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Gradient elution chromatography is frequently used for the preparative separation of peptides and proteins. Separations at high loadings are often avoided because peaks become asymmetrical and saturate the detector. However, non-linear interactions which become important at high loadings may actually improve the separation with greater concentrations of the product being extracted. In this study, the separation of a mixture of two chemotactic peptides N-formyl-met-phe (X~phe) and N-formyl-met-trp (X~trp) was considered using reversed-phase (RP) chromatography. These runs were limited by the solubility of the peptides. The effects of solubility and feed conditions on the chromatograms were examined. Because of the poor solubility of the peptides in low organic solvent concentration, runs where feed conditions are different from the column inlet conditions were examined. Since these two components exhibit low selectivity on the RP column, separation is difficult. Productivities were obtained and compared for the preparative purification of these compounds using isocratic, stepwise and gradient

elution with acetonitrile/buffer and methanol/water mobile phase conditions. Selectivity reversal was present in methanol/water conditions. Selectivity reversal implies that the order of retention of the feed compound changes as the mobile phase composition varies. Curved gradient shapes were used in an attempt to improve the separation under selectivity reversal limitation. Using a combination of solubility methods and non-linear interactions at high loadings, simultaneous concentration and purification was achieved.

Preparative Purification of Chemotactic Peptides by Gradient Elution in Reversed-Phase Chromatography

by

Billy Kim

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Billy Kim, Author

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SYMBOLS

A	axial dispersion process term of the Van Deemter equation, [s^{-1}]
a_P, a_T	isotherm parameters of the X~phe and X~trp adsorbates corresponding to the initial slope of the Langmuir competitive isotherm when c_P, c_T=0
B	eddy diffusion term of the Van Deemter equation, [m]
b_P, b_T	parameters of the X~phe and X~trp of the Langmuir competitive isotherm
C_i	coefficients of pore diffusion (internal mass transfer) of the Van Deemter equation
C_f	coefficients of film mass transfer (external mass transfer) of the Van Deemter equation
C_k	coefficients of binding/sorption kinetics of the Van Deemter equation
C_M	modulator concentration, [%]
c_P, c_T	concentration of X~phe and X~trp adsorbates, [mg/ml]
C_{lumped}	coefficient of the overall mass transfer, by representing band broadening effects into a single lumped value
D	column diameter [cm]
d_p	average particle diameter, [μm]
H	height equivalent to theoretical plate, [m]
H_{lumped}	plate count obtained by lumping band broadening processes into one mass transfer process, [m]
k'	capacity factor
K	partition coefficient, Henry's law constant, equilibrium distribution coefficient, $q=Kc$
k_{MT}	mass transfer coefficient, [m/s]
L	column length, [cm]
m_1, m_2	masses measured on the column experimentally for two different concentrations of modulator, [mg]
$m_{98\%}$	mass of peptides collected at 98% purity, [mg]
$m_{95\%}$	mass of peptides collected at 95% purity, [mg]
P	productivity, [mg/ml·hr]
q	stationary phase concentration, [mg/ml]
q_j	SP concentration of the j^{th} adsorbate at equilibrium, [mg/ml]
q_P, q_T	SP concentration of the X~phe and X~trp adsorbates, [mg/ml]
s	is the slope of the modulator k' as a function of the modulator concentration
t_c	adsorbate elution time at concentration c, [min]
t_{cyc}	cycle time or time that it takes to purify the sample and regenerate the column for the next purification cycle, [min]
t_{delay}	time delay of gradient, [min]
t_P	retention time of acetone pulse, [min]

SYMBOLS (continued)

t_{pur}	time of preparative purification run, [min]
t_{reg}	time of regeneration of column, [min]
u_0	void velocity, [m/s]
u_c	adsorbate velocity at concentration c, [m/s]
V_c	adsorbate elution volume at concentration c, [ml]
V_{ec}	empty column volume ($=\pi(d_p/2)^2 \cdot L$), [ml]
V_{inj}	feed or injection volume, [ml]
V_R	total volume of mobile phase required to elute an analyte through the column, [ml]
V_{MP}	volume in the mobile phase, [ml]
V_{SP}	volume in the stationary phase, [ml]
V_0	void volume, [ml]
w_c	mass of column, [mg]

Greek letters

α	separation factor ($\alpha = k'_T/k'_P$)
$\delta c_i/\delta t$	accumulation of component i in the mobile phase, [mg/ml·min]
ε_t	void fraction of column. Can be calculated from the phase ratio ($\phi = (1-\varepsilon_t)/\varepsilon_t$)
ϕ	phase ratio (ratio of MP volume to SP volume)
$\phi\, \delta q_i/\delta t$	accumulation in the stationary phase, [mg/ml·min]
ρ_1 , ρ_2	density of solvent mixtures at two different modulator concentrations.
Λ	saturation capacity, [mg/ml]
ν	adsorbate velocity, [m/s]

ABBREVIATIONS

ACN	acetonitrile
HPLC	high performance liquid chromatography
HIC	hydrophobic interaction
IEX	ion exchange
MeOH	methanol
MP	mobile phase
RP-HPLC	reversed phase high performance liquid chromatography
SCI	single component isotherm
SEC	size exclusion
SP	stationary phase
TFA	trifluoroacetic acid
X~ala	N-formyl-methionyl-alanine
X~phe	N-formyl-methionyl-phenylalanine
X~trp	N-formyl-methionyl-tryptophan
X~val	N-formyl-methionyl-valine

PREPARATIVE PURIFICATION OF CHEMOTACTIC PEPTIDES BY GRADIENT ELUTION IN REVERSED-PHASE CHROMATOGRAPHY

1 INTRODUCTION

Separation processes leading to highly pure substances are essential to the pharmaceutical, biotechnology and food industries. The starting material is usually the effluent from fermentation, consisting of a desired product or products along with many impurities. The bioseparation steps needed to isolate the desired products are referred to as downstream processing. These steps involve purification by various methods from filtration, centrifugation and extraction, to electrophoresis and chromatography. New separation technologies are constantly appearing (Gupta and Mattiasson 1994), but due to its high separation efficiency, high performance liquid chromatography (HPLC) remains the method of choice for the separations of peptides and proteins requiring a high degree of purity. Therapeutics, for instance, require 99.997% purity (Wheelwright 1991) and proteins used in research and development require purity between 95-99.9% (Jungbauer 1993).

