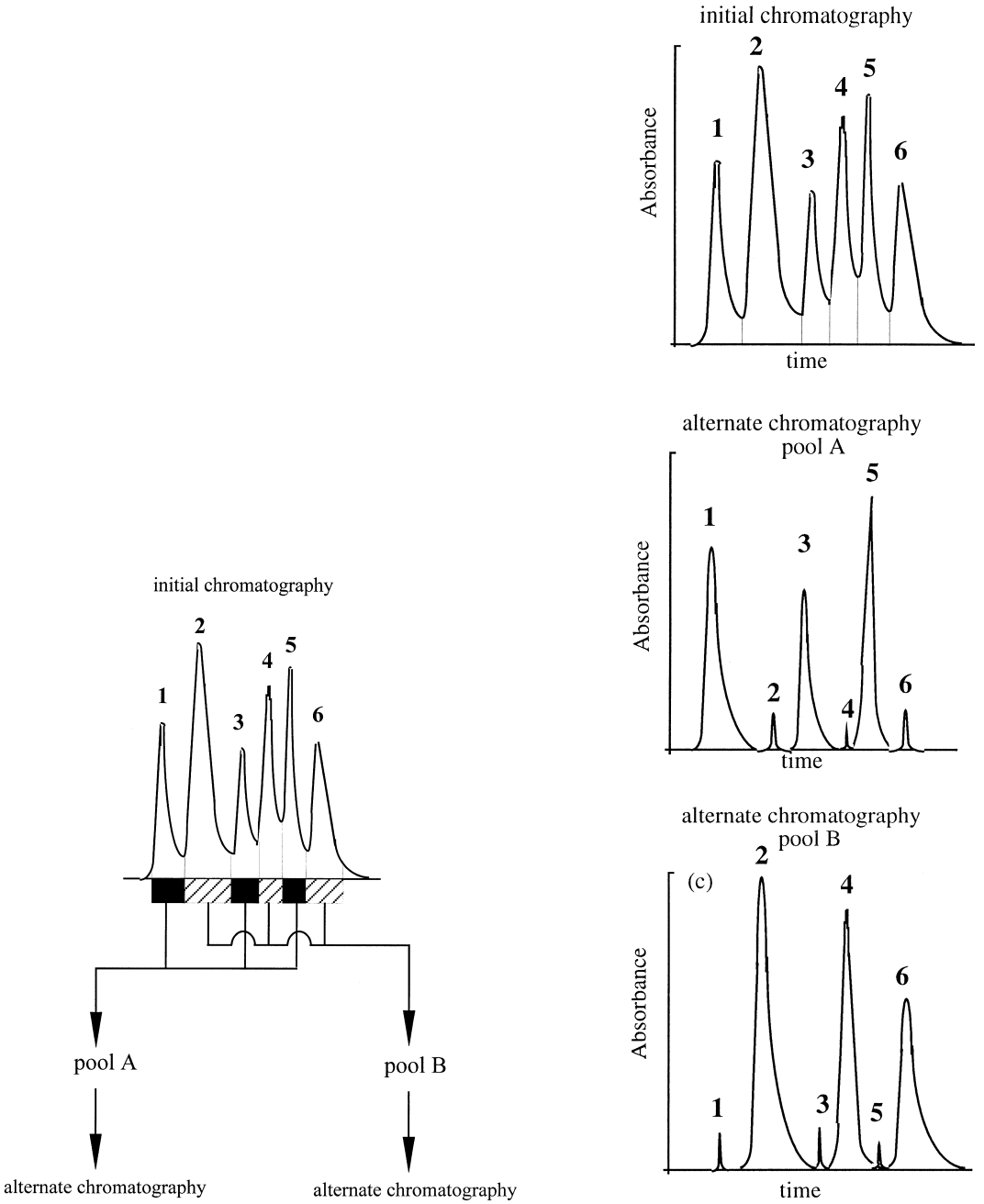

Fig. 3. (Left) Alternate pooling. Following the initial separation, the collected fractions are systematically pooled in two groups in such a manner that the two neighbors of each peak ($n-1$ and $n+1$) will be added to the other pool. For example, pool A will contain peaks 1, 3 and 5 while pool B will contain peaks 2, 4 and 6. (Right) Initial chromatography and alternate second chromatographies under the same conditions as used for the initial chromatography, with pool A and pool B.

