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Preparative high-performance liquid chromatography of echinocandins

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Abstract

The isolation of fermentation derived small bioactive molecules remains extremely challenging due to the presence of many analogues with similar physicochemical behavior. Removal of analogue impurities typically involves crystallization and/or preparative HPLC. The normal-phase preparative HPLC for the purification of fermentation derived echinocandins is described. Resolution of key impurities from the product of interest, pneumocandin B_o, is accomplished using a ternary ethyl acetate–methanol–water mobile phase with silica gel as the sorbent. Plate counts, measured with small molecules, show that the column efficiency is excellent under the operating conditions despite the use of an irregular silica and unusually high levels (greater than 6%) of water in the mobile phase. The results of optimization studies indicate the product solubility, retention and resolution of key analogue impurities are strong functions of the ternary mobile phase composition. The normal-phase HPLC process was optimized by carrying out eluent flow-rate (linear velocity) and column loading studies. The results of these experimental studies indicate that both yield and productivity are a function of linear velocity and product loading and that a tradeoff exists between these two parameters.

Keywords: Preparative chromatography; Echinocandins; Lipopeptides; Peptides; Pneumocandins

1. Introduction

Echinocandins are a class of naturally occurring lipopeptides with antifungal activity. The structures of three closely related members of the subclass known as pneumocandins are given in Fig. 1. They are cyclic hexapeptides with multiple hydroxyl groups and a hydrophobic dimethylmyristate tail connected via an amide bond to the α-amino group of the (hydroxylated) ornithine residue. The three compounds in the figure differ from each other only in subtle modifications to the side chain of the proline residue adjacent to the ''ornithine''. Indeed, pneumocandins B_o and C_o differ only in the position of the hydroxyl group on this side chain, whereas pneumocandin A_o has an additional methyl group [1].

The large-scale isolation of pneumocandin B_o from cultures of the fungus *Glarea lozoyensis* is a significant technical challenge. The organism can produce, in addition to the desired compound, a dozen or more echinocandins [2,3] including pneumocandins A_o and C_o. Furthermore, the lipopeptides have unique solubility properties: they are essentially insoluble in water and most pure solvents, but dissolve in alcohols and some aqueous–organic solvent mixtures [4]. Moreover, they are difficult to purify by crystallization.

The present work outlines some aspects of the development and scale-up of a large-scale high-

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performance liquid chromatography (LS-HPLC) step for purification of pneumocandin B_o, extending earlier reports on low pressure silica-gel chromatography of the pneumocandins [4–6]. The feasibility of pneumocandin purification by HPLC has been demonstrated [7]. The use of bare and functionalized silica-gel media in the purification of many low-molecular-mass compounds has been repeatedly documented in the literature [8–12] and summarized in a recent review [13]. Typical normal-phase separations are performed with bare silica, either spherical or irregular, employing a binary mobile phase composed of a weakly polar solvent combined with an alcohol (e.g. EtOAc and MeOH). However, balancing the solvent polarity of a mobile phase via the use of ternary solvent system for preparative normal-phase separations has only seen limited utilization [14].

This work discusses reasons for selection of the normal-phase system, choice of the stationary phase, and selection and optimization of the mobile phase, which is a ternary solution of ethyl acetate (EtOAc), methanol, (MeOH) and water with an unusually high water content of about 7%. Although much speculation exists on the effect of water on the separation efficiency and compound adsorption in normal-phase chromatography, in particular in a ternary mobile phase, few conclusive experimental studies have been presented in the literature [15]. The question of whether water content impacts column efficiency is examined with the help of plate number measurements with tracer compounds at different water levels.

Translating the research performed at analytical to preparative scale, including the importance of balancing yield and productivity, [16] has been the focus of significant research. Previous investigators have described the important parameters associated with the successful development and scaleup of preparative HPLC processes [17] including the packing and operation of dynamic axial compression columns [18–25].

The maximization of preparative HPLC throughput is also examined here with experiments carried out at different loads and flow-rates, using pneumocandins A_o and C_o as critical markers of the quality of separation. Finally, results of scaling up from the laboratory to a 15-cm I.D. column in our pilot plant are presented.

2. Experimental

2.1. Materials

All experimental studies employed irregular silica from Amicon (Cherry Hill, MA, USA) designated Grade 631, Si-60 of nominal particle size 20 μm. Analytical scale columns (250×4.6 mm) were obtained prepacked from Amicon (Beverly, MA, USA). All solvents employed in the ternary mobile phase (ethyl acetate, methanol and water) were HPLC grade from Fisher Scientific (Pittsburgh, PA, USA). Mobile phases employed for the preparative scale chromatography were prepared from MeOH (99.9%, w/w, purity) from Enron (Houston, TX, USA), and urethane grade ethyl acetate from Eastman (Perth Amboy, NJ, USA) and low ion water produced in the laboratory using a purification unit from Osmonics (Minnetonka, MN, USA). Volumetric compositions of solutions are designated as volumes prior to mixing and do not account for non-ideal mixing effects. For example, an EtOAc–MeOH–water solution designated as 84:9:7 was prepared by mixing 84 volumes of EtOAc, 9 volumes of MeOH and 7 volumes of water.

Tracer solutions for both analytical and preparative scale were prepared by adding 2.5% (v/v) methylethyl ketone (MEK; 2-butanone) and 0.5% (v/v) toluene to the ternary mobile phase. The MEK was ACS grade from Fisher Scientific and the toluene was GR grade (99%) from EM Science (Gibbstown, NJ, USA).

Feedstock for the preparative separation experiments was derived from an isolated solid intermediate containing the product of interest, pneumocandin B_o, in addition to more than ten other analogue impurities. A method for isolation of the feedstock material from a fermentation broth using solvent extractions and a precipitation is described elsewhere [1,6]. The structure of the product and two other marker analogue impurities is presented in Fig. 1. The feed to the preparative scale HPLC experiments contained the product at a concentration ranging from 10 to 30 g/l and was prepared via dissolution in a proprietary solvent blend.

2.2. Equipment

Analytical scale experiments were performed on a

Hewlett-Packard (Waldbronn, Germany) HP-1100 HPLC system composed of a quaternary pump, column thermostat and variable-wavelength detector (VWD) with detection monitored at 278 nm. The data analyses were performed using CHEMSTATION Software Rev. A.05.04 and a Windows 95 (Microsoft, Eugene, OR, USA) operating system. All mobile phases were prepared via blending of solvents off-line to HPLC operations. Injection volumes for tracer experiments were performed at volumes from 5 to 20 μl. Analyses of detailed fractions from preparative HPLC runs were performed with the HP-1100 system.

Preparative experiments were performed at two different scales of operation employing a 6-cm I.D. semipreparative column and a 15-cm I.D. preparative column. The semipreparative experiments employed a Dorr Oliver Model C (1 l/min pumping skid, Biotage Division of Dyax) and a 6-cm I.D. (0.5-m length column) axial compression column from Prochrom (Indianapolis, IN, USA). The preparative experiments employed a Dorr Oliver Model B (5 l/min pumping skid, Biotage) and a 15-cm I.D. (1.7-m length) axial compression column from Prochrom. A limited number of column efficiency measurements are reported using a Biotage KP 3000 (12 l/min pumping skid) and a 30-cm I.D. dynamic axial compression column from Prochrom. Detection for all systems was at 278 nm.

Tracer solutions were injected via a sample pump for the 6- and 15-cm I.D. columns and via a syringe for the 30-cm I.D. column. Owing to the design limitations for the 15-cm system, a minimum injection volume of 2 column volume percent was required. Data obtained from the laboratory experiments that explored the effect of tracer injection volume on column efficiency were subsequently employed to normalize all column efficiency measurements to a fixed tracer volume injection of 0.5 column volume percent (Appendix B).

