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Displacement chromatography of chemotactic peptides[1]

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Abstract

Displacement chromatography was successfully used to separate a binary peptide mixture, *n*-formyl-Met–Phe and *n*-formyl-Met–Trp, on a reversed-phase column. Displacement parameters such as choice of displacer, displacer concentration, mobile phase organic level, and flow-rate were critically examined in the context of maximizing productivity. Since the feed composition was limited by solubility, optimal productivity was sought as a function of feed volume. The impurities contained in the commercial displacers used in this study did not seem to affect the overall separation quality. In most cases the final pattern of contiguous rectangular bands was not attained; nevertheless, separations of high productivity were achieved using benzethonium chloride and tris[2-(2-methoxyethoxy)ethyl]amine as displacers. In some cases further increase in productivity was not possible only because of solubility constraints. Loading of feed at low initial organic modulator level coupled with displacements at higher modulator level was found to give efficient separations.

Keywords: Displacement chromatography; Preparative chromatography; Peptides

1. Introduction

Preparative and process scale separations of biomolecules has been of considerable interest in recent years [1]. In this context, displacement chromatography, an intrinsically nonlinear mode, has several advantages including the enrichment of valuable feed components. Displacement, invented by Tiselius [2] in 1943, was first used for the separation of amino acids and peptides using activated carbon adsorbents by Synge and Tiselius [3]. Concurrently, Bendall et al. [4] reported displacement separation of amino acids on an ion-exchange column. The displacement of biomacromolecules, such as proteins, was also attempted by Hall and Tiselius [5] with limited success, mainly due to the unavailability of efficient stationary phases. First reports on the separations of an antibiotic polypeptide was published by Poráth [6,7], and oligosaccharides and branched fatty acids by Claesson [8]. After 20 years of dormancy, displacement chromatography was revived by Horváth [9], and has since found many applications including separation of peptides on reversed-phase columns [10–15]. Displacement separation of proteins on an ion-exchange system has been investigated by several groups [16–19] in recent years. A detailed discussion on the evolution, theory and applications of displacement is found in Frenz and Horváth [20].

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Optimization in preparative chromatography has received increased attention recently [1,21–23]. Productivity is used in this study as a measure of the effectiveness of displacement in separating a binary peptide mixture. Classically, the objective in displacement chromatography is to attain the final pattern of contiguous rectangular bands of feed ahead of the displacer front. Although such final patterns give high yields, they may not result in high productivities. As pointed out by Gadam et al. [24] and Zhu and Guiochon [25], maximum productivity is likely to be achieved by mixed bands that are far from their corresponding final patterns.

In this paper, displacement was used to separate a pair of closely related peptides, *n*-formyl-Met–Phe (denoted by P) and *n*-formyl-Met–Trp (denoted by T) on a reversed-phase column using acetonitrile (ACN) as the mobile phase modulator. These peptides are implicated in bacterial chemotaxis [26,27]. Solubility of P and T in the mobile phase was limited and acted as a major constraint in optimization. P was found to be weakly soluble at the low pH values used in typical reversed-phase runs. It was also observed that the solubility of P increased in the presence of T. Hence, P and T were first suspended in ACN–phosphate buffer solution; only after they were completely dissolved was trifluoroacetic acid (TFA) added. This kept the P in solution; by contrast, adding P directly to ACN–phosphate buffer–TFA solution does not dissolve all the P. Detailed discussions on the solubility studies for this system were reported by Kim [28]. The retention properties (k' versus modulator plot) and the single-component isotherms for these closely related peptides were reported in Kim and Velayudhan [29]. P and T showed converging retention behavior with increasing modulator levels. The Langmuir formalism adequately described the single-component isotherm data.

Productivity was calculated for each feed separately using the expression:

$$\text{Productivity} = \frac{c_f V_f Y}{t_c V_{sp}} \quad (1)$$

where c_f and V_f are respectively concentration and volume of the feed, Y is the yield at 98% purity, t_c is the cycle time and V_{sp} is the adsorbent solid volume (excluding pore volume). The cycle time is the sum of the separation time and the time required for column regeneration. Operating parameters such as displacer concentration and flow-rate were varied systematically to obtain optimal productivity. The consequences of changing the modulator level associated with the displacer are also reported. Commercial displacers invariably contain impurities; these impurities may affect the separation [30,31]. The effect of displacer impurities on the separation of P and T was quantified for one case.

2. Experimental

2.1. Materials

The peptides P and T were obtained from Sigma (St. Louis, MO, USA). Sodium monobasic phosphate and sodium dibasic phosphate was purchased from Mallinckrodt (Paris, KY, USA). HPLC-grade ACN was obtained from EM Science (Gibson, NJ, USA), and sequanal-grade TFA from Pierce (Rockford, IL, USA) respectively. Water was distilled and deionized using the Milli-Q ultrapure water system (Millipore, Bedford, MA, USA). Tris[2-methoxyethoxy)-ethyl]amine (TMEEA) and benzethonium chloride were purchased from Aldrich (Milwaukee, WI, USA). Other candidates (5-nonanone, 4-heptanone, 3,4 dimethoxybenzyl alcohol, *n*-benzylbenzamide, diethyldodecamide, heptoxybenzyl chloride) were obtained from the Department of Chemistry at Oregon State University (Aldrich).

2.2. Apparatus

All preparative separations and subsequent analysis of fractions were performed on a Waters (Milford, MA, USA) HPLC system, which consisted of a quaternary pump (Model 600), UV detector (Model 486), and an autosampler (Model 717 plus). All the units were controlled by a DEC (Nashua, NH, USA) personal computer using Waters Millennium software. The feed mixture for all preparative separations was loaded using Model 7125i injector (Rheodyne, Cotati, CA, USA). Displacement separations by different displacers were carried out on the same Nova-Pak C_{18} column (Waters) with dimen-

sions of 150 mm×3.9 mm I.D. The average particle size of the packing was 4 μm with an average pore size of 60 Å.

2.3. Procedures

2.3.1. Sample preparation

The peptides P and T were dissolved in ACN–phosphate buffer–TFA with either 10:90:0.1 (v/v/v) or 15:85:0.1 (v/v/v) composition. Phosphate buffer was made by preparing a 10 m*M* dibasic solution to which 10 m*M* monobasic solution was added until the pH reached 7. For all feed preparations TFA was added after the feed was completely solubilized in the organic–buffer mixture. In all preparative runs the mobile phase composition of feed was identical to the initial state of column, since previous work [29] has shown that otherwise the separation quality suffered.