Such purities are routinely achieved by liquid chromatography. In general terms, chromatographic separations depend on the differential migration of sample components, or "adsorbates," flowing through a column made of a stationary phase (SP) and mobile phase (MP). The SP is a particulate "adsorbent" and the MP or "eluent" is a fluid that moves or "elutes" through the column. As the sample components are eluted at different rates through the column, they become separated and emerge at different times.

Ion exchange (IEX), reversed phase (RP), hydrophobic interaction (HIC), normal phase, affinity/biospecific interactions, and size exclusion (SEC) are all different modes of interactions available for chromatographic separations. IEX separations are based on the binding of electrically charged adsorbates onto an ion-exchange SP. RP and HIC rely on the hydrophobicities of the eluting adsorbates to the hydrophobic stationary phase, whereby the less polar adsorbates will bind more strongly to the SP, and the more polar adsorbates will prefer the MP. RP and HIC differ in their degree of hydrophobicity, with the RP stationary phase having a greater hydrophobicity. Normal phase chromatography uses a polar stationary phase and a non-polar eluent. SEC is based on size only and is less effective at separating very similar complex biomolecules than any of the other modes. Affinity chromatography depends on using specific interactions between the desired products and the SP.

1.1 Chromatographic theory

Although the theory of chromatography applies to many other types of chromatography (gas chromatography, liquid-liquid chromatography, etc.), the following discussion will deal in terms of solid-liquid chromatography. Chromatography is modeled according to two fundamental assumptions, representing the thermodynamic effects of the components binding to the SP, and the kinetic effects that are due to the diffusion and mass transfer of the components through the column.

The thermodynamic effects can be linear or non-linear. In a linear system, adsorbates do not interact with each other because their concentrations are so low that they bind according to the linear portion of their adsorption isotherm. An adsorption isotherm represents the concentration distribution of the component molecules between the solid and liquid phases. Non-linearity occurs when adsorbate concentrations are sufficiently high that competition for binding sites occur, giving rise to a non-linear adsorption isotherm. If kinetic effects, such as peak bandspreading, are not present, the chromatographic system is said to be ideal, otherwise it is non-ideal. A chromatographic system can then be conveniently classified into four types of models: linear and ideal, linear and non-ideal, non-linear and ideal, and non-linear and non-ideal (Table 1.1).

	Ideal chromatography	**Non-ideal chromatography**
Linear isotherm	too idealized, not much use	Analytical chromatography
Non-linear isotherm	Good first approximation of preparative chromatography	General case, very complex, numerical solutions needed

Table 1.1: Classification of chromatographic models. Ideal, non-ideal, linear, non-linear definitions.

Linear, ideal chromatographic models are too simplified to be of much use. Extensive and well-understood models under linear, non-ideal conditions are used in the field of analytical chromatography. Analytical separations deal with components under low concentrations and therefore only consider bandspreading effects. Complete

solutions can be obtained for linear, non-ideal separations, using either a rate model or a plate model (Kucera 1965).

In preparative runs, the non-linearity by itself can cause tailing and peak shape distortions. For instance, when bandspreading effects are minor, such as in the case of well-packed columns using very small particles, the shape distortions are dependent primarily on the curvature of the adsorption isotherms of all the molecules present in the run (which can also include the organic solvent adsorption isotherm itself).

Non-linear, ideal chromatographic models are a good first approximation of preparative runs, and have worked well for predicting preparative runs of small molecules such as alcohols (El Fallah and Guiochon 1991). A review of solutions to the ideal model of chromatography is given by Guiochon and Golshan-Shirazi 1994. Non-ideal, non-linear models, on the other hand, are very complex. Various models have been summarized by Velayudhan et al. (1992).

Numerical models are needed for the study of preparative non-linear, non-ideal chromatography. In modeling a non-linear, non-ideal chromatographic system, the mass balance, non-linearity and non-ideality must be satisfied. The mass balance of the j^{th} component in a column is represented by (Velayudhan et al. 1995, Ruthven and Ching 1993):

$$\frac{\delta c_j}{\delta t} + \upsilon \frac{\delta c_j}{\delta x} + \phi \frac{\delta q_j}{\delta t} = 0 \qquad (1.1)$$

where $\delta c_j/\delta t$ is the accumulation in the mobile phase, $\phi\delta q_j/\delta t$ is the accumulation in the stationary phase, and $v\delta c_j/\delta x$ is the convective flow. The non-linearity is represented by an adsorption isotherm:

$$q_j^* = f_j(c_1, c_2, ..., c_n) \qquad (1.2)$$

where q^*_j is the concentration of the j^{th} component in the SP at equilibrium, and depends in principle on the mobile-phase concentrations of all the components.