2.3. Silica gel defining protocol/column packing

Packing for preparative scale columns was defined using the following protocol derived from technical discussions with Prochrom, the preparative column vendor. The dry silica powder was slurried in an equal volume of mobile phase followed by gravity settling for a minimum of 3 h. The supernatant was decanted and additional mobile phase, equal to the original silica-gel volume, was added to the wetted silica and the step repeated. Following completion of the silica-gel de-fining operation, the silica gel was reslurried in the mobile phase and poured or pumped into either a 6-, 15- or 30-cm I.D. Prochrom axial compression column. The silica-gel slurry was then rapidly compressed with a packing pressure in the range of 4.14 to 4.48 MPa. Column volume was calculated by multiplying the column length, determined immediately after completion of packing, by the internal diameter of the column.

3. Results and discussion

Two key marker compounds (Fig. 1), pneumocandin A_o and C_o, are employed throughout the work to demonstrate the sensitivity of the isolation process to the parameters investigated. Note that these two compounds differ only slightly from pneumocandin B_o, the compound of interest. Pneumocandin A_o contains an additional methyl group at the 4 position in the hydroxyproline functional group. One key analogue impurity, pneumocandin C_o, is a positional isomer of the product, and represents a shift in a hydroxyl group from the 4 to the 3 position on the hydroxyproline section of the polar hexapeptide core.

The goal of these experimental studies was the development of a purification process for pneumocandin B_o from various other analogue impurities present in the feedstock solution. The research is composed of two parts: the first consists of a background/overview section which provides the rationale for why chromatography was pursued to isolate the product of interest and the second explores the process development aspect of the project.

3.1. Choice of normal-phase silica-gel HPLC

The first technical question is — why explore chromatography to purify pneumocandin B_o? The answer to this question lies in the complex nature of the compound. The amphiphilic nature of these compounds gives rise to unusual partitioning and solubility properties, and makes them extremely difficult to purify via conventional crystallization,

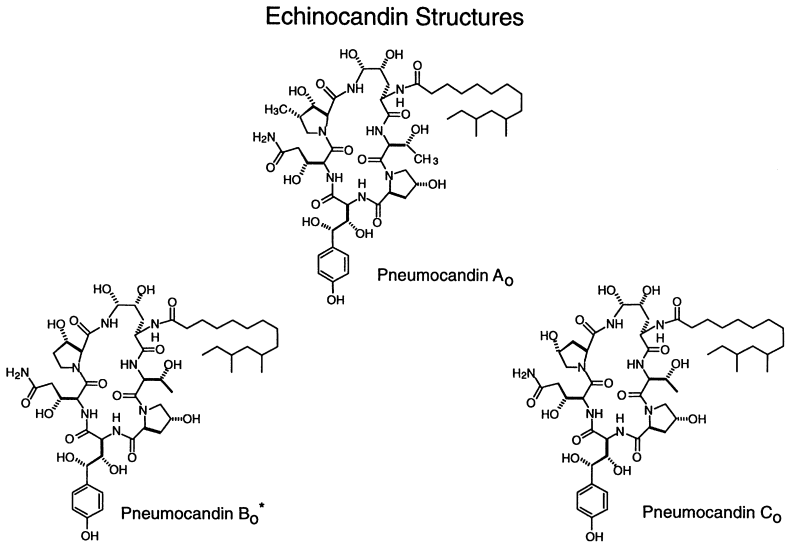

Fig. 1. Structures of three pertinent pneumocandins including the product of interest, pneumocandin B_o. Analogue impurity pneumocandin A_o represents the addition of a methyl group at the 4 position on the hydroxyproline functional group in the polar hexapeptide core. Pneumocandin C_o is a positional isomer of the product representing a shift in the hydroxyl group from the 3 position (pneumocandin B_o) to the 4 position (pneumocandin C_o) of the same hydroxyproline group. For more details on pneumocandin B_o structure and details, see [1–6]. *Reference: Schwartz et al. US Pat. 5202309 and 5194377 (1993).

making preparative chromatography an attractive alternative.

Once preparative chromatography has been identified as the purification strategy, several parameters remain to be determined including; (a) the type of chromatography (reversed-phase or RP-HPLC, normal-phase, ion-exchange), (b) the mode of chromatography (HPLC vs. low pressure liquid chromatography), (c) the type and size of sorbent (spherical or irregular) and (d) the mobile phase composition. Once these parameters have been defined, practical issues pertaining to productivity optimization can be pursued.

The type of sorbent for the isolation of pneumocandin B_o is dictated by the resolution of the many analogue impurities, including pneumocandin A_o and pneumocandin C_o. Silica-gel chromatography exploits the subtle variations in the hydroxyl rich hexapeptide core among the analogues. Silica-gel chromatography is the only method that allows for the resolution of pneumocandin C_o from the product of interest. The presence of pneumocandin C_o at significant concentrations (up to 10% relative to pneumocandin B_o) in the feed to the isolation step dictates the choice of NP-HPLC over RP-HPLC. Although RP-HPLC has been successfully used for the isolation of many other small- and intermediate-molecular-mass compounds at high yield and purity, it is clearly not the optimal choice for the isolation of pneumocandin B_o.

3.2. Choice of mobile phase composition: ternary versus binary

The key goal of mobile phase selection and optimization is to maximize the overall productivity of the step. Typical normal-phase separations are performed with bare silica, either spherical or irregular, employing a binary mobile phase composed of a weakly polar solvent combined with an alcohol (e.g. EtOAc and MeOH). However, due to the unique solubility and retentive properties of pneumocandins, a ternary mobile phase consisting of EtOAc, MeOH and water is required for the chromatography [1].

Control of product retention and resolution of impurities is obtained by varying the ratio of these three components in the mobile phase. The results from sensitivity and optimization studies revealed that product retention and resolution are strong functions of the mobile phase composition. Fig. 2

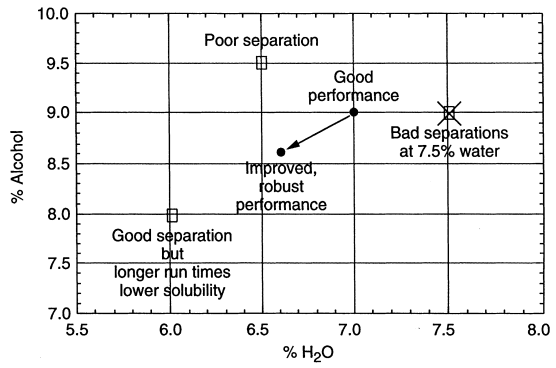

Fig. 2. Results of sensitivity analyses exploring the affect of changes in mobile phase solvent composition on pneumocandin B_o chromatography. Both alcohol and water percent compositions are based on volumes of solvent prior to mixing. The balance of solvent composition is provided by EtOAc. For example, the poor separation observed at 7.5% water and 9% alcohol corresponds to a mobile phase composition of 83.5% EtOAc, 9.0% (v/v) MeOH and 7.5% (v/v) water.

represents the effect on NP-HPLC performance with respect to changes in the alcohol and water content in the mobile phase. The base case for the study is a blend containing 84% (v/v) EtOAc, 9% (v/v) MeOH and 7% (v/v) water which allows for resolution of the two key marker analogue impurities, pneumocandin A_o and C_o at reasonable yield of greater than 80%.

A slight change in the mobile phase composition (EtOAc–MeOH–water) from the base case of 84.0:9.0:7.0 to 84.8:8.6:6.6 increases resolution of the marker impurities resulting in a slight increase in yield (~5%). Moving further along this path of reduced alcohol and water compositions provided for further improvements in resolution of impurities (e.g. 86.0:8.0:6.0).