2.3.2. Displacement runs

The reversed-phase adsorbent was first equilibrated with ACN–phosphate buffer–TFA (10:90:0.1, v/v/v) mobile phase mixture. The feed containing P and T in the same mobile phase composition was then loaded into the sample loop. After the end of feed introduction the displacer solution was fed continuously into the column. Fractions were collected at the outlet of the UV detector in 15- or 30-s fractions.

2.3.3. Column regeneration

After the emergence of peptides and displacer, the column was washed three times using a gradient cycle from 10% to 95% ACN in 15 min followed by equilibrating the column at 20% ACN for analysis of fractions.

However, we were also able to clean the column by stepping up to 100% ACN and washing at 2 ml/min for 10 min. Then, the mobile phase composition was stepped down to 20% ACN and equilibrated at this level for 10 min. The entire regeneration step lasted 20 min; the latter regeneration time was used for all productivity calculations.

2.3.4. Peptide analysis

The peptide fractions from preparative runs were diluted to fall within their calibration curves, and were analyzed using the same column, with ACN–phosphate buffer–TFA (20:80:0.1, v/v/v) for mobile phase under isocratic conditions. The peptides were monitored using the UV detector at 214 nm. The data from all the fractions was used to reconstruct the chromatograph.

2.3.5. Displacer analysis

Benzethonium chloride was analyzed under isocratic conditions using ACN–phosphate buffer–TFA mixture (60:40:0.1, v/v/v) for mobile phase and detected at 214 nm. Benzethonium chloride was only 97% pure, and the remaining 3% impurities consisted of at least two or three different components, as seen in the frontal runs at various concentrations. However, only one other peak was seen under analytical conditions. Since the impurities were just ahead of the displacer, the other peak was considered the main impurity, and quantified in the same way as benzethonium chloride.

The absorbance of TMEEA was highly nonlinear: while it is easily detected beyond 2 mg/ml, concentrations below this level gave very low peaks. This makes it difficult to analyze collected fractions. Attempts to quantify TMEEA are discussed later.

3. Results

3.1. Displacement of peptides by benzethonium chloride

Benzethonium chloride was recently shown [32] to be a useful displacer in hydrophobic interaction chromatography; here its potential as a displacer in reversed-phase is examined. Frontal runs of benzethonium chloride in ACN–phosphate buffer–TFA (15:85:0.1, v/v/v) were carried out at various concentrations, and shown in Fig. 1a. The single-component isotherm was constructed from the shocks in the frontal runs, and is shown in Fig. 1b. Based on the single-component isotherms of benzethonium chloride and of the feed components, displacer concentrations of 7.5 mg/ml and 10 mg/ml were chosen. Displacement runs were carried out for both concentrations at increasing feed volumes until the productivity dropped for either component. In some

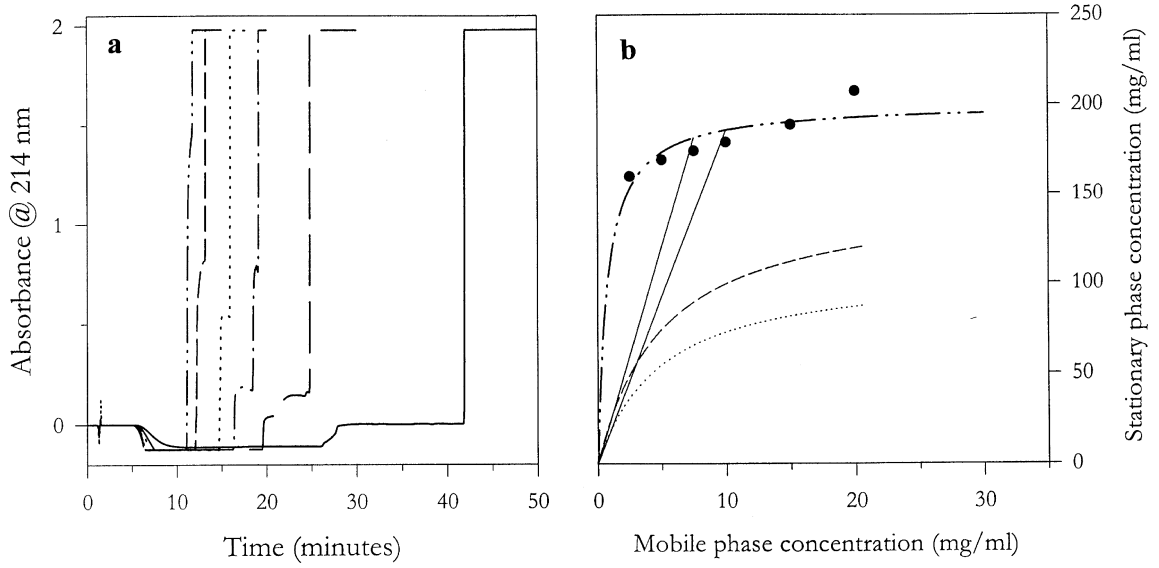

Fig. 1. (a) Frontal runs at various benzethonium chloride concentrations [2.5 mg/ml (solid line), 5 mg/ml (long dash), 7.5 mg/ml (dash–dot), 10 mg/ml (dots), 15 mg/ml (dash), 20 mg/ml (dash–dot–dot)]; displacer suspended in ACN–phosphate buffer–TFA (15:85:0.1, v/v/v). (b) Single-component adsorption isotherm of benzethonium chloride (dash–dot–dot) constructed from the frontal runs in (a). Operating lines for 7.5 mg/ml and 10 mg/ml shown (solid lines). Single-component isotherms for P (dots) and T (dashes) from Ref. [29] are also shown.

cases, further increase in feed volume was not possible due to solubility constraints, even though the productivity was still increasing. From previous studies on solubility for the P+T system [28], it is known that P in particular had very limited solubility. Hence, when the effluent concentration of P reached 2.5 mg/ml, runs with larger feed volumes were not attempted.

The column was first equilibrated at 10% ACN followed by loading of feed containing 10% ACN. The displacement step, where the displacer was suspended in 15% ACN, started 0.5 min after the end of feed introduction. Fig. 2(a, b and c) shows the displacement results at 7.5 mg/ml benzethonium chloride for feed volumes of 3.9, 5.0, and 6.5 ml, respectively. For convenience, the feed input into the column is also shown, starting at $t=0$. The maximum concentration of P increased with increasing feed volume. In all cases, P showed a right-triangle pattern with tailing into T, suggesting that P elutes. The increase in concentration of the early fractions is then attributed to the generation of a concentrated band of P during loading (''roll-up'') [33]. The amount of mixing increased steadily with loading; however, the productivity of P reached a maximum at 6.5 ml. Further increase in feed volume to 7.4 ml (figure not shown) resulted in decreased productivity since the amount recovered at 98% purity decreased. In all cases, the last fraction of T is significantly more concentrated than the others. The rectangular pattern for T was more developed for low feed volume runs (3.9 and 5.0 ml) than for higher feed volume runs (6.5 and 7.4 ml). This is because of greater mixing between P and T at higher feed volumes. The inset in all diagrams shows the quantification of the most strongly retained impurity of benzethonium chloride. The first fraction of this impurity in Fig. 2(a and b) is highly concentrated. However, this impurity was found to degrade quickly at room temperature in separate experiments, which could explain its low level in Fig. 2c. Separations beyond 7.4 ml feed volumes for 7.5 mg/ml benzethonium chloride concentration were not carried out since the productivity for both P and T dropped.