A and B were submitted to lyophilization and then fractionated separately using the same preparative column and the same gradient elution as previously (Fig. 1b). It should be noted that an optimal utilization of the gradient is thus performed during the second chromatography compared to the case where fractions are re-injected individually and only a small portion of the gradient is involved in the separation. An additional advantage is the limiting of the effect of non-specific adsorption, aggravated when a small quantity is loaded on a column.

Following the second chromatographic run, each collected fraction was submitted to the same analytical evaluation as indicated above, analytical RP-HPLC and CZE. The identity of each component was assessed by its retention time during analytical RP-HPLC. Table 1 presents the relative proportions of the major species in each fraction as estimated by the integration of the peak area following the initial and the alternate second chromatography. The evolution of the major component was representative of the progress of the purification process. For 15 of the 22 fractions, 5, 6, 7, 8, 10, 11, 14, 15, 17, 18, 19, 20, 21 25 and 27, which contained at least two components at the end of the initial chromatography, the re-injection was beneficial. The degree of purity of some of these fractions was slightly improved without complete removal of the contaminants. For instance, fractions 10 and 11 were contaminated by 12% and fraction 11 by 24% of impurities (Fig. 4). Judging by the retention time of the major component of both fractions, the contamination of fraction 10 came from fraction 11 and vice versa. Following the alternate second chromatographic run, peak 11 decreased from 12 to 7% in fraction 10 and peak 10 from 24 to 12% in fraction 11. So, less than half of the impurities were removed from these fractions following the alternate second chromatographic run. Fractions 7, 8 and 17 were also slightly improved. Best results were obtained for the fractions 5, 6, 14, 18, 19, 20, 21 and 25, which were clearly enriched in their own component following the alternate second chromatographic run. The apparent impurities of fractions 19 and 21 revealed by analytical RP-HPLC were removed (Fig. 5c,d and g,h). A similar improvement was also observed for fraction 5. The contaminants of fractions 6, 14, 18, 20 and 25 did not totally disappear, but a significant increase in the degree of purity, was observed. Fig. 4 displays the RP-HPLC analytical profiles of fractions 14 (a, b); 20 (e, f) and 25 (i, j) before and following the alternate second chromatographic run. For these five fractions the contaminating species decrease at least two-thirds following the alternate second chromatography. In order to evaluate the method for fractions 19 and 20, both were submitted to Edman sequencing following each chromatographic step (Fig. 6). The efficiency of Edman degradation depends upon the proteins; for both contaminated fractions the sequence determination of the major component remained difficult at the end of the initial

Table 1
Percentage of major component in each fraction following the initial and alternate RP-HPLC chromatography. The percentage is estimated by integration of the analytical RP-HPLC profiles. Experimental conditions: column 150×4.6 mm I.D., C_{18} Nucleosil reversed phase, 5 μm, 300 Å; flow-rate, 1.0 ml/min; elution gradient, 0 to 60% B over 30 min; detection, UV 215 nm; temperature, 50°C

Fraction number	RP-HPLC	
	Initial	Alternate
1	100	100
2	100	100
3	h[a]	h
4	h	h
5	89	100
6	78	92
7	90	94
8	83	88
9	h	h
10	88	93
11	75	85
12	73	74
13	h	h
14	66	96
15	35	57
16	100	100
17	82	88
18	74	93
19	79	100
20	60	88
21	91	100
22	83	83
23	100	100
24	62	62
25	68	92
26	100	100
27	40	60

[a] h, heterogeneous.