However, two distinct disadvantages result from reducing the alcohol and water concentration; reduced product solubility and longer run times. Both of these effects result in reduced productivity for the step versus the base case.

The sensitivity of the separation is further revealed by additional sensitivity analyses that also involved varying the water and alcohol levels and examining the effect on the chromatography performance. Increasing the alcohol level while reducing the water (EtOAc constant) resulted in poor separation as evidenced by the 84:9.5:6.5 experimental point. Moving in the opposite direction (increased water and decreased EtOAc) also results in poor separations (e.g. 83.5:9.0:7.5).

One interpretation of these sensitivity analyses is that the alcohol level in the mobile phase controls the retentivity of the compound and the water level controls the resolution of the impurities. These results clearly indicate that a balance of the three solvents is critical to the success of the chromatography.

3.3. Choice of sorbent and column efficiency studies

Preliminary screening experiments indicated that silica gel of ~20-μm particle size provided ample resolution of analogue impurities when coupled with the ternary mobile phase. Economic considerations dictated that an irregular silica be employed in the process. Experiments were subsequently performed with an analytical scale column packed with the same nominal 20-μm material as used for preparative experiments, to determine column efficiency as a function of linear velocity, tracer loading level and mobile phase composition.

The results of the column efficiency measurements are presented in Fig. 3 as a plot of reduced plate height (h) versus reduced velocity (ν), Van Deemter curves, including the typical LS-HPLC operating range (see Appendix A). In addition to examining the effect of reduced velocity on column efficiency, the effect of water content was also examined. Note that the base case mobile phase consists of a volumetric blend of EtOAc–MeOH–water of 84:9:7. The effect of water was determined by preserving the ratio of EtOAc to MeOH at 84:9 and independently varying the water level from anhydrous (84:9) to 84:9:7. A reduced plate height of 2.0 to 6.0 corresponds to 27 600 to 15 400 plates/m.

The data for the entire range of water concentrations indicate that although relatively inexpensive irregular silica is employed as the sorbent, the column separation efficiency is excellent over the range of linear velocities. The analytical column is well packed as indicated by the reduced plate height of two achieved at the lowest reduced velocity measured, independent of water concentration in the mobile phase. The effect of water on separation

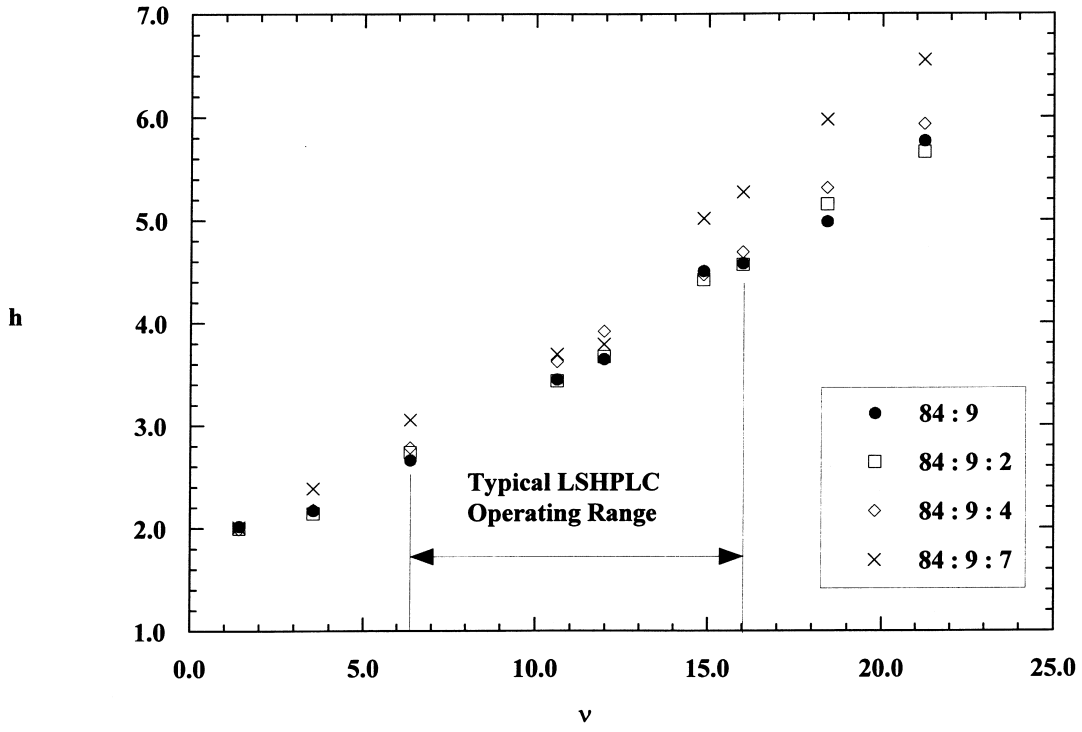

Fig. 3. Reduced plate height (h) versus reduced velocity (ν) over a range of mobile phase compositions from anhydrous, (84:9) (EtoAc–MeOH) to the base case composition of 84:9:7 (EtoAc–MeOH–water). All column efficiency measurements were performed using an analytical scale column of nominal dimensions 250×4.6 mm. Reduced velocity range typically employed in operating the large scale HPLC (LS-HPLC) for this process is also illustrated on the plot.

efficiency appears to be almost insignificant over the typical LS-HPLC operating range corresponding to reduced velocities in the range of 6 to 16 (equivalent to flow-rates of 1000 to 2400 ml/min in the 15-cm I.D. column). However, the effect of water becomes more pronounced at higher velocities (e.g. reduced velocity of 22). Note that these column efficiency determinations were based on tracer retention studies of a relatively small compound, MEK. However, the influence of water on the column efficiency for a larger compound such as pneumocandin B_o may be more significant and is currently under investigation.

4. Case study of process optimization

Once the key parameters, i.e. sorbent and mobile phase composition were defined (Section 3.3) optimization and scaleup experiments commenced. Practical process optimization was performed by performing the parametric examination of: (a) the effect of product loading and separation efficiency on yield, (b) productivity as a function of eluent flow-rate and (c) scaleup from 6 to 15 cm.

4.1. Effect of product loading on separation efficiency and yield

Initial experiments to examine the effect of product loading and separation efficiency on yield were performed on a 6-cm semipreparative scale. These initial experiments were carried out at a fixed mobile phase superficial velocity of 9.3 cm/min (0.16 cm/s) using a 750-ml bed volume. The goal of these initial experiments was to determine the column loading conditions and fraction recovery strategy prior to scaleup to the 15-cm preparative system.

The column efficiency for the 6-cm scale was determined to be 10 000 plates/m or 2500 plates/column (25-cm length) via tracer solution injection at

0.5 column volume percent loading. For ease of experimentation, the linear velocity was fixed at 9.3 cm/min which corresponds to a reduced velocity of 4.9 and the mobile phase composition was held constant at the base case of 84:9:7. The typical feedstock analogue profile, target concentration in the rich cut and purification factor required for the preparative HPLC step are presented in Table 1. Although more than ten other analogue impurities are present in the feed and removed during the preparative HPLC separation for reasons of brevity only three marker analogue impurities are presented in Table 1, pneumocandins A_o, C_o and D_o. Pneumocandin D_o is analogous to pneumocandin B_o with an additional hydroxyl group located at the 4 position of the hydroxyproline functional group in place of the hydrogen present in pneumocandin B_o. Although pneumocandin D_o is present in significant concentration in the feed to the preparative HPLC step, it is readily resolved to the target level of 0.1% in the product rich cut fraction.