Fig. 3 shows the chromatograms for 3.9, 6.5 and 7.9 ml feed volumes for 10 mg/ml benzethonium

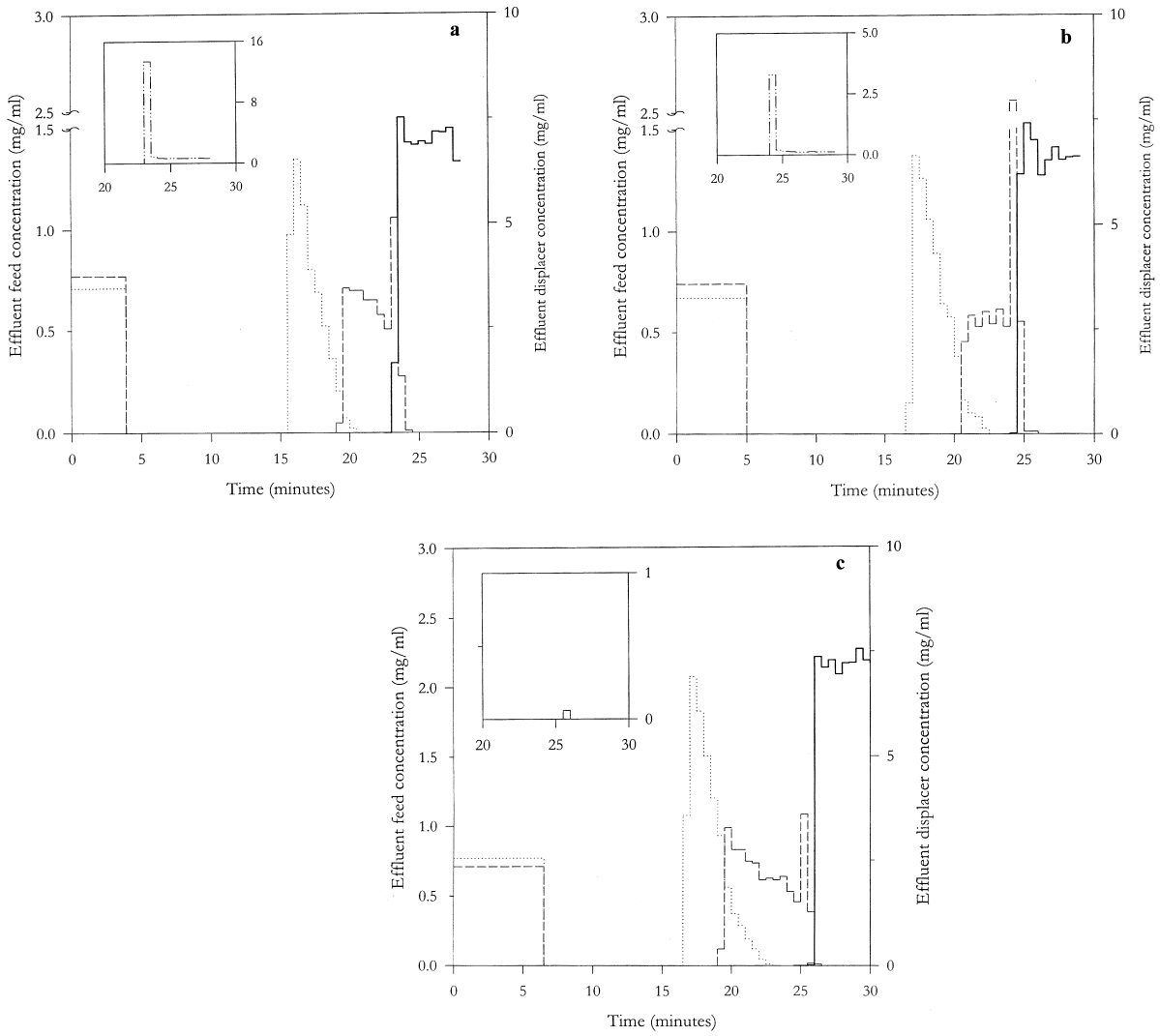

Fig. 2. Displacement chromatogram using 7.5 mg/ml benzethonium chloride as displacer in 15% ACN. (a) Feed: 0.71 mg/ml P (dots) and 0.77 mg/ml T (dashes) in 3.9 ml at 10% ACN; displacer, benzethonium chloride (solid line); flow-rate, 1 ml/min; fraction size, 0.5 ml each. (b). Feed: 0.67 mg/ml P and 0.74 mg/ml in 5.0 ml feed volume. (c) Feed: 0.76 mg/ml P and 0.71 mg/ml T in 6.5 ml feed volume.

chloride. Again, P shows similar behavior, indicating elution. However, T formed a narrow concentrated band in the 3.9 ml run. As the feed volume increased, so did the mixing, resulting in lower concentrations of T further ahead of the displacer front. Feed volumes beyond 7.9 ml were not used because of solubility constraints on P, even though productivity was still increasing. Relatively low levels of the impurity were found, which is again attributed to degradation. A detailed comparison of productivities will be made in Section 4.6.