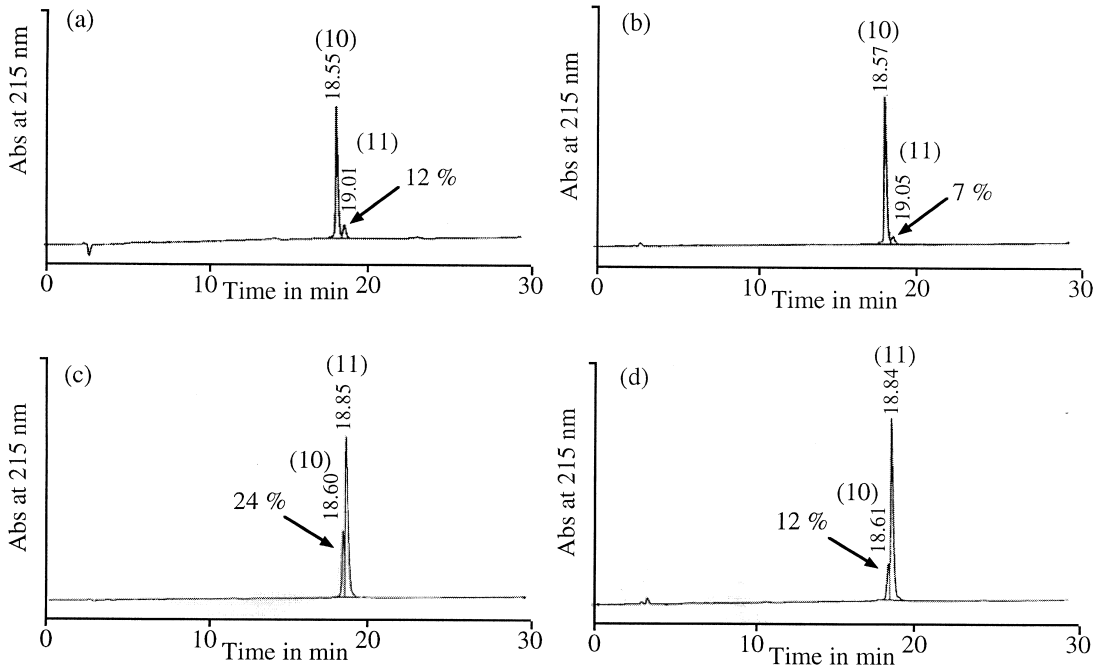

Fig. 4. Comparison of the analytical RP-HPLC profiles of fractions 10 (a, b) and 11 (c, d) following the initial chromatography and the alternate second chromatography. Experimental conditions as in Fig. 2.

chromatographic run. As displayed in the chromatogram of the first cycle of Edman degradation, two and three PTH-amino acid derivatives in fractions 19 and 20, respectively, were released with a similar ratio preventing the direct determination of the first residue of each major component following the initial chromatographic run. By the end of the second chromatographic run, the decrease in PTH-Met in sample 19 indicated that the first amino acid residue of the major component was a leucine residue. In the case of fraction 20, the PTH-Met and the PTH-Leu in the first cycle of Edman degradation disappeared following the second chromatographic run which led to the determination of an arginine as the first amino acid residue of the major component of fraction 20. In the same manner, the second residue of each component was unambiguously determined following the second chromatographic run. It should be noted that Edman sequencing revealed remaining contaminant in fraction 19 following the alternate second chromatography which was not detected by analytical RP-HPLC and CZE, where only a single peak was detected following each chromatographic run.

Although the benefit of the alternate second chromatographic run on fractions 15 and 27 was not clear, interesting results were obtained. Both fractions were severely contaminated by their closest neighboring fraction, which were recovered in higher yield than the major component following the initial chromatographic run. Fig. 7 displays the chromatographic profile of fractions 15 and 16. Following the first chromatographic run, peak 16 was almost twice as large as peak 15 in fraction 15. This was not surprising when the strong overlap of peak 16 upon peak 15 in the initial preparative chromatography is considered. Following the second run, peak 16 decreased significantly, rendering peak 15 the larger of the two. Then, the second chromatographic run clearly enriched fraction 15 with its own component. A complementary method of separation could take advantage of this improvement. Similar data were obtained with fraction 27, which was contaminated with peak 26.