Clearly from the information presented in Table 1, the targets for this purification step are typical for a preparative HPLC process. To ensure the success of the scaleup, initial experiments involved collection of detailed fractions that were analyzed by analytical HPLC to determine the analogue impurity profiles. The analogue profiles in the preparative HPLC chromatograms were then reconstructed based on these analytical results. The detailed fraction information allowed for an accurate determination of the yield associated with the desired purification factors. The results of product loading experiments, at 1.2 and 3.0 g product/l bed, are presented in Fig. 4a and b, respectively. Note that the injection volume was fixed at 10 column volume percent (75 ml) and the concentration of product in the feed was increased from 11.7 to 29.9 g/l to affect the higher column loading.

The results of the experiment (Fig. 4a) at 1.2 g/l bed loading indicate that pneumocandin A_o is essentially completely resolved from the product peak with the maximum concentration of the analogue impurity at less than 100 mg/l in the front shoulder of the product peak. Resolution of pneumocandin C_o from the product is also excellent with concentrations of this analogue in the tail of the product peak comparable to pneumocandin A_o in the front of the peak. Increasing the product loading to 3.0 g/l bed (Fig. 4b) results in the melding of pneumocandin A_o peak into a front shoulder of the product peak. Resolution of the other marker analogue, pneumocandin C_o, is also significantly diminished with concentrations as high as 250 mg/l in the final fractions of the main peak. Note that the analogue impurity concentration in the tail corresponds to ~10% of the maximum concentration of the product in the main peak.

Fractions were selected to achieve a rich cut pool meeting target purity specifications shown in Table 1. The fraction collection range and associated yield for the two loading experiments is presented in Fig. 5. The data clearly indicate that increasing the loading of the product on the column to 3.0 g/l results in only a modest decrease in yield (~4%) and a more concentrated product rich cut. Judicious fraction collection allows for this high yield of 93% while still maintaining the target purification factors of 2 and 7 for pneumocandin A_o and C_o, respectively. However, shifting the collection point for the fractions by only a slight degree results in a significant increase in the concentration of either of these

Table 1
Purification targets for analogue impurities

Compound	Feed concentration (%)	Target concentration (%)	Purification factor required
Pneumocandin A_o	1.5	<1	<2
Pneumocandin C_o	12.4	2	7
Pneumocandin D_o	7.5	0.1	75

Analogue impurity profile/target concentration in the rich cut and purification factor required for a typical feed to the HPLC step. Note that pneumocandin D_o represents an analogue of pneumocandin B_o with an additional hydroxyl group at the 4 position on the hydroxyproline functional group of the molecule. Although pneumocandin D_o is present at a relatively large concentrations in the feed it elutes significantly after the product and does not appear in any of the assayed fractions from the preparative HPLC experiments.

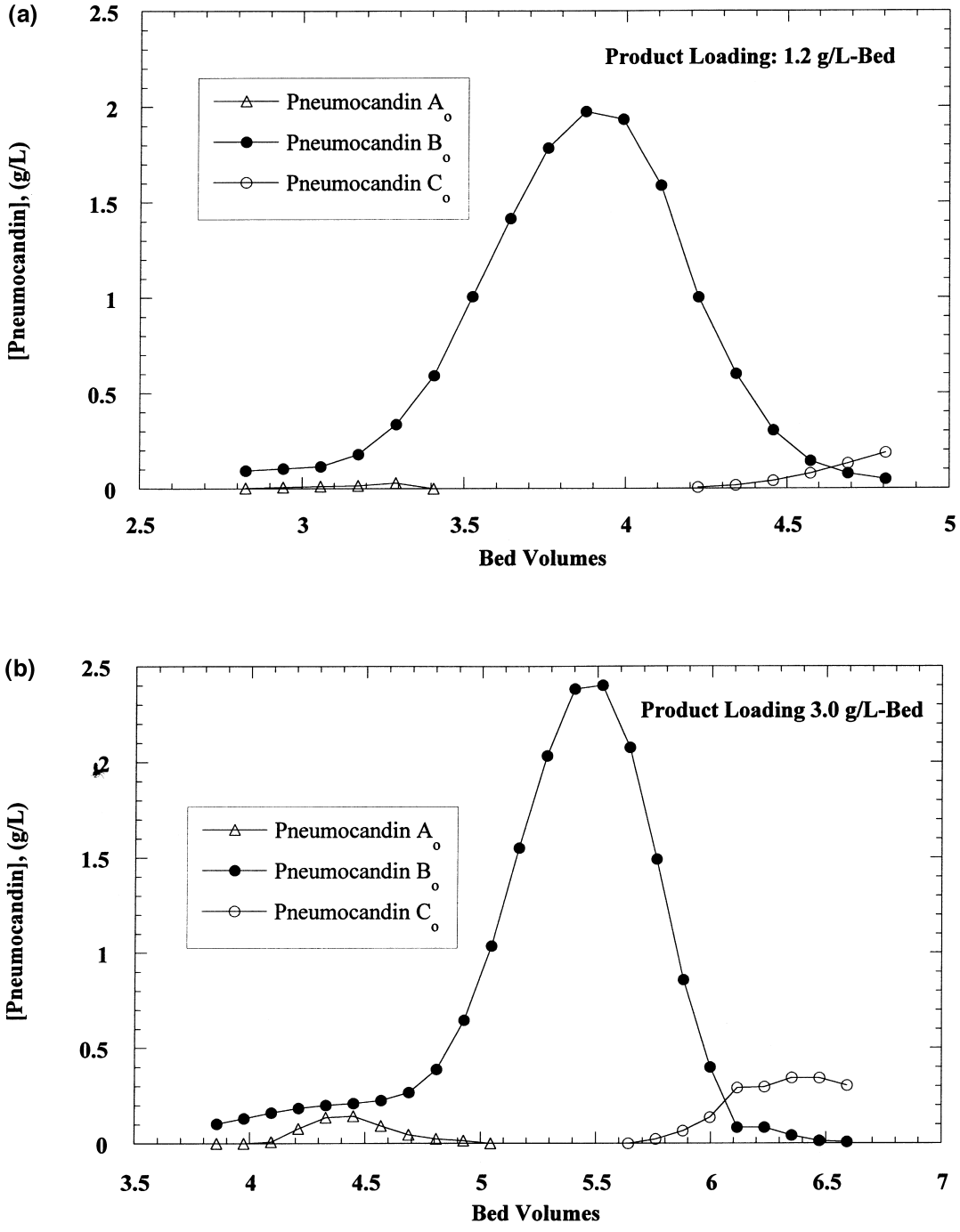

Fig. 4. Reconstructed semipreparative (6-cm system) chromatograms at column loadings of (a) 1.2 and (b) 3.0 g product/l bed. Injection volume of feed solution was constant at 75 ml or 10% bed volume for all experiments. Column loading (g product/l bed) was controlled by varying product concentration in the feed solution. Chromatograms reconstructed based on analytical NP-HPLC assays of detailed fractions. Note: Initial column loading experiments were performed prior to complete mobile phase sensitivity analyses. The change in retention time of the product peak from 3.9 column volumes (1.2 g product/l bed) to 5.4 column volumes (3.0 g product/l bed) is attributable to a slight change in the mobile phase composition and is not a function of increased product loading on the column.