3.2. Displacement of peptides by TMEEA

Frontal runs of TMEEA in 15% ACN were carried out at different concentrations, and are shown in Fig. 4a. In order to measure the linear retention (k') of TMEEA (so as to describe the initial slope of the

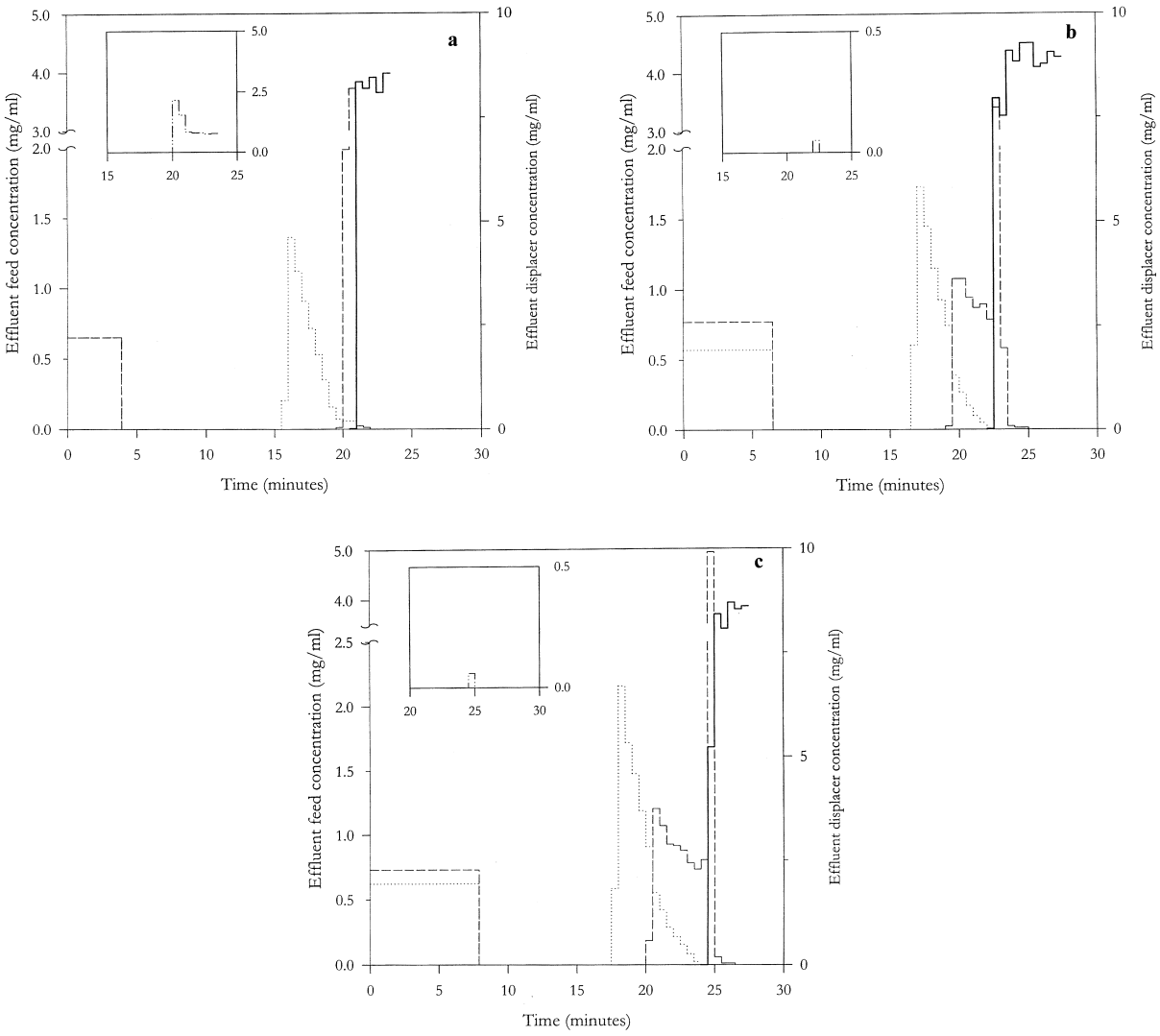

Fig. 3. Displacement chromatogram using 10 mg/ml benzethonium chloride as displacer in 15% ACN. (a) Feed: 0.65 mg/ml P (dots) and 0.65 mg/ml T (dashes) in 3.9 ml at 10% ACN; displacer, benzethonium chloride (solid line); flow-rate, 1 ml/min; fraction size, 0.5 ml each. (b) Feed: 0.57 mg/ml P and 0.77 mg/ml T in 6.5 ml feed volume. (c) Feed: 0.62 mg/ml P and 0.73 mg/ml T in 7.9 ml feed volume.

single-component isotherm), gradient runs (steep and shallow) were carried out under analytical conditions, following the method of Quarry et al. [34]. However, it was found that TMEEA did not confirm to linear-solvent-strength (LSS) behavior (i.e., linear retention did not vary exponentially with modulator level). Hence, another approach was taken: gradients of varying slopes starting at 15% ACN were performed. A double exponential fit was used to fit the relationship between k' and gradient slopes, and was then extrapolated to estimate the isocratic retention time at 15% ACN. The single-component isotherm for TMEEA was obtained by combining the retention factor estimate with the location of shocks from the frontal runs, and is shown in Fig. 4b. The single-component isotherm was well fitted by a double Langmuir expression, shown by the dash–dot–dot curve in Fig. 4b.

Fig. 5(a, b and c) shows displacement runs for feed volumes of 3.4, 3.9 and 5.0 ml respectively. The

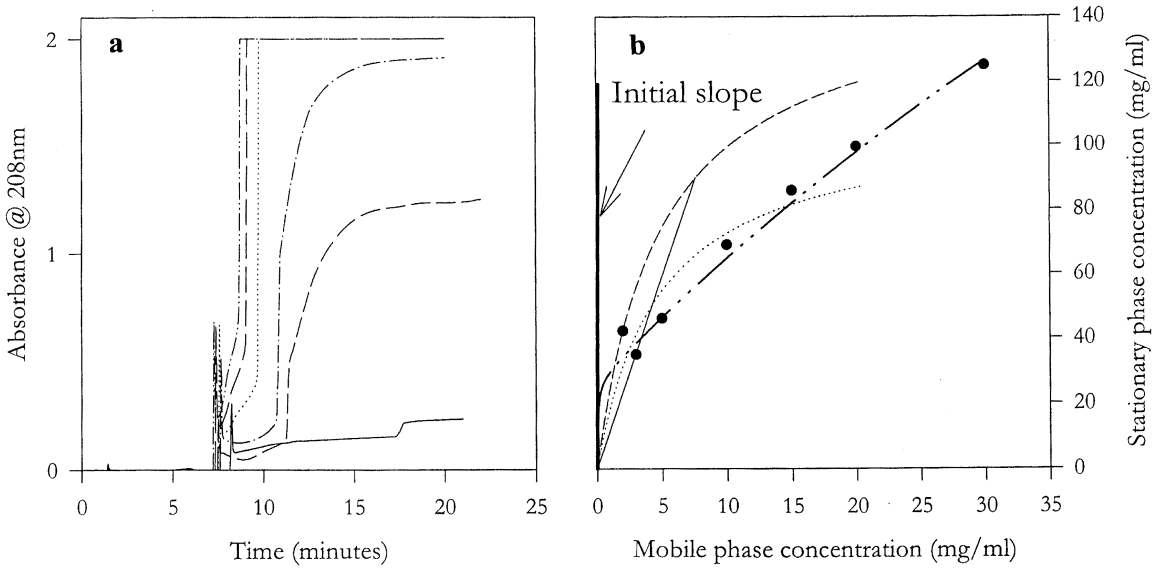

Fig. 4. (a) Frontal runs at various TMEEA concentrations [2 mg/ml (solid line), 3 mg/ml (dashes), 5 mg/ml (dash dot), 10 mg/ml (dots), 15 mg/ml (long dash), 20 mg/ml (dash–dot–dot)]; displacer suspended in ACN–phosphate buffer–TFA (15:85:0.1, v/v/v). (b) Single-component isotherm for TMEEA (dash–dot–dot). Operating line for 3 mg/ml shown (solid line). Single-component isotherms for P (dots) and T (dashes) from Ref. [29] are also shown.