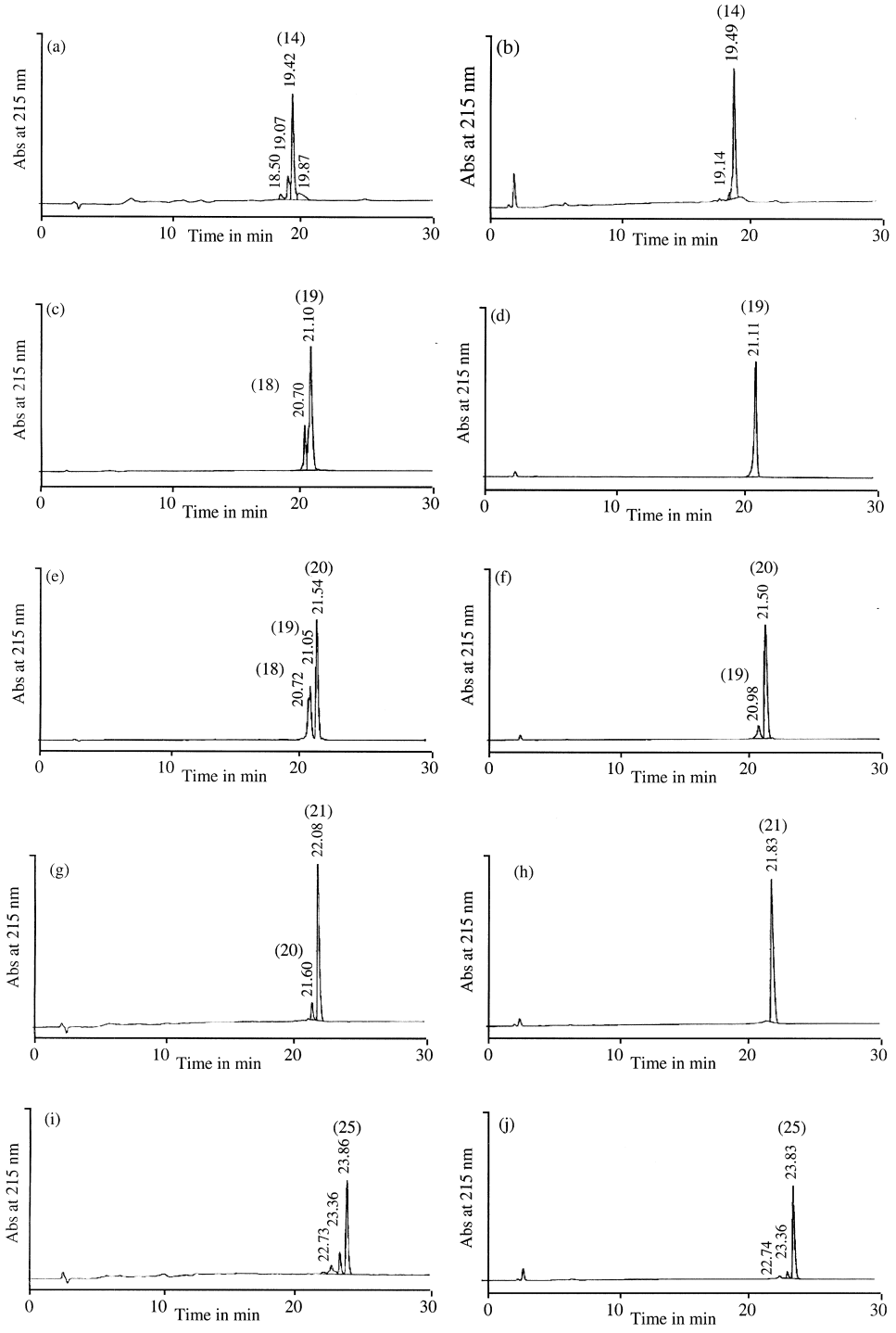

Fig. 5. Comparison of the analytical RP-HPLC profiles of fractions 14 (a, b), 19 (c, d), 20 (e, f), 21 (g, h) and 25 (i, j) following the initial chromatography and the alternate second chromatography, respectively. Experimental conditions as in Fig. 2.