To take into account the non-ideality of a system, the semi-ideal chromatographic model can be used, such as the "lumped" model (Velayudhan et al. 1995). This model accounts for all non-idealities by representing the overall bandspreading through a lumped mass-transfer coefficient:

$$\frac{\delta q_j^*}{\delta t} = k_{MT,j}(q_j^* - q_j) \qquad (1.3)$$

where q_j is the j^{th} adsorbate concentration in the SP at any time t, q_j^* is the j^{th} adsorbate concentration in the SP at equilibrium, and k_{MTj} is the overall mass transfer coefficient accounting for all non-ideal phenomena. The k_{MTj} term is obtained from a lumped equation on the bandspreading term. Non-ideal chromatographic behavior involve peak broadening processes, such as pore diffusion (internal mass transfer, C_i), film mass transfer (external mass transfer, C_f), binding/sorption kinetics (C_k), axial dispersion (A/v) and eddy diffusion (B). The overall bandspreading is given by the sum of all these effects, as expressed in the Van Deemter equation. The Van Deemter equation relates the band spreading to the theoretical plate height of the column(H), which is a measure of a column's bandspreading efficiency:

$$H = \frac{A}{v} + B + C_i v + C_f v + C_k v \qquad (1.4)$$

In a lumped model, the plate height is "lumped" into a single value representative of all the bandspreading effects. All bandspreading effects (H) are lumped into a single mass transfer effect:

$$H_{lumped} = C_{lumped} v \qquad (1.5)$$

where C_{lumped} is related to the overall mass transfer effects k_{MT}. The models are summarized in Table 1.2. Neglecting the mass transfer effect gives back the equilibrium model of non-linear chromatography.

	Equilibrium Model	Lumped Non-Equilibrium Model
Mass Balance	$\frac{\delta c_j}{\delta t} + v\frac{\delta c_j}{\delta x} + \phi\frac{\delta q_j}{\delta t} = 0$	$\frac{\delta c_j}{\delta t} + v\frac{\delta c_j}{\delta x} + \phi\frac{\delta q_j}{\delta t} = 0$
Isotherm	$q^*_j = f_j(c_1, c_2, \ldots, c_n)$	$q^*_j = f_j(c_1, c_2, \ldots, c_n)$
Mass Transfer	$q_j = q^*_j$	$\frac{\delta q^*_j}{\delta t} = k_{MT,j}(q^*_j - q_j)$

Table 1.2: Comparison of ideal and non-ideal chromatographic models.

1.2 The single component Langmuir isotherm

Adsorption isotherms are essential to predicting chromatographic behavior. The non-linearity of the isotherm has a major effect on the peak shape. Considering the

single component isotherm (SCI), whether the isotherm is linear, concave down, or concave up has an effect on the shape of the chromatographic peak. A linear isotherm gives a Gaussian chromatographic peak, concave down isotherms tend to front and convex isotherms tail (Figure 1.1).

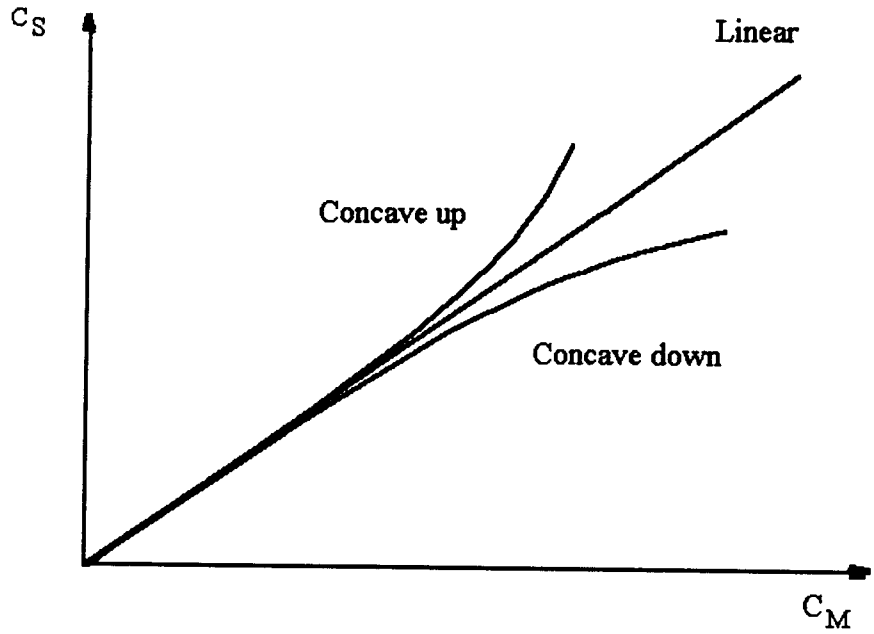


Figure 1.1: Plot of concave, linear and convex isotherms.

One of the simplest adsorption isotherms (see equation 1.2) is the Langmuir form. If we consider the stationary phase of a reversed-phase system as composed of homogeneous hydrophobic patches, then the single "patches" of hydrophobicity on a single molecule will bind to the free "patches" on the stationary phase:

$$c + I \Leftrightarrow q \qquad (1.6)$$

where c is the concentration of the adsorbates in the MP and q the concentration of adsorbates in the SP, and I the concentration of free patches on the SP. If Λ is the total

concentration of patches (known as the saturation capacity) on the SP, and K is the equilibrium constant, then