column was initially at 10% ACN, as was the feed; the displacer was dissolved in 15% ACN. Touching-band separations with very low levels of mixing for both feeds were achieved for 3.4 ml and 3.9 ml runs. The concentrations of both peptides were substantially higher than in the feed. Although slightly greater mixing was found for the 5.0 ml run (Fig. 5c) the productivities of both P and T increased. However, the maximum concentration of P was close to the maximum level of 2.5 mg/ml prescribed by its limited solubility. Hence the feed volume could not be further increased.

TMEEA did not absorb in UV at low concentrations, and hence its concentrations could not be estimated by direct analysis of fractions. In fact, UV absorption of TMEEA was extremely nonlinear, as can be seen from the frontal runs (Fig. 4a). The run for 2 mg/ml was much lower than that for 3 mg/ml. The fractions collected from displacements at 3 mg/ml were diluted during analytical isocratic runs to the point where the TMEEA peaks could not be clearly identified. Hence, the location of the TMEEA shock was sought by repeating the preparative experiment with no feed in the sample loop. The fronts from these runs were overlaid on the corresponding separations, and quantified on the secondary *y*-axis in Fig. 5. It is evident from all the plots that TMEEA is immediately behind T. Additionally, the rear of T was very sharp, making it likely that it is being displaced.

In this study, experiments were performed with TMEEA from two different stocks. All the experiments shown here were from an older supply of TMEEA. The frontal run for the newer TMEEA (not shown), although qualitatively similar, was quantitatively somewhat different from that of the older TMEEA. Nevertheless, upon repeating the displacement run for 3.9 ml feed volume (Fig. 6) with the newer stock, the separations were similar. Comparing Fig. 6 with Fig. 5b shows that the breakthrough of P and the rear of T occur at the same location, although the amount of mixing was higher when using the newer TMEEA.

4. Discussion

4.1. Choice of displacer

When choosing a displacer for reversed-phase

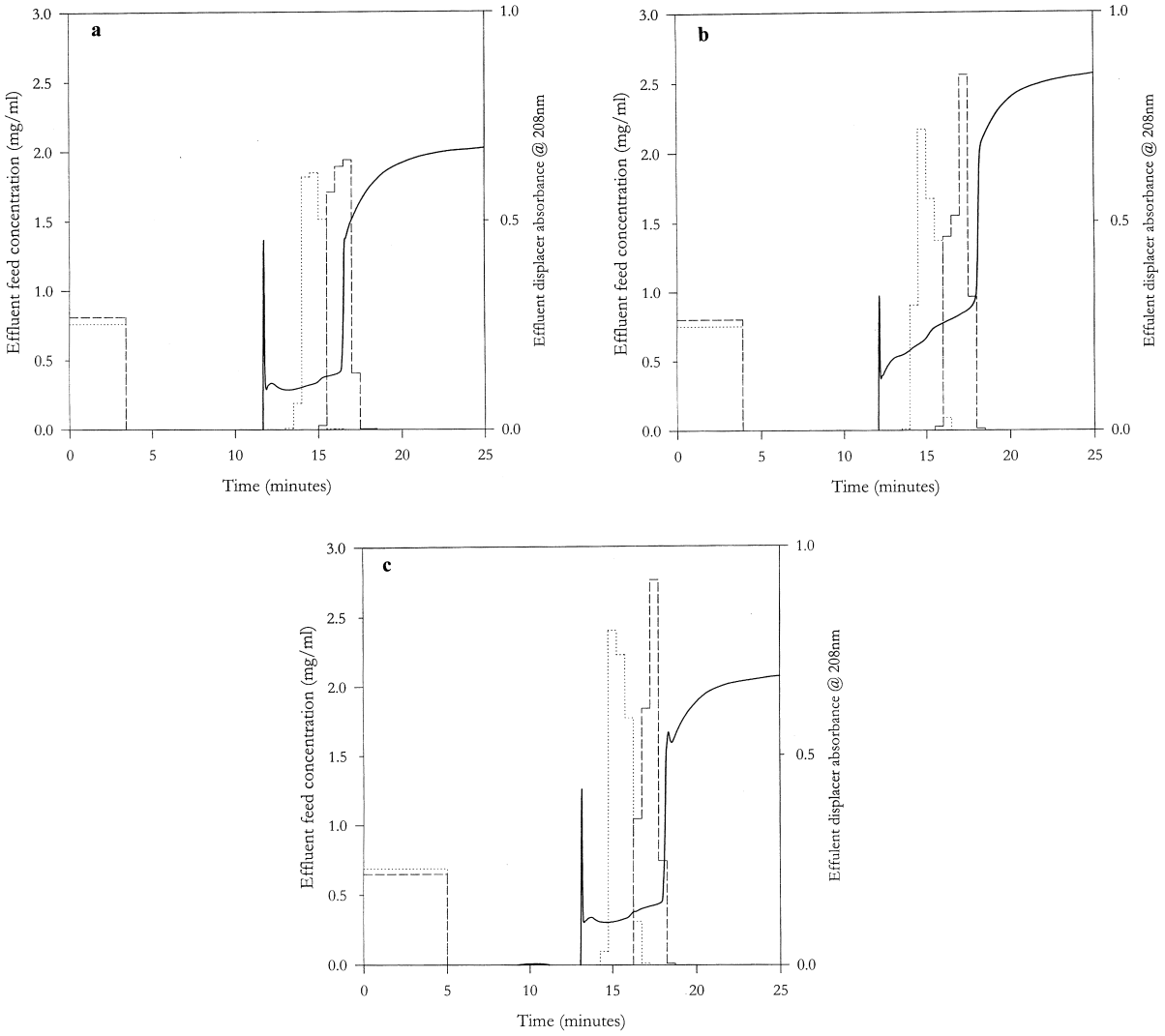

Fig. 5. Displacement chromatogram using 3 mg/ml TMEEA as displacer in 15% ACN. (a) Feed: 0.76 mg/ml P (shown by dots) and 0.81 mg/ml T (shown by dashes) in 3.4 ml feed volume in 10% ACN. (b) Feed: 0.75 mg/ml P and 0.80 mg/ml T in 3.9 ml feed volume. (c) Feed: 0.69 mg/ml P and 0.65 mg/ml T in 5.0 ml feed volume. All other conditions as in Fig. 3.

chromatography a compromise has to be made between analytical retention, saturation capacity (which may be regarded as a measure of nonlinear retention), and solubility. In addition, since the displacer produces an enriched zone of modulator next to the displacer front [35], complex retention changes could occur in the microenvironment within the column due to the differences in modulator levels, which could potentially change the overall separation.