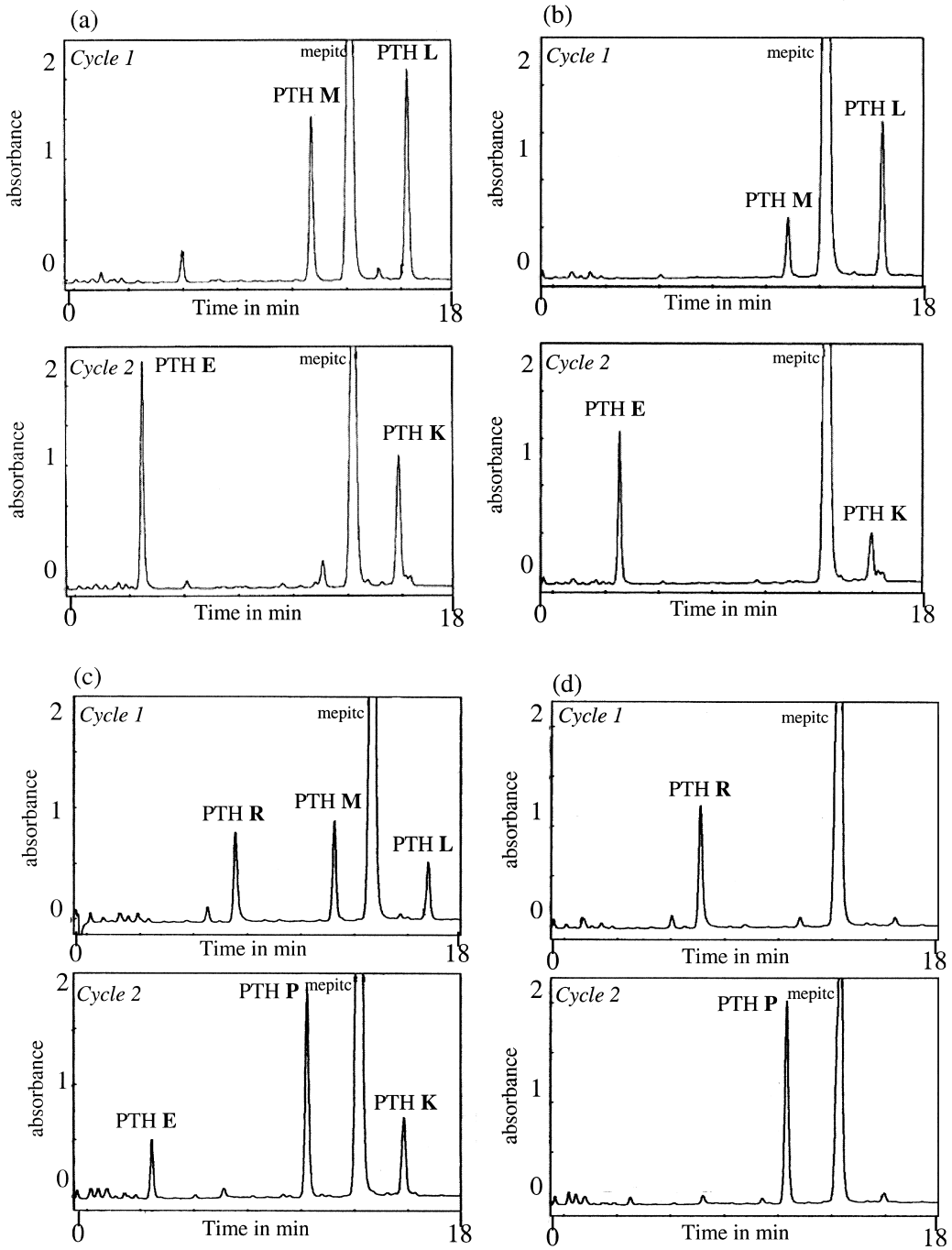

Fig. 6. First and second cycle of Edman degradation performed on fraction 19 (a, b) and 20 (c, d) following the initial chromatography and the alternate second chromatography. PTH-R (phenylthiohydantoin-arginine), PTH-M (phenylthiohydantoin-methionine), PTH-L (phenylthiohydantoin-leucine), PTH-E (phenylthiohydantoin-glutamic acid), PTH-P (phenylthiohydantoin-proline), PTH-K (phenylthiohydantoin-lysine). PTH-Amino acid were separated and quantified by reversed-phase HPLC over a Brownlee Spheri-5 PTH 5 μm, C_{18} column, 220×2.1 mm I.D. Mepitc: methylphenylthiocarbamate.