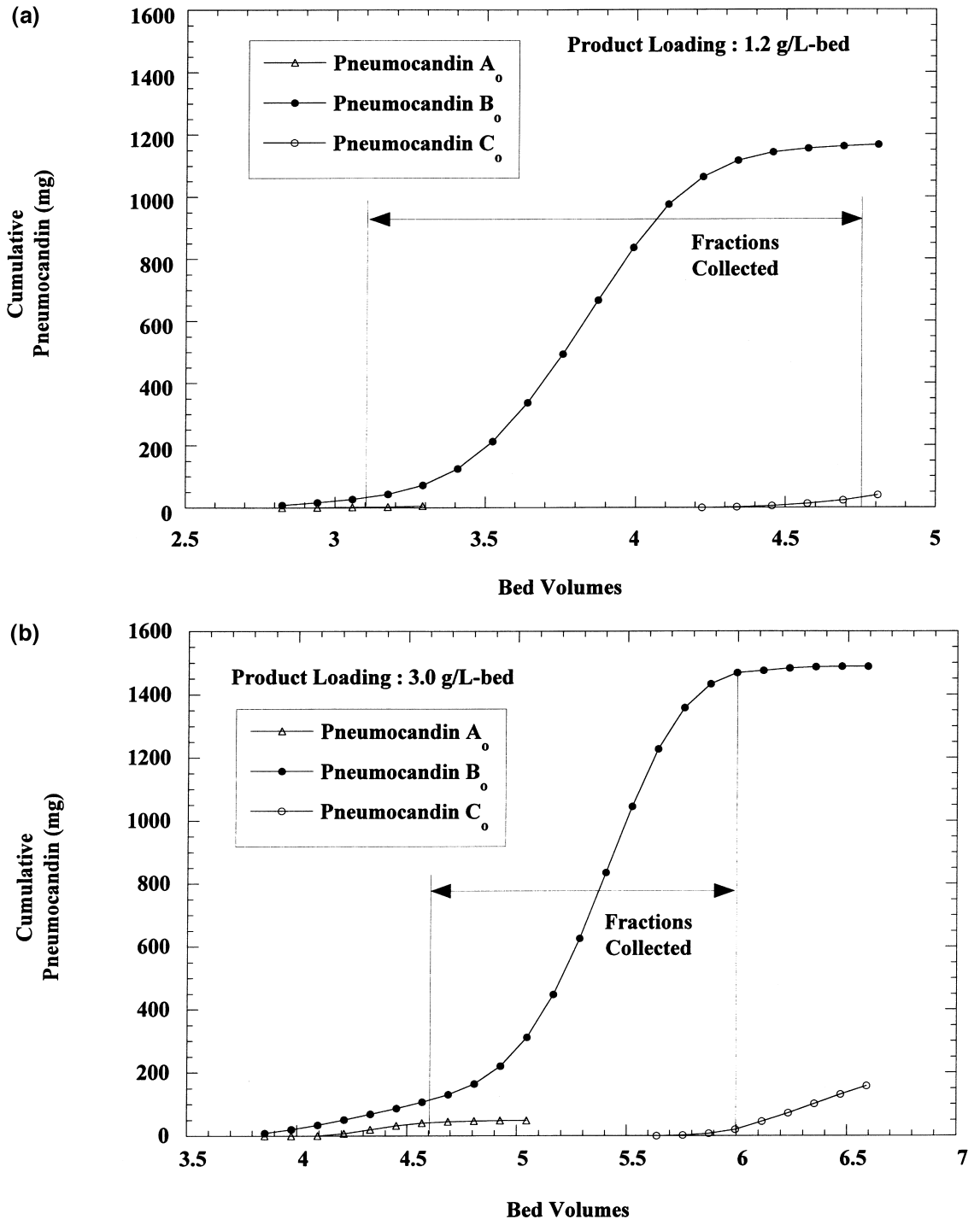

Fig. 5. Analogue impurity profiles from semipreparative experiments indicating fraction selection and yield as a function of column loading. Adjusted yields of 97% and 93% were obtained at the two column loadings at column loadings of (a) 1.2 and (b) 3.0 g product/l bed, respectively.

two analogue impurities. Although additional optimization could increase the robustness of the process, owing to project time constraints the promising results of the 6-cm semipreparative were employed as a template for scaling to the 15-cm preparative system.

4.2. Scaleup from 6-cm (semipreparative) to 15-cm (preparative)

A linear scaleup was performed from the parameters employed for the 6-cm scale experiment to the 15-cm preparative HPLC system. The product loading, linear velocity and column length were all held constant for the scaleup. Fractions were collected for the initial 15-cm scale experiment in the same manner as the 6-cm scale experiments and assayed by analytical scale NP-HPLC. A comparison of the reconstructed chromatograms from the two systems is presented in Fig. 6. Resolution of the two marker analogues, pneumocandin A_o and C_o, are equivalent in the two systems. Only slight differences exist between the two scales including a subtle difference in peak maxima for the main product peak.

Following successful scaleup of the initial HPLC process to the 15-cm scale, additional optimization/sensitivity experiments were performed to ensure process robustness. Recall that the experimental results from the 6-cm scale (25-cm column length) indicated that the analogue impurity profile in the product rich cut was quite sensitive to the fraction selection strategy. A slight shift in the position of the fraction selection on either side of the product peak would result in unacceptably high levels of analogue impurity in the product cut. This observation implies that the 25-cm length column provided only the minimum number of theoretical plates to affect the resolution of the analogues. Therefore, in the final scaleup experiments the column length was increased ~3-fold to afford better analogue resolution, reduce the sensitivity of the product cut to fraction selection, and increase the yield for the step. In addition, the feed volume and linear velocity were increased to help offset the decrease in productivity associated with the increased column length. The final scaleup conditions are presented in Table 2.

4.3. Tradeoff between yield and productivity

One practical issue associated with the development of a manufacturing scale process, including preparative HPLC, is productivity. Often the focus of laboratory development activities is the step yield with only modest regard given to the influence of cycle time or productivity. Although some comment has been made in the literature on the importance of optimizing the combination of productivity and yield [16], this case study underscores the importance of these practical considerations.

The results of experiments to determine the potential effect of eluent flow-rate on yield and productivity are presented in Table 3. Tracer experiments were performed on the 15-cm column (76-cm length) using injections of 2.0 column volume percent. Note that a separate set of experiments indicated a linear relationship between the column volume loading of tracer solution to plate count (see Appendix B for details). For consistency with other data presented herein, the plate count data obtained for the 15-cm column at tracer injection amounts of 2.0 column volume percent have been normalized to a standard tracer injection of 0.5 column volume percent for presentation in Table 3.

For comparison, the productivity which would be achieved at the very low flow-rate of 500 ml/min (ν of 3.3) was calculated assuming a yield of 100%. Even if such a high yield could be achieved by operating at this low flow-rate, overall productivity would be quite low at 7.7 g product/h. The standard operating case for the experiment was the lowest eluent flow-rate of 1000 ml/min at which 10 375 plates were present in the column and a reasonable yield of 85% was obtained with a productivity of 13.1 g product/h.