Benzethonium chloride was found to have good potential: it was highly soluble, strongly retained, and easily detected by UV (because of the benzyl rings). Other candidates tested were 5-nonanone, 4-heptanone, 3,4 dimethoxybenzyl alcohol, *n*-benzylbenzamide, diethyldodecamide, heptoxybenzyl

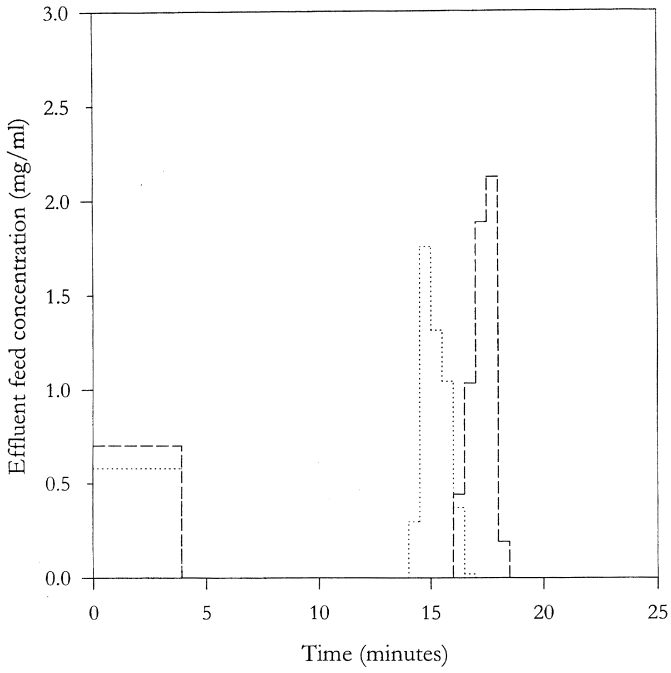

Fig. 6. Displacement chromatogram using 3 mg/ml ''newer stock'' TMEEA as displacer in 15% ACN. (a) Feed: 0.58 mg/ml P (shown by dots) and 0.70 mg/ml T (shown by dashes) in 3.9 ml feed volume in 10% ACN. All other conditions as in Fig. 3.

chloride, and TMEEA. Amongst these compounds only TMEEA, a tertiary amine, was found to satisfy both solubility and retention requirements

4.2. Choice of modulator level

The retention of many molecules decreases exponentially with increasing organic modulator level [36]. Hence, minor differences in modulator levels both in the initial feed state and in the displacer could cause significant differences in retention. High initial modulator levels could potentially help in reducing the separation time; however, they may also result in less efficient separation because of decreased selectivity. On the other hand, when low initial modulator levels are used, the molecules bind strongly to the stationary phase, thereby increasing the loading capacity, but increasing the separation time. Hence, the highest productivity, reflecting the balance between loading and selectivity on the one hand and the separation time on the other, will be obtained by a judicious choice of modulator levels in the feed state and in the displacer (these need not be equal).

Separations using TMEEA as displacer suspended in 15% ACN for a 3.4 ml feed volume where the initial state of column was also at 15% ACN are shown in Fig. 7 (hereafter, 15 initial/15 displacement). By comparing this to the 3.4 ml run (Fig. 5a) when the displacer solution was fed at 15% ACN level keeping both the initial state of column and the feed state at 10% ACN (10 initial/15 displacement), it can be observed that the latter resulted in complete separation, but had a greater separation time. The 10 initial/15 displacement resulted in concentrated and roughly rectangular bands. In the 15 initial/15 displacement run, the feed components moved too fast for the displacer to affect them appreciably, resulting in lower productivities. A third choice, where both displacer and initial state of the column were at 10% ACN (10 initial/10 displacement) is shown in Fig. 8. While the separation was good, the substantially longer separation time resulted in lower productivities. It can be concluded from this series of

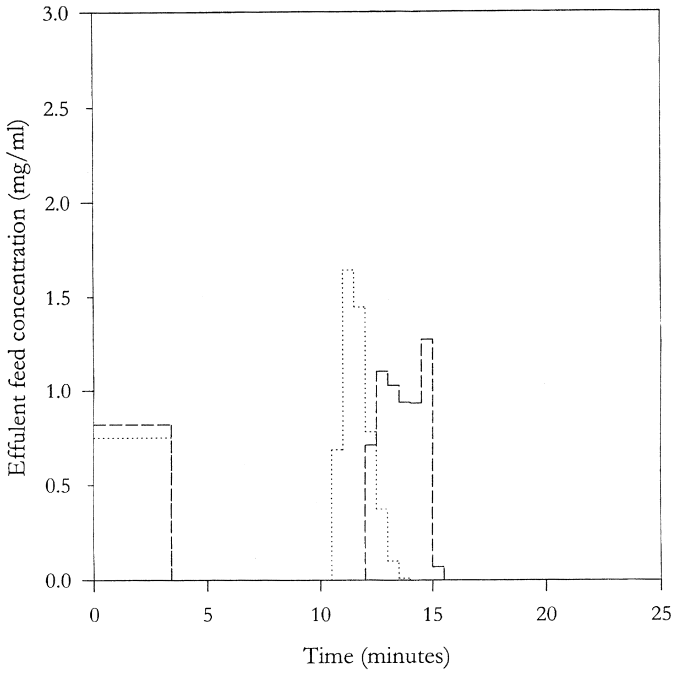

Fig. 7. Displacement chromatogram using 3 mg/ml TMEEA as displacer in 15% ACN. Feed: 0.75 mg/ml P (shown by dots) and 0.82 mg/ml T (shown by dashes) in 3.4 ml. The initial state of column was 15% ACN. All other conditions as in Fig. 3.