$$I = \Lambda - q \tag{1.7}$$

$$K = \frac{q}{c \cdot I} = \frac{q}{c(\Lambda - q)} \tag{1.8}$$

Therefore, the single component isotherm is

$$q = \frac{\Lambda \cdot K \cdot c}{1 + K \cdot c} \tag{1.9}$$

which gives an explicit expression of q in terms of c. If we remain in the linear portion of the isotherm, then $K^*c << 1$ and therefore $q \cong \Lambda^* K^* c$. K is the Henry's law constant, and is the slope of the isotherm at c=0. Under conditions where the saturation capacities (Λ) of the SCI of each components are identical ($\Lambda_1 = \Lambda_2 = \Lambda_3 = ...$), then the isotherm can be extended to multiple components (Antia and Horvath 1989, Quinones and Guiochon 1996):

$$q_j = \frac{\Lambda_j K_j c_j}{1 + \sum_{i=1}^{m} K_i c_i} \tag{1.10}$$

This form is also used even when $\Lambda_1 \neq \Lambda_2 \neq \Lambda_3 \neq ...$, because of its simplicity. The "modulator" can play a significant role on the isotherm in overloaded gradient elution. In IEX, the modulator is a salt, and in RP, the modulator is an organic solvent such as acetonitrile or methanol. The modulator is used to adjust, or "modulate", the polarity of the mobile phase in RP. Just as salt modulators influence the distribution of proteins (Kaltenbrunner 1996), the organic modulator has an effect on the adsorbate binding to

the RP stationary phase and must be taken into account in the isotherm (Velayudhan and Ladisch 1991). The organic modulator can be considered to be one of the binding adsorbates considered in equation 1.10. The Langmuir isotherm for X~phe and X~trp accounting for the modulator is (Antia and Horvath 1989):

$$q_P = \frac{a_P e^{-s_P C_M} c_P}{1 + b_P e^{-s_P C_M} c_P + b_T e^{-s_T C_M} c_T}$$
$$q_T = \frac{a_T e^{-s_T C_M} c_T}{1 + b_P e^{-s_T C_M} c_P + b_T e^{-s_P C} c_T} \qquad (1.11)$$

where C_M represents the modulator concentration, q_P and q_T the stationary phase concentration of the X~phe and X~trp adsorbates respectively, a_P, a_T, b_P and b_T the corresponding isotherm parameters, and s is the slope of the modulator k' as a function of the modulator concentration. The exponential terms account for the logarithmic binding behavior of the modulator in reversed-phase chromatography (Snyder 1980).

1.3 Analytical vs. preparative chromatography

Depending on the purpose of separation, analytical or preparative chromatography is used. Analytical chromatography is used for sample identification and quantitation. It requires as many sample solutes as possible be separated. In contrast, maximum recovery of a solute or solutes for a given purity is desired in preparative chromatography (Guiochon 1986). As a result the desired solute(s) are isolated in relatively large amounts and at a high purity. One fundamental goal of

preparative chromatography is to optimize productivity. The productivity relates the throughput (amount of the desired adsorbate purified at a given purity) to the time it took to complete the purification, and to the amount of stationary phase material that was used in the process.

When designing large-scale production, preparative chromatography is initially attempted under low loadings on analytical-scale columns. Then once throughput and other relevant parameters have been optimized, columns are then simply scaled up. It is therefore crucial to optimize the productivity at the analytical scale. Scale-up theory using the plate model and the rate model of chromatography have been widely examined (Knox and Pyper 1986, Velayudhan and Ladisch 1993, Whitley et al. 1993, Heuer et al. 1996).

Present preparative chromatographic purification, however, is typically performed under linear isotherm conditions, and as a result the stationary and mobile phases, which contribute the major separation costs (Felinger and Guiochon 1994), are not being used to their fullest potential (Ruthven and Ching 1993, Hodges et al. 1993). For instance, the preparative purification of a binary system is done by optimizing their analytical separations and then increasing the injected quantity until right before the two adsorbate peaks begin to overlap (Colin 1993, Knox and Pyper 1986). This type of separation does not take advantage of competitive interactions that can occur at higher loads (Colin 1993). With this type of separation, the sample loading (how much can be put onto the column) being used is so low that the different adsorbates in the sample bind to the adsorbent independently from one another as well as from the other adsorbate species.

If the sample load is increased until the adsorbates begin to compete against one another for sites, the binding ceases to be linear, and operation conditions become complex. However, throughput can be significantly improved and these added complications can be overcome with further understanding of these non-linear processes.

1.4 Gradient elution under non-linear conditions

Preparative improvements of purification using non-linear adsorption is dependent on the type of operational mode that is being used. Operational modes include isocratic elution, gradient and stepwise elution, frontal chromatography and displacement chromatography. The operational modes considered here are isocratic, gradient and step-wise elution. In isocratic elution, the concentration of the modulator in the MP remains constant throughout the run. In gradient elution the concentration of the modulator in the MP is increased with time. As the modulator concentration increases, polarity of the MP decreases, and the adsorbates are less likely to bind to the SP and elute faster. Stepwise gradient elution is when the modulator concentration is abruptly increased to a higher modulator level using a step. Thereafter the modulator is kept constant as in isocratic elution.

Gradients are beneficial in preparative purifications as a result of several effects: (1) Peak focusing effect: Because of the initial low modulator concentration, the feed components bind strongly to the inlet of the column. However, the increase in

modulator concentration eventually cause the adsorbates to elute earlier than if it was kept at isocratic conditions. This means that the adsorbates elute with a smaller k' (less retained). The decreased retention reduces the band broadening. This usually results in the concentration of the eluting adsorbate peaks (Snyder and Kirkland 1979). In addition, a decrease in k' means that the equilibrium distribution coefficient (K) of the adsorbates decreases, and under overloaded conditions, nonlinear adsorbate isotherms then become linear (Figure 1.2), a phenomenon known as gradient linearization (Frey 1990).