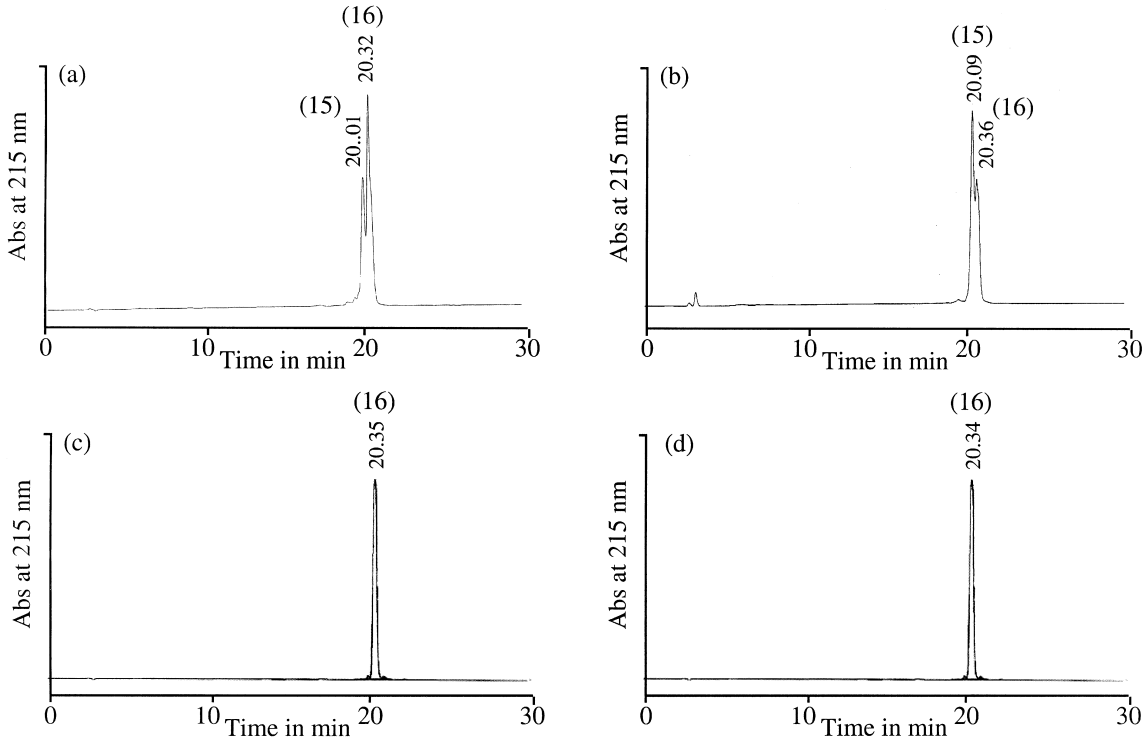

Fig. 7. Analytical RP-HPLC profiles of fractions 15 (a, b) and 16 (c, d) following the initial and the alternate second chromatography, respectively. Experimental procedure as in Fig. 2.

For seven of the heterogeneous fractions following the initial chromatographic run, no improvement was observed. Although the contaminant of the major component of fraction 12 was resolved during the analytical RP-HPLC (Fig. 8a,b), no decrease of this contaminant was observed after the second chromatographic run. Fraction 12 was large and still overlapped its contaminant following the second preparative chromatographic run. In this case it may be necessary to sacrifice sample load to expect elimination of the contaminating compound. As for fraction 12, fraction 22 was not improved. Fraction 13 contained several major components which could not be resolved during the initial chromatography and had been collected simultaneously, preventing them from being alternatively pooled (Fig. 8c,d). The same phenomenon was observed for fractions 4, 9 and 24. In the case of fraction 3, the contaminants co-eluted in analytical RP-HPLC and could only be revealed using the orthogonal CZE method (Fig. 8e,f), indicating that no separation by RP-HPLC could be expected and another purification method should be found.

Finally, the fractions which could be improved by the alternate second chromatographic run were improved, making eventual further purification steps easier.

4. Conclusion

The process of alternate pooling is very simple, allowing us to achieve optimal utilization of the chromatographic system (gradient and column) in a minimum time, avoiding exponential multiplication of collected fractions and limiting the risk of losses of material by non-specific adsorption which occurs when small quantities are injected alone during further purification steps. The direct coupling of the second runs with mass spectrometry can be envis-