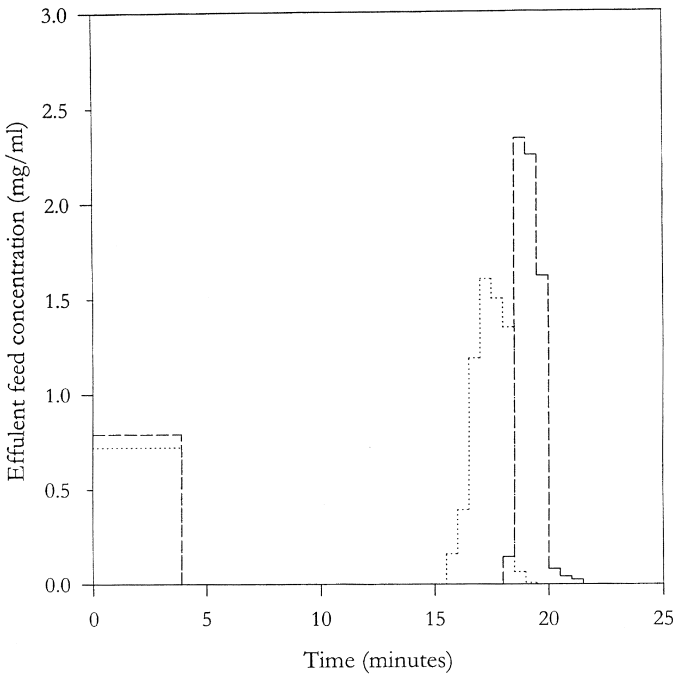

Fig. 8. Displacement chromatogram using 3 mg/ml TMEEA as displacer in 10% ACN. Feed: 0.72 mg/ml P (shown by dots) and 0.79 mg/ml T (shown by dashes) in 3.9 ml. The initial state of column was 10% ACN. All other conditions as in Fig. 3.

experiments that the best results are achieved when the feed is loaded at low modulator levels (thereby exploiting the discrimination of stationary phase for the feed components) and displacements carried out with higher modulator levels in the displacer solution, producing rapid separations. The potential difficulty with high modulator level in the displacer is that the feeds may elute in the enriched modulator band formed ahead of the displacer. However, that does not occur in the TMEEA runs.

4.3. Choice of flow-rate

It has been found that low flow-rates can result in improved displacement separations [37]. In order to test the effect of flow-rate on separation of P and T, displacements at low flow-rates (0.1 ml/min for benzethonium chloride and 0.1 ml/min and 0.75 ml/min for TMEEA) were attempted for both displacers. The low flow-rate run for benzethonium chloride could not be carried out because P crystallized immediately after elution from the column. Experiments carried out with TMEEA at 3 mg/ml for 3.4 ml feed volume at 0.1 ml/min and 0.75 ml/min are shown in Fig. 9. In comparison with the run at 1 ml/min (Fig. 7), the separations, including the extent of mixing, were quite similar. Thus, the flow-rate is not a limiting factor in these runs.

4.4. Choice of displacer concentration

The single-component isotherms of feed components and displacer serve as the starting point for selecting the displacer concentration. The classical final-pattern concentrations for the feed components are found by drawing a chord between the origin and a given displacer concentration (operating line), and identifying the points of intersections of the feed single-component isotherms with this operating line (see Figs. 1 and 4). For systems where feed solubility is a constraint, lower displacer concentrations are often useful. However, lower concentration fronts move slower than higher concentration fronts, thereby increasing separation time. The frontal run for 7.5 mg/ml benzethonium chloride resulted in a displacement front at 19 min. From the gradient runs at 15% ACN [29] for a 2.4 ml feed volume, both P and T eluted by 20 min. Also, results from the corresponding isocratic runs [29] suggested that P would elute when using 7.5 mg/ml benzethonium chloride.

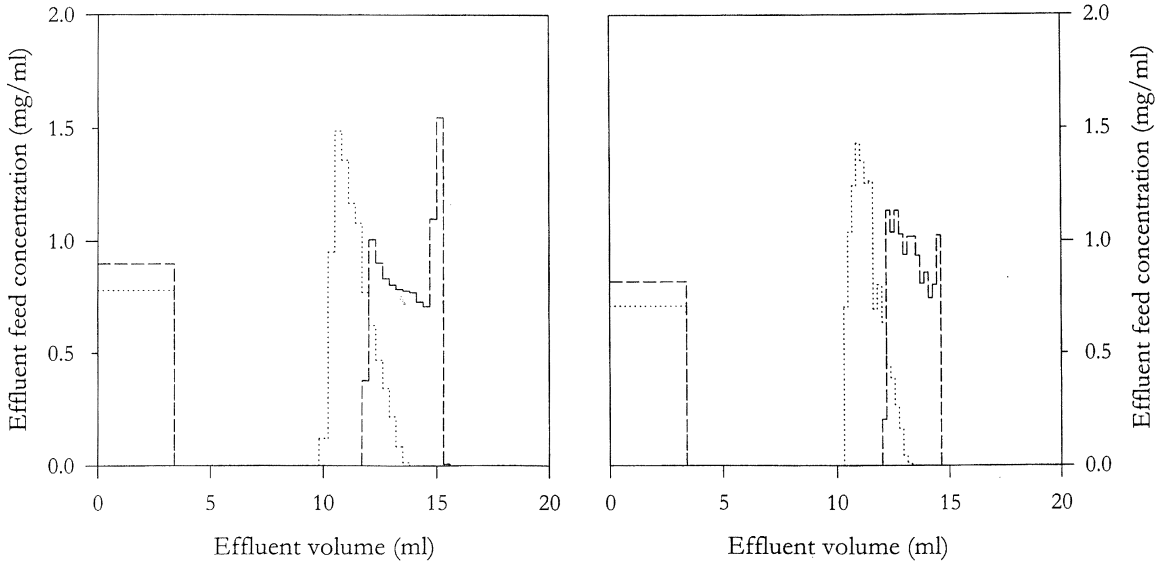

Fig. 9. Displacement chromatogram using 3 mg/ml TMEEA as displacer in 15% ACN. Feed: 0.78 mg/ml P (shown by dots) and 0.90 mg/ml T (shown by dashes) in 3.4 ml. (a) Flow-rate was 0.1 ml/min, (b) 0.75 ml/min. All other conditions as in Fig. 3.

The elution of P is desired because of its limited solubility.

The single-component isotherm for TMEEA closely overlay the single-component isotherm of feed components in the region of operation at 3 mg/ml, where a frontal run at for the same concentration resulted in a shock at 12.5 min. This is another useful approach when feed solubility is limited, because the feeds are unlikely to be highly concentrated in the final pattern. Hence, 7.5 mg/ml of benzethonium chloride and 3 mg/ml of TMEEA were chosen as starting points for displacement experiments, and were observed to give good productivities without unduly concentrating the P band. A 10 mg/ml concentration of benzethonium chloride was also tried, and the results were discussed in Section 3.1. In general, changing the displacer concentration as well as the modulator levels in both feed and displacer are needed to maximize productivity.