One way to increase productivity is to increase flow-rate, if this can be done without changing yield. Increasing the flow-rate clearly increases productivity for a fixed yield via reducing cycle time; for a fixed yield via reducing cycle time. Indeed for this process increasing the eluent flow-rate from 1000 to 2100 ml/min results in a only modest decrease in the column efficiency (32%) and product yield (3%) but a strong decrease in cycle time (56%); the large reduction in cycle time offsets the loss of product to

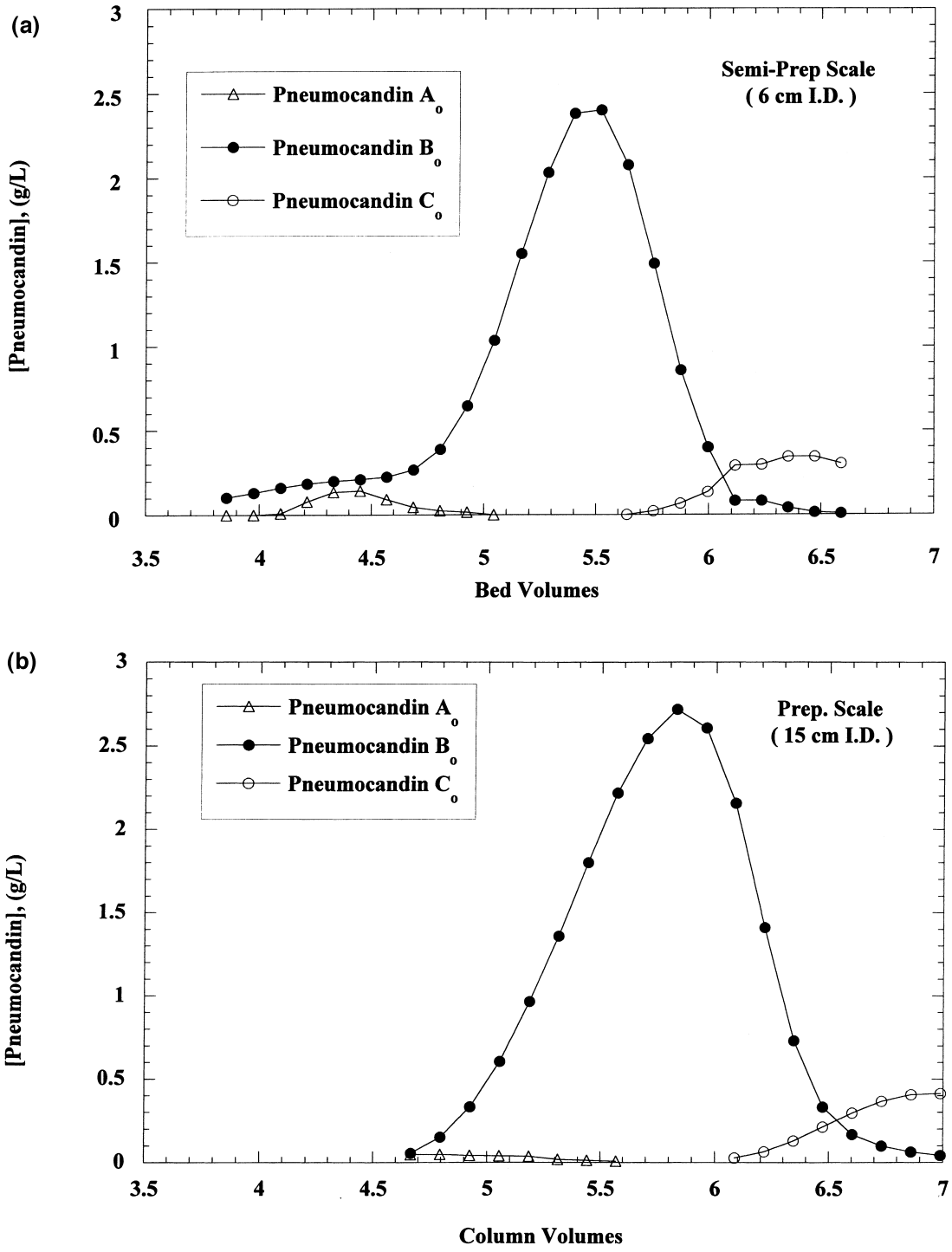

Fig. 6. Comparison of reconstructed chromatograms indicating the excellent agreement obtained on the 6-cm (semipreparative, 750-ml column volume) and 15-cm I.D. (preparative, 6.2-l column volume) scale chromatographies at a fixed column loading of 3.0 g product/l bed.

Table 2
Scaleup of the preparative normal-phase HPLC — final scaleup parameters

Parameter	Semipreparative (6-cm I.D.)	Preparative (15-cm I.D.)
Column length (cm)	25	76
Column volume (*L*)	0.7	13.4
Linear velocity (cm/min)	9.3	10.2
Feed volume (% column volume)	10	13
Feed concentration (g product/l)	~30	~30
Column loading (g product/l bed)	3.0	3.0
Product load (g)	2.12	40.2

Summary listing of the final scaleup parameters following completion of optimisation studies. Key parameter changes include increased column length (76 vs. 25 cm) and increased linear velocity (10.2 vs. 9.3 cm/min).

the reduced yield ultimately resulting in improved productivity. Productivity for the 15-cm LS-HPLC reaches a maximum at an eluent flow-rate of 2100 ml/min at 28.7 g product/h.

The breakpoint is actually reached at 2400 ml/min eluent flow-rate where a precipitous drop is observed in column efficiency as well as yield; product yield falls by 15% while cycle time decreases by 15%. These results indicate a classic tradeoff between productivity and yield as presented in Fig. 7. The optimal productivity for the process occurs at the intermediate eluent flow-rate of 2100 ml/min. Under these conditions, the only means of increasing productivity for this process is to increase the scale of the equipment.

The issue as to why such a marked breakpoint was observed in the column efficiency and productivity at 2400 ml/min flow-rate. The effect is also observed if one compares the column efficiency measurements performed on the preparative scale to those on the analytical scale (as shown in Fig. 8).

At the highest reduced velocity measured for the 15-cm column, ν equal to 16, a significant difference exists between the analytical and preparative data. The main difference between the analytical and preparative scale systems is the column length, 25 cm for the analytical scale and 76 cm for the preparative scale. This difference in column length results in significant differences in the operating pressures for the two systems at elevated eluent flow-rates. The 15-cm I.D. system operating at a reduced velocity of 16, corresponding to an eluent flow-rate of 2400 ml/min, has an associated pressure drop of ~4.5 MPa across the column. This pressure drop is essentially equivalent to the packing pressure of the column and at the limit of the sustainable pressure for the irregular silica. In an axial compression system, when elution pressure exceeds the

Table 3
LS-HPLC productivity evaluation

Eluant flow-rate (ml/min)	N[a] (plates per column)	Product cycle time (min)	Yield (%)	Productivity (g product/h)
500	N/A	312	100[b]	7.7[b]
1000	10 375	156	85	13.1
1800	8440	87	80	22.0
2100	7040	69	82	28.7
2400	4870	60	67	26.8

Experimental parameters included the use of a 15-cm I.D. preparative scale column packed with a 13.5 l bed with a product loading of 3.0 g/l bed volume. The effect of eluent flow-rate (linear velocity) on column efficiency (as demonstrated by tracer tests), yield and productivity are presented. Data presented for the column efficiency measurements are normalised to a tracer loading level of 0.5 column volume percent.
[a] Theoretical plates determined via tracer solution of MEK and toluene; normalised to injection volume.
[b] Assume maximum yield of 100% for 500 ml/min eluent flow-rate.