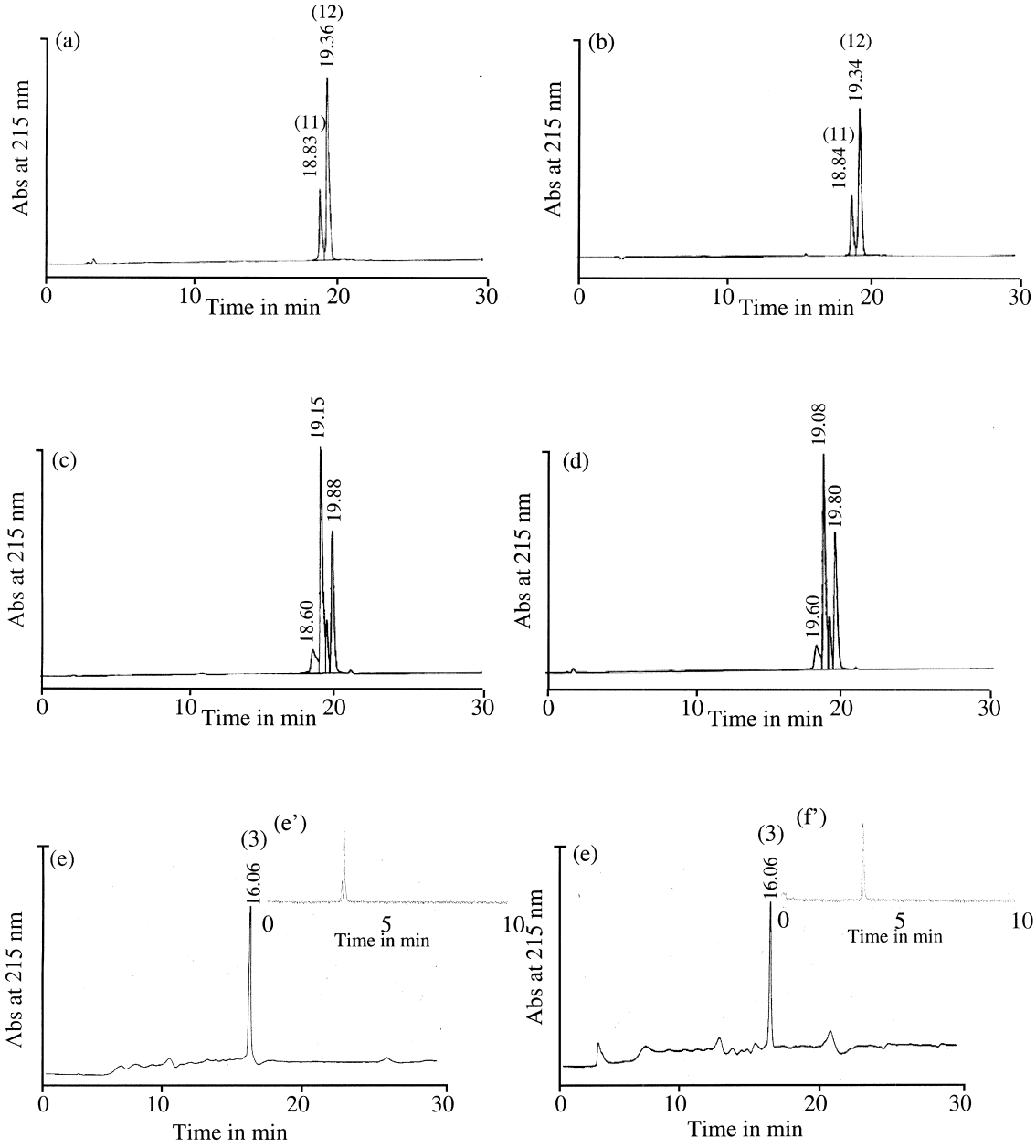

Fig. 8. Analytical RP-HPLC profiles following the initial and the alternate second chromatography of fraction 12 (a, b), fraction 13 (c, d) and fraction 3 (e, f). Experimental conditions as in Fig. 2. Capillary electrophoretic profiles following the initial (e′) and the alternate second chromatography (f′). Experimental conditions: 50 cm×50 μm I.D. capillary; buffer, 20 m*M* sodium citrate pH 2.5; electrophoretic migration, 10 min; detection, UV at 200 nm; temperature, 30°C.

aged. More generally, this method will allow the partitioning of natural extracts or other complex mixtures of peptides or organic compounds in a format that can be useful for systematic parallel analytical determination, and inclusion in high throughput screening for pharmacological evaluation.

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