(2) Self and mutual interference effect: Under high feed concentration, there may not be enough binding sites available on the stationary phase. As a result, the adsorbates compete for sites, and the peak shape is dependent on the non-linearity of the component's isotherm. In the presence of other eluting component species, the competitive binding gives rise to multicomponent isotherms, and the resulting effect is the mutual interference effect (Velayudhan 1995).

(3) Band compression effect: As the gradient increases, the peak is sharpened because the adsorbates at the tailing end find themselves under higher modulator concentrations causing them to elute faster, catching up to the adsorbates at the front end which are at lower modulator concentration. As a result, a chromatographic peak which would normally tail under isocratic conditions may become Gaussian in a gradient (Snyder and Kirkland 1979).

Using gradient elution, peaks can be concentrated to more than its original concentration in the feed sample (Snyder et al. 1989). The solutes collected as a result

are enriched. In general, preparative chromatography in isocratic conditions are performed under linear conditions. These chromatographic separations are simpler to deal with, and linear isocratic elution has been extensively studied. Retention mechanisms of isocratic elution are well understood (Martin 1988, Snyder and Kirkland 1979) and scale up of optimized isocratic systems have been done using various models (Gibbs and Lightfoot 1986, Ladisch and Velayudhan 1995).

Improvements in HPLC technology have led to shorter separation times, improvements in gradient manipulations, more reproducible results and greater

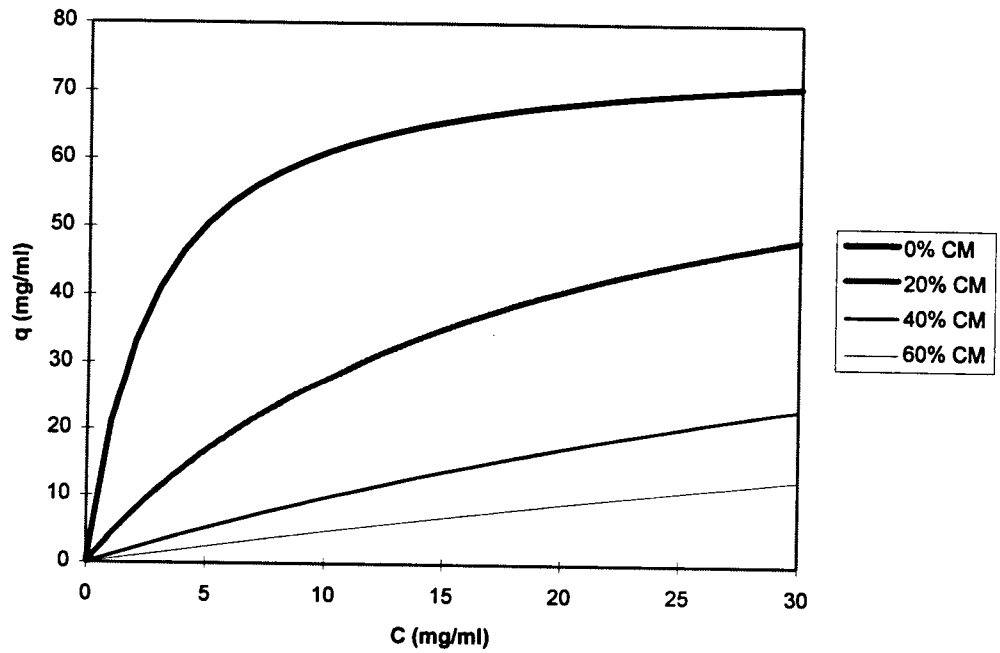


Figure 1.2: Gradient linearization with increasing modulator concentration (C_M).

efficiency (e.g., higher plate count). Improvements in HPLC peptide separations have been done by changing particles (e.g., particle size, pore diameter, bonded-phase composition), or by changing mobile phase composition, therefore improving band shape, adsorbate recovery and resolution (Stadalius et al. 1984). Therefore, the instrumental improvements and technological innovations of HPLC have improved biomolecule purification.

However, fundamentally, preparative purification under linear conditions is very inefficient, as mentioned before. Preparative separations are commonly done in industry by increasing the concentrations of the mixtures to be purified, until the lowest acceptable resolution is reached. The system is then scaled-up by running larger feed volumes into proportionally larger columns. Although this type of purification method makes it relatively easy to scale up to production levels, which may mean gram quantities of materials being produced, the column is not being overloaded because the adsorbate conditions are not high enough to produce appreciable isotherm non-linearities. As a result, the concentration of adsorbates obtained relative to the amount of solvent being used is very low (Felinger and Guiochon 1994, Colin 1993).

1.5 Reversed phase chromatography

Of particular interest is the RP mode because of its common usage by research and industry and its effectiveness in separating similar compounds. RP is efficient at separating complex molecules; for instance, RP chromatography can separate

diastereomeric peptides that contain more than 10 amino acid residues (El Rassi et al. 1990). RP also has excellent reproducibility (Dolan et al. 1987). However, RP is not frequently used for larger protein purification as denaturation problems occur (El Rassi et al. 1990). Although there are attempts to model the effects of denaturation (Whitley et al. 1994) and there are sometimes ways to avoid denaturation such as starting at low solvent strength (Cox 1993), proteins are most often purified using ion-exchange chromatography (IEX), while peptides are usually separated by reversed-phase chromatography (RP). However, RP was used to purify proteins by intentionally denaturing and renaturing it (Knuth and Burgess 1987). Nevertheless, in general, RP should be used for separating polypeptides with molecular weights < 10000 (Welinder et al. 1991).