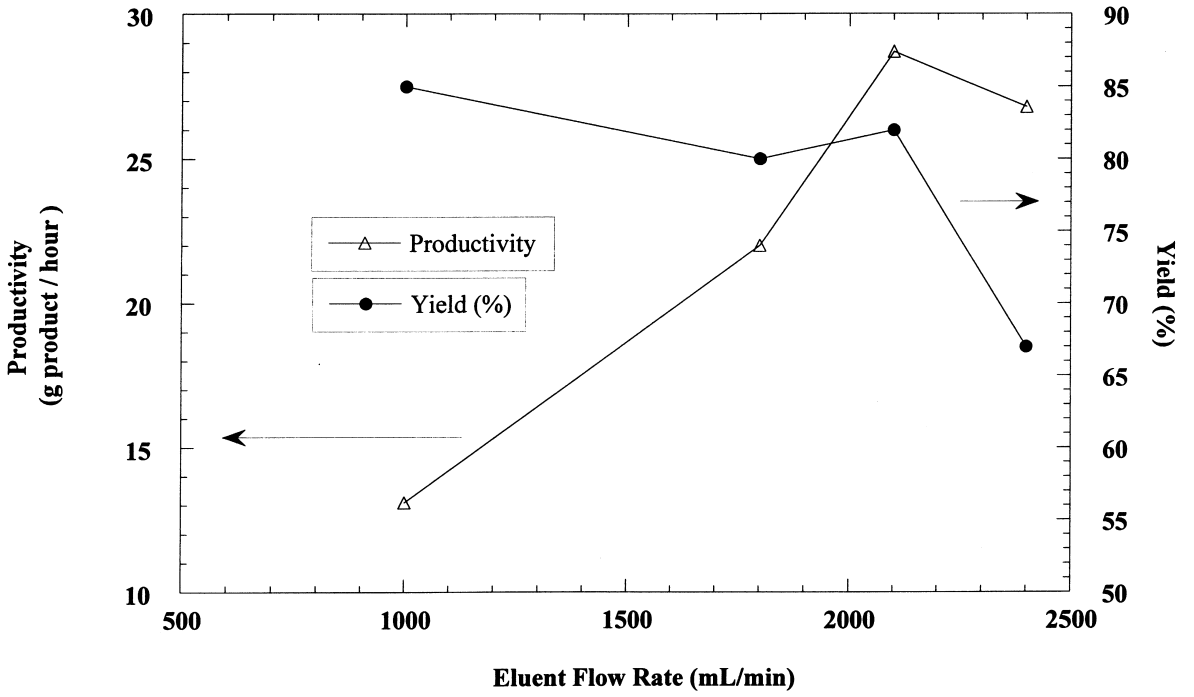

Fig. 7. Tradeoff between productivity and yield as a function of eluent flow-rate at the preparative scale (15-cm I.D. LS-HPLC). Note that for this preparative separation the optimum flow-rate for operation is 2100 ml/min.

packing pressure, the column piston can ''float'', causing loss of performance. Thus, the practical limit to the productivity of the system is the fragile nature of the irregular silica. This limitation is not revealed at the analytical scale due to the shorter column length.

Additional data are presented in Fig. 8 from a 30-cm I.D. system packed with the same media, column length and mobile phase composition as the 15-cm column. The only difference between the two preparative systems, 15- and 30-cm I.D. is the scale. As shown in the figure, data points for the 30-cm column lie on or about the same line as for the 15-cm column and analytical scale data, indicating that performance is independent of scale for dynamic axially compressed columns.

5. Conclusions

This research provides a case study of the challenges associated with preparative HPLC development for an intermediate-molecular-mass amphiphilic compound. Resolution of a key isomeric analogue, pneumocandin C_o from the product of interest, pneumocandin B_o, required the use of normal-phase HPLC to exploit the subtle change in position of a hydroxl group. Initial efforts helped to define the necessity of the use of a ternary mobile phase in order to afford product solubility and selectivity. Subsequent optimization/sensitivity studies clearly demonstrated that a balance in the composition of the ternary mobile phase was required in order to control the product solubility, retention and resolution of key analogues. Additional experimental results confirmed that these three variables were a strong function of the composition of the mobile phase.

Practical issues relating to scaleup and optimization provided the framework for the remainder of the case study. For this experimental system, yield is a strong function of column loading and linear velocity. In addition, productivity is dictated by loading and linear velocity. Therefore, this ex-

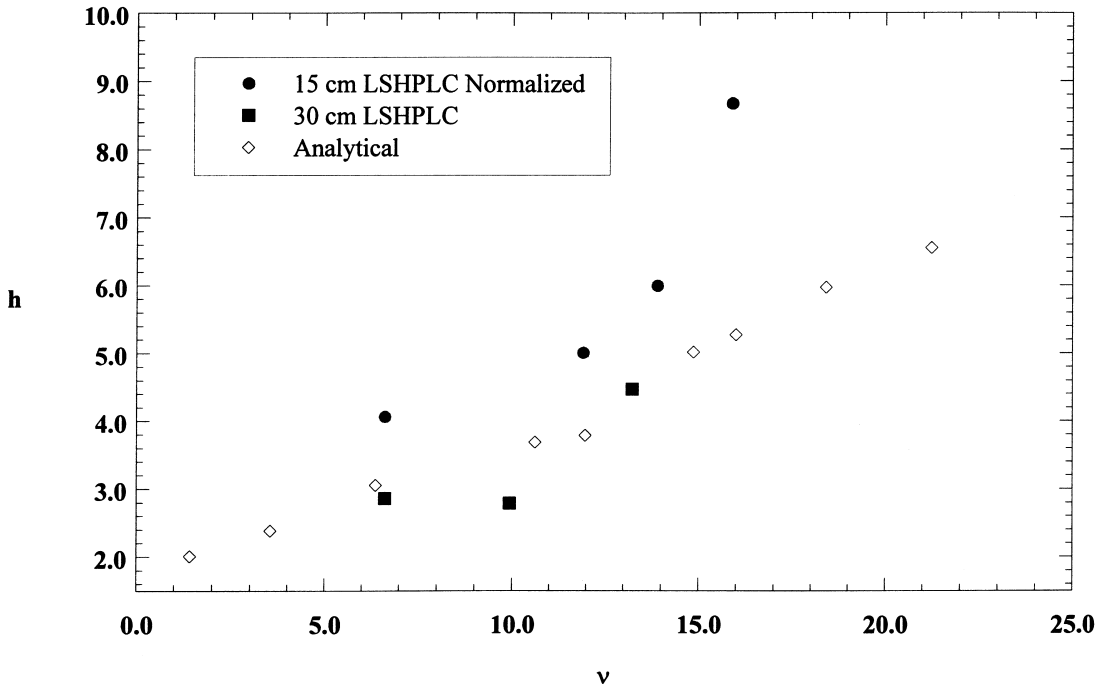

Fig. 8. Effect of reduced velocity (ν) on column efficiency as measured by reduced plate height (h) for the 15- and 30-cm preparative HPLC systems as compared with the analytical HPLC system. Note that the column length for the preparative systems (76 cm) is ~3-fold longer than the analytical system (25 cm) resulting in a higher overall pressure drop. The jump discontinuity at a ν value of 16 for the 15-cm system occurs when the operating system pressure drop equals the packing pressure for the adsorbent. Column efficiency determined via toluene–MEK tracer solution.

perimental system represents a classic tradeoff between yield and productivity. For a high value product, this tradeoff becomes significant. Despite all of the challenges, the process was successfully scaled from the analytical scale (4.6-cm I.D.) to the semipreparative (6-cm I.D.) to the preparative scale (15- and 30-cm I.D.)

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Appendix A

Calculation of reduced plate height and reduced velocity

Determination of the reduced column efficiency parameters for the silica-gel chromatography requires values which cannot be readily experimentally determined, including the diffusivity of the tracer solvents in the mobile phase and the column void fraction. Under the circumstances where a parameter required for a given calculation was not available, empirical correlations obtained from the literature were employed.