4.5. Effect of displacer impurity

The commercial displacers used in this study contained impurities. Benzethonium chloride is 97% pure, and TMEEA 95% pure. Since the displacer is continuously fed into the column, impurities, although present in small levels, could potentially affect the overall separation quality. Frontal runs for TMEEA (Fig. 4a) show a peak well ahead of the main front, which is likely to be a weakly retaining impurity. Previous reports on displacer impurities by Zhu et al. [30] and Jen and Pinto [31] show that a weakly retained impurity could form a sharp peak followed by a plateau before the displacer front. This impurity is almost unretained, and is unlikely to affect the separation quality. In the case of benzethonium chloride, as evident from the frontal runs (Fig. 1), there were several impurities; one impurity that was quantifiable in analytical runs was found to occur at significant levels in the fraction just ahead of the displacer front itself. Hence, it is reasonable to assume that this impurity's adsorption was similar to that of benzethonium chloride. However this impurity degraded rapidly in solution at room temperature, and was often not seen under analytical conditions. By contrast, benzethonium chloride was quite stable under the same conditions.

4.6. Comparison of productivities

Productivity for P and T was calculated using Eq. (1) and listed in Table 1 for the various runs. The cost of separating the displacer from purified T was not accounted for. TMEEA gave a maximum productivity of 9.0 mg/(ml·h) for P and 7.2 mg/(ml·h) for T. Enrichments of P and T increased with increasing feed volumes. Solubility constraints on P prevented further increase in feed volume, which otherwise could have increased the productivity of P. However, productivity of T went through a maximum, and hence further increases in feed volume were not useful. Productivity for displacement runs using benzethonium chloride at 7.5 mg/ml went through a maximum for both P and T. Enrichments for P increased with increasing loading, while enrichment for T went through a maximum. Benzethonium chloride at 10 mg/ml gave maximum productivities of 9.9 mg/(ml·h) and 7.5 mg/(ml·h). Solubility constraints on P limited further increase in loading. However, the productivity of T went through a maximum, making further increases in loading pointless. In all cases the yield of T dropped faster than for P since high concentrations of T, at the front of the T band, become mixed with low concentrations of P, at the rear of the P band.

Recent results in our laboratory gave comparable productivities for the P+T separations in isocratic, stepwise and gradient elution. For example, productivities for a 5 ml feed under isocratic conditions at 15% ACN were 7.2 mg/(ml·h) for P and 3.3 mg/(ml·h) for T. Similar results for stepwise elution from 10–15% ACN in 0.5 min gave 10.3 mg/(ml·h) for P and 5.6 mg/(ml·h) for T, and a gradient run from 10–40% in 30 min gave 7.6 mg/(ml·h) for P and 6.0 mg/(ml·h) for T. A detailed comparison of operating conditions resulting in optimal productivities for P+T in all the chromatographic modes is underway in our laboratory.

In order to assess the efficacy of these displacement separations, productivities were estimated from the literature for displacements of other biomolecules on reversed-phase adsorbents. The regeneration time for all separations was assumed to be same as that of the P+T system (20 min). Additionally, the total porosity (interstitial+intraparticulate) for all the columns was assumed to be 0.7. Productivity estimates for the displacement separations of melanot-

Table 1
Productivity table for all the displacement separations

Feed (ml)	*n*-Formyl-Met–Phe				*n*-Formyl-Met–Trp			
	Loading (%)	Productivity (mg/ml h)	Yield (%)	Enrichment	Loading (%)	Productivity (mg/ml h)	Yield (%)	Enrichment
TMEEA: 3 mg/ml								
3.4	4.0	7.2	100	1.4	3.1	7.1	100	1.1
3.9	4.3	7.2	99	1.5	3.4	7.2	98	1.6
5.0	5.3	9.0	95	2.4	3.6	7.0	83	2.1
Benzethonium chloride: 7.5 mg/ml								
3.4	3.4	5.6	98	0.8	2.9	5.2	89	0.7
3.9	4.2	7.0	98	1.0	3.3	6.7	99	0.8
5.0	5.1	7.8	94	0.9	4.1	6.8	85	0.9
6.5	7.6	10.5	84	1.8	5.1	5.3	54	0.7
7.4	7.1	7.9	68	2.0	5.8	4.3	40	0.6
Benzethonium chloride: 10 mg/ml								
3.4	3.4	5.7	98	0.9	2.8	6.1	100	0.9
3.9	3.9	6.2	98	0.8	2.8	6.0	100	0.9
5.0	5.2	7.5	88	1.5	4.3	6.5	73	1.5
6.5	5.7	8.2	87	1.9	5.6	7.5	68	1.1
7.9	7.5	9.9	82	2.2	6.4	7.3	59	1.3

Loading factor [23] is calculated by $\left(\frac{V_f c_f}{V_{sp} \Lambda_i}\right) \cdot 100$, where V_f is the volume of feed, c_f is concentration of feed, V_{sp} is the stationary phase volume (0.6 ml), and Λ_i is the saturation concentration of the i^{th} component (108 mg/ml for P and 150 mg/ml for T). Enrichment is the ratio of the average concentration across all the acceptably pure fractions to the feed concentration.

ropins [10] on a laboratory-made octadecylsilica column using ACN–water as mobile phase and benzyldodecyldimethylammonium bromide as displacer (hereafter the details are listed as: column; mobile phase; displacer) was 5.1 mg/(ml·h). Separation of oligomycins [38] (LiChrosorb RP-18; methanol–water; palmitic acid) resulted in 5.2 mg/(ml·h). Viscomi et al. [11] report scaling-up displacement separations without significant loss in productivity for a peptide fragment of human interleukin-β (LiChrosorb RP-18; water–TFA; benzyltributylammonium chloride). The productivity obtained on a 250×4 mm column gave 12.6 mg/(ml·h); scaling up by a factor of 20 gave productivity of 10.6 mg/(ml·h). The productivities obtained for P+T were comparable to, and some cases substantially higher than, the estimates for other biomolecules in spite of stringent solubility limitations.

5. Conclusions

Displacement chromatography was successfully used to separate a binary peptide mixture. Flow-rates similar to those typical of elution modes were used effectively. Displacer impurities did not affect separation quality in these runs. Good productivities were obtained despite the limited solubility of the feed components. Maximum productivities were achieved under non-isotachic conditions. For this system, feed loading should be at low modulator levels followed by displacements at higher modulator concentrations. For feeds with limited solubility, displacers with single-component isotherms that are only slightly higher than those of the feeds are preferable.

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