A RP system utilizes the hydrophobic nature of the adsorbates as a basis for separation. The SP is composed of a matrix with carbon chain ligands attached. Ligands chains of 18 carbons in length attached to silica based particles are commonly used. The MP is composed of a mixture of water and an organic solvent such as methanol or acetonitrile. As the adsorbates are injected into the RP column, the less polar (more hydrophobic) adsorbates will bind to the SP, and the more polar adsorbates will prefer the MP and elute first. That is why an organic (non-polar) solvent such as methanol or acetonitrile is added to reduce the polarity of the MP, in turn reducing the retention of adsorbates, which may otherwise be irreversibly adsorbed.

Reversed-phase HPLC is used with for the purification of many compounds. Peptides used in the study of learning and memory have been preparatively purified

using RP-HPLC (Gasc 1982). Industrial production of the antibiotic cephalosporin is well developed (Kodama et al. 1995). Preparative liquid chromatography has also been used for quality assurance for impurity determination such as impurities in arbidol and SI-5 drugs (Miller and Bergeron 1994). Hormones are commonly separated using RP-HPLC (Welinder et al. 1991). The high purity that can be obtained by RP-HPLC makes it suitable for cell membrane molecules such as the lipid ganglioside(Menzeleev et al. 1994). Other substances purified by RP-HPLC include sugars, antibiotics, lipids, amino acids, peptides, and enzymes.

1.6 Loading and throughput

Preparative purification is used in laboratory scale purification of peptides for further lab scale experimental uses and in industrial scale up for commercialization purposes. Sample loads are typically around 0.001 to 0.1 gram of sample/gram of packing (McDonald and Bidlingmeyer 1987). A measure of the productivity of a purification process is therefore essential. The measure of productivity depends on the purity desired. At 98% and 95% purity, the productivities (P) are defined as

$$P_{98\%} = \frac{m_{98\%}}{t_{cyc} * V_{SP}} \quad \text{or} \quad P_{95\%} = \frac{m_{95\%}}{t_{cyc} * V_{SP}} \tag{1.12}$$

where $m_{98\%}$ is the mass collected at 98% purity and the $m_{95\%}$ is the mass collected at 95% purity, t_{cyc} is the cycle time or the time that it takes to purify the sample plus the time it takes to regenerate the column for the next cycle, and V_{SP} is the volume of the

stationary phase of the column. In determining the cycle time, 20 minutes was chosen as the column regeneration time. It was assumed that one regeneration was needed for each gradient elution, and two isocratic runs could be performed before regeneration of the column was needed; therefore 20 minutes was added to the cycle time for gradient runs and 10 minutes was added for isocratic runs. A list of some other examples of purified biological products with their estimated productivities (according to equ. 1.12) is given in Table 1.3.

1.7 Model mixture: chemotactic peptides

An analytical scale RP column was preparatively used under overloaded gradient conditions for peptide purification. The chemotactic dipeptides N-formyl-met-trp, N-formyl-met-phe, N-formyl-met-val and N-formyl-met-ala were selected for their similarity in structure and their biological uses, such as in the study of chemotaxis in bacteria. *Escherichia coli* and *Salmonella typhimurium* are known to form regular patterns when grown. This occurs because these bacteria are able to excrete a chemical attractant, and aggregate according to the gradient of that attractant (Budrene and Berg 1995). These chemotactic substances have been identified as peptides (Marasco et al. 1984).

Of the four peptides selected, N-formyl-met-trp and N-formyl-met-phe were eventually selected for their close separation factor (tested to be $\alpha \leq 1.2$, see section 3.2.1). Preparative runs were performed with both ACN and MeOH as modulators and

the results of isocratic and gradient elution productivities were compared. Limiting conditions turned out to be the solubility of the N-formyl-met-phe peptide and selectivity reversal in methanol conditions. The effect of feed conditions on the chromatograms were also examined.

Reference	product	column dimensions $L \times D$ (cm x cm)	approx. V_{SP} (ml)	approx. t_{cyc} (min)	approx. m_p (mg)	$P=m_P/(t_{cyc} \cdot V_{SP})$ (mg/hr·ml)
Wolfe et al. 1984	human interleukin-2	6.6 x 2.2	17.56	50	0.01	0.00068
Welinder et al. 1987	human growth hormone	25.0 x 0.4	1.26	80	0.016	0.0096
Kodama et al. 1995	cephalosporin C	15.0 x 0.6	2.97	150	0.38	0.051
Kalman et al.1996	phe-pro isomers	25.0 x 0.46	1.66	30	1.0	1.0
	tyr-pro-phe isomers	25.0 x 0.47	1.73	35	1.0	1.0
Wu and Greenbladt 1995	lysozyme	25.0 x 0.46	1.66	30	2.0	2.4
	ribonuclease	25.0 x 0.46	1.66	30	2.0	2.4
	BSA	25.0 x 0.46	1.66	30	2.0	2.4
Menzeleev et al. 1994	ganglioside proteins	25.0 x 2.14	35.97	25	3.1	0.21
Minkiewicz et al. 1996	bovine κ-casein	25.0 x 2	47.12	50	8.0	0.20
Erhard et al. 1987	leukotriene B4	25.0 x 2.12	35.30	40	33.8	1.4
Kroeff et al. 1989	insulin	15.0 x 0.94	7.29	260	153.0	4.8
Feng et al. 1996	α-amylase	25.0 x 2.2	57.02	110	200.0	1.9
Edwards et al. 1996	microcystins	15.0 x 7.5	530.14	50	415.0	0.94
Bishop et al. 1980	tetrapeptide	30.0 x 5.7	535.87	40	1000.0	2.8

Table 1.3: Typical productivities in bioproduct purification using RP-HPLC.