The first example is the estimation of the diffusivity of the tracer solutions, methylethyl ketone (MEK) and toluene in the ternary mobile phase consisting of

ethyl acetate, methanol and water. An estimate of diffusion coefficient (cm^2/s) for small concentrations of compound A in a solution of B can be derived from the Wilke–Chang equation [26–28] as described in Eq. (1).

$$D_{AB} = \frac{7.4 \cdot 10^{-8}(\psi_B M_B)^{0.5} T}{\mu V_A^{0.6}} \quad (1)$$

in which ψ_B is an association parameter for the solvent B, M_B is the molar mass for solvent B, T is the absolute temperature in degrees Kelvin, μ is the viscosity of the solution in centipoise, V_A is the molar volume of the solute A in $cm^3\ g\ mol^{-1}$ as liquid at its normal boiling point. Data for molar volumes of toluene and MEK, and viscosities of ethyl acetate, methanol and water were obtained from the literature [29]. The experimental conditions employed a temperature of 25°C or 298 K. The molecular mass employed for the ternary mobile phase, M_B, is the average of the three solvents in the mobile phase normalized to the volume contributions of the solvents as described in Eq. (2).

$$M_B = M_1 \underline{v}_1 + M_2 \underline{v}_2 + M_3 \underline{v}_3 \quad (2)$$

where M_1, M_2, M_3 and $\underline{v}_1$, $\underline{v}_2$, $\underline{v}_3$ are the molecular masses and volume fractions of ethyl acetate, methanol and water, respectively. The volume fractions of EtOAc, MeOH and water employed were the base case of 0.84, 0.09 and 0.07, respectively. The viscosity of the ternary mobile phase was determined in a weighted linear combination of the volume fractions of the three solvents employing literature values [29]. A value for ψ_B, the association parameter, was determined as a weighted linear combination of the volume fractions of the three solvents employing values [26] of 1.5 for EtOAc, 1.9 for MeOH and 2.6 for water. Application of these parameters to Eq. (1) yields diffusion coefficients of $3.17 \cdot 10^{-5}$ and $3.40 \cdot 10^{-5}$ cm^2/s for toluene and MEK, respectively, in the ternary mobile phase solution.

Once the values of the diffusion coefficients for toluene and MEK were obtained, the reduced velocity, ν, could be calculated from the experimental data according to Eq. (3).

$$\nu = u d_p / D_{AB} \quad (3)$$

where u is the superficial velocity (cm/s) of the mobile phase, d_p is the average particle size, and D_{AB} is the diffusion coefficient for MEK or toluene. Based on data provided by the manufacturer, a d_p value of 18 μm or $1.8 \cdot 10^{-3}$ cm was employed for the nominal 20-μm material. A void fraction of 0.75 was employed for the silica media. Note that the flow-rate corresponding to the LS-HPLC reduced velocities indicated in Fig. 3 are 1000 to 2400 ml/min in the 15-cm I.D. system.

The number of plates for both analytical and preparative columns was calculated based on the results of tracer injections according to a standard half-height measurement per Eq. (4).

$$N = 5.54(t_R / w_{1/2})^2 \quad (4)$$

where t_R is the retention time of the tracer species (s) and $w_{1/2}$ is the width of the tracer peak at half height. The plate height, H, was then calculated by dividing the column length, L, by the number of plates, N, determined according to Eq. (4). The reduced plate height, h, was subsequently determined according to Eq. (5).

$$h = H / d_p \quad (5)$$

Appendix B

Effect of volume loading on column plate count

Experiments were performed on an analytical scale column (250×4.6 mm) at 25°C and mobile phase composition of 84:9:7 to determine the potential affect of varying the loading of tracer solution (Section 2.1) on the measured column efficiency. Experiments examined a range of tracer column volume loading from 0.5 to 2.0 column volume percent, which translates to injection amounts ranging from 10 to 80 μl. The potential affect of tracer loading was also evaluated over the same range of reduced velocity, 1.4 to 21.2, as examined in the previous experiments.

The results of the tracer loading experiments and the least squares linear regression analyses of the data are presented in Fig. 9. The analyses of the

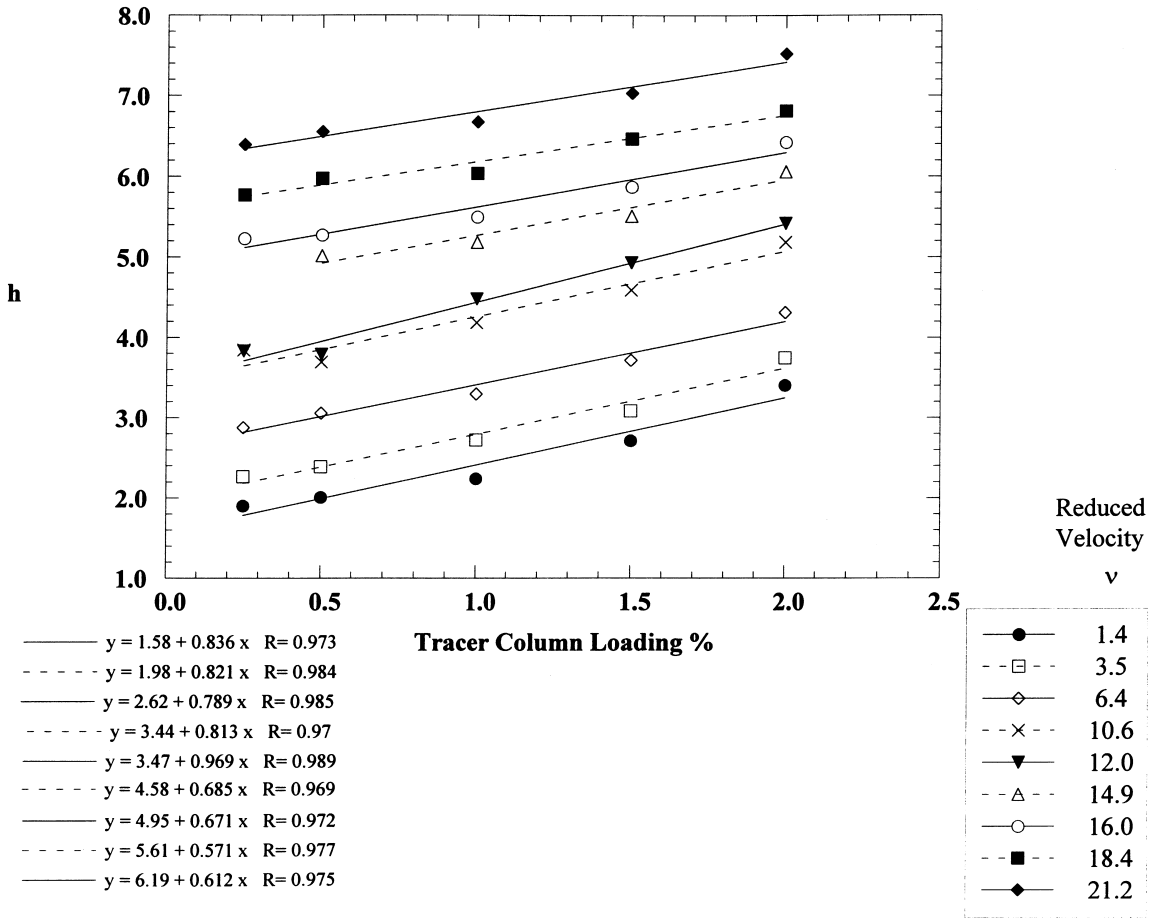

Fig. 9. Tracer loading experiments indicating reduced plate height as a function tracer column loading percent and reduced velocity. Least squares linear regression analyses are also indicated. See Appendix B for details.

experimental results clearly indicate a linear relationship between reduced plate height and tracer loading level at each reduced velocity examined. These results translate into an inverse linear relationship between plate number and tracer loading levels. Note that due to system limitations, the tracer experiments were performed on the 15-cm system at loading levels of 2.0 column volume percent. The linear relationship (for each reduced velocity) between reduced plate height and tracer loading was then employed to calculate the normalized reduced plate height for the 15-cm system equivalent to a 0.5 column volume percent loading.

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