2 MATERIALS AND METHODS

2.1 Equipments and protocol

The adsorbates considered are small peptides which have a relevant biological purpose and activity. The following bioactive peptides were purchased from Sigma (St. Louis, MO):

N-formyl-methionyl-alanine (X~ala)

N-formyl-methionyl-valine(X~val)

N-formyl-methionyl-phenylalanine (X~phe)

N-formyl-methionyl-tryptophan (X~trp)

Preparative injections were performed using a Waters 600 pump (Milford, MA). The procedure is described in Figure 2.1. Briefly, it involved injecting a sample mixture into the column, running an isocratic or gradient elution, then manually collecting fractions at 15 or 30 second intervals. These fractions were then re-injected under analytical conditions, using a Waters 717plus autosampler, so that concentrations could be obtained using a previously generated calibration curve (see Figure 2.2). Concentrations were often so high that they saturated the UV detector. Thus, the fractions had to be collected, diluted and then re-run analytically.

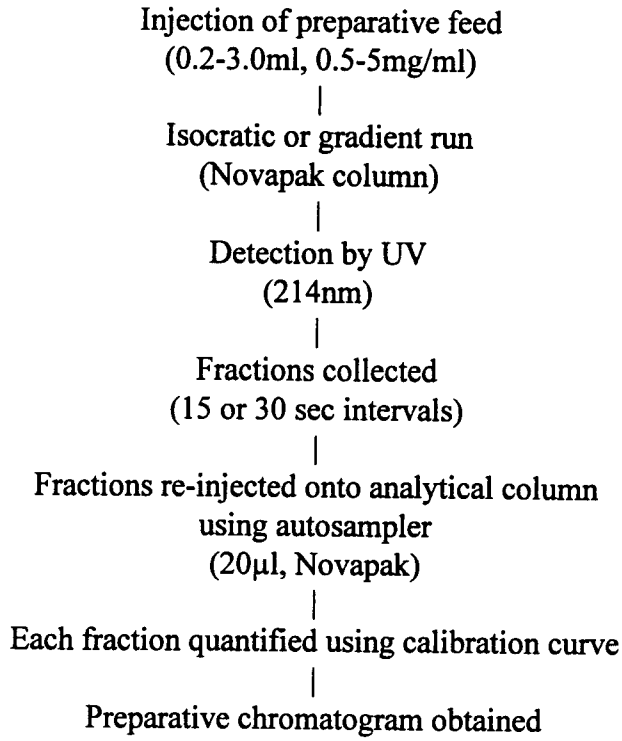


Figure 2.1: Schematic of preparative run and analysis of fractions.

The Waters (Milford, MA) millenium chromatography software was used throughout. All adsorbates were detected with a Waters (Milford, MA) 486 UV absorbance spectrophotometer at 214 nm. Three columns were used:

1. A Waters (Milford, MA) Novapak C18 reversed-phase column 150 x 3.9 mm I.D., 60A pores with 4µm average particle diameter was used for preparative injections and analyses of fractions of the dipeptides using acetonitrile as the organic modifier.

2. A second Waters (Milford, MA) Novapak C18 reversed-phase column 150 x 3.9 mm I.D., 60A pores with 4μm average particle diameter was used for preparative injections of the dipeptides using methanol as the organic modifier.

The preparative runs were done at room temperature. The Novapak C-18 columns were run at 1ml/min flow rate.

Adsorbate concentrations for the binary mixtures of dipeptides in the feed varied from 0.4 to 1mg/ml (see results section). Feed volumes ranged from 0.5ml to 3.0ml.

The X~phe and X~trp dipeptides were calibrated using the Novapak C18 at 20% acetonitrile isocratic conditions at 1ml/min flow rate and 20μl injection volume. The calibration curve ranged in concentration from 0.01mg/ml to 0.38mg/ml (Figure 2.2).

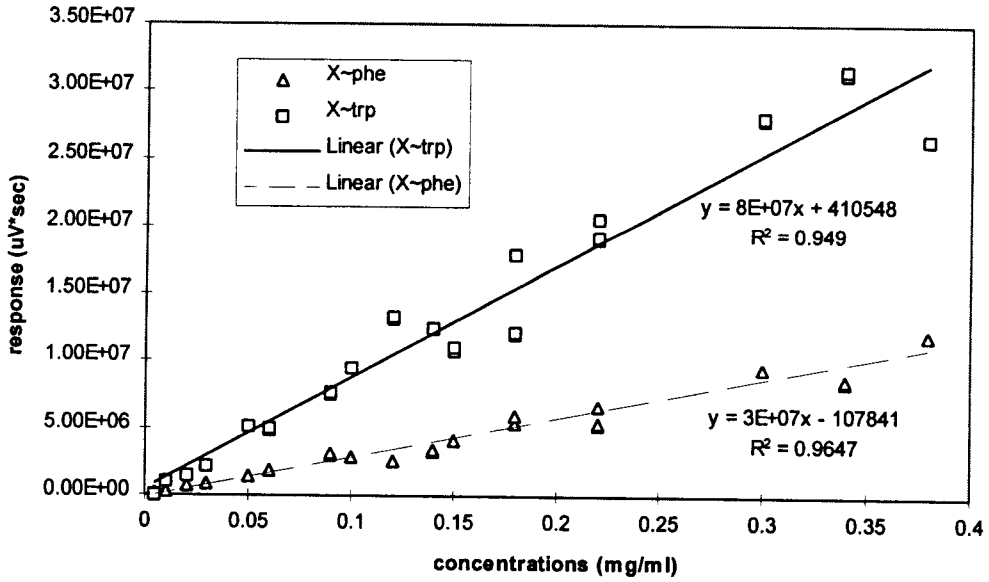


Figure 2.2: Calibration curve for X~phe and X~trp.

All solvents were mixed with 0.1% trifluoroacetic acid (TFA) except for the studies done on the effect of feed (see section 3.2.3). Sequanal grade TFA was obtained from Pierce (Rockford, IL). HPLC grade acetonitrile was purchased from E.M. Science (Gibston, NJ) and the water was distilled-deionized using the Corning Megapure System (Corning, NY). A mixture of monobasic sodium phosphate and dibasic sodium phosphate (Mallinckrodt, Paris, KY), adjusted to pH=7 was used as buffers.

The sodium phosphate buffer solution was made as follows:

(1) One solution of 10mM monobasic and another solution of 10mM dibasic solutions were made from powder:

(a) dibasic (pH>7) solution: 1.42g of Na_2HPO4 was added to 1L of distilled water (141.96g/mol $Na_2HPO_4 \cdot 0.01M = 1.42g/L$).

(b) monobasic (pH>7) solution: 1.38g of NaH_2PO_4 was added to 1L of distilled water (1.37.99g/mol $NaH_2PO_4 \cdot 0.01M = 1.38g/L$).

(2) Then using a pH meter (Corning, NY) the monobasic sodium phosphate was gradually added to the dibasic sodium phosphate solution, until the pH = 7, while maintaining a constant stirring.

2.2 Adsorbate retention factors and phase ratio

The adsorbate retention factor k' is obtained from:

$$k' = \frac{V_R}{V_0} - 1 \qquad (2.1)$$

where V_R is the adsorbate residence volume, and V_0 is the column void volume, or the volume that is required to elute the adsorbate if it is unretained. An adsorbate retention plot is obtained by calculating the various k' for different concentrations of organic solvents, from V_R obtained experimentally. V_R is obtained by measuring the retention times (t_R) of the solutes at various modulator concentrations ($V_R = t_R \cdot F$). Obtaining the void volume V_0 is described below.

For the Novapak column used for the preparative runs, V_0 can simply be calculated by measuring the mass and weight of the column, and using the result:

$$m = w_c + \rho V_0 \qquad (2.2)$$

where m is the mass measured on the column experimentally at a certain concentration of modulator, w_c is the weight of the column and ρ is the density of the solvent mixture at that modulator concentration. Since the density of the solvent mixtures at different concentrations of modulators is known, several measurements can be made for a more accurate result (Table 2.1). The modulator used here was MeOH.

The linear regression (Figure 2.3) gave:

$$m = 1.19\rho + 62.7 \qquad (2.3)$$

Therefore, the void volume is simply the slope, V_0 = 1.19 ml.

Concentration MeOH (%v/v)	Mass of column *m* (g)	density of MeOH ρ (g/ml)*
10	63.85	.982
30	63.8	.952
50	63.77	.916
70	63.72	.872
90	63.65	.820

*Perry and Green 1985

Table 2.1: Measurements of column and modulator parameters for void volume calculations.

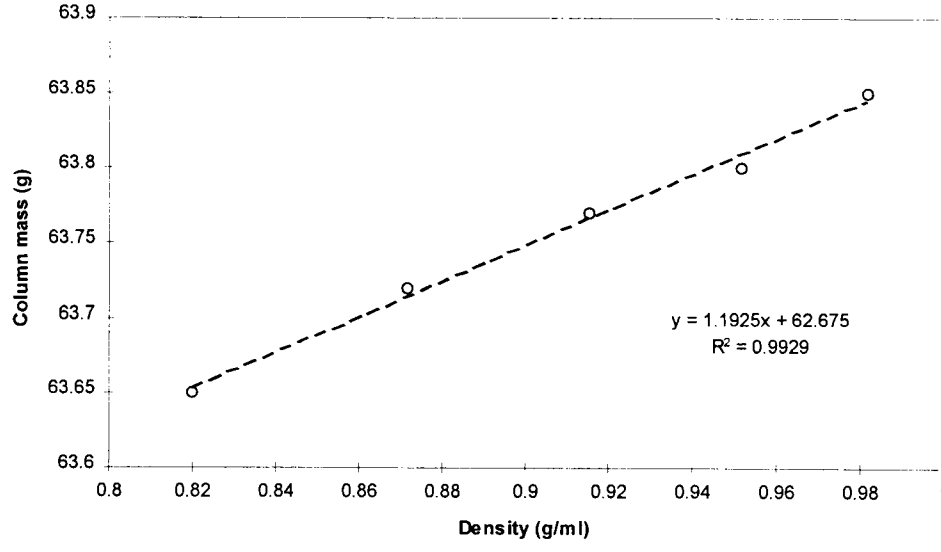


Figure 2.3: Regression plot of column mass and density of mixture.

The phase ratio (ϕ) is the ratio of the volume of SP to the volume of MP. If we assume that all pores are accessible, then the void volume equals the mobile phase volume ($V_0 = V_{MP}$), and the phase ratio is:

$$\phi = \frac{V_{SP}}{V_{MP}} = \frac{V_{SP}}{V_0} \tag{2.4}$$

where V_{SP} is the stationary phase volume and V_{MP} is the mobile phase volume. The volume of the stationary phase is simply the difference between the empty column volume and the void volume of the column:

$$V_{SP} = V_{ec} - V_0 \tag{2.5}$$

The phase ratio is therefore:

$$\phi = \frac{1.79ml - 1.19ml}{1.19ml} = 0.50 \tag{2.6}$$

2.3 Measurement of adsorption isotherms

To determine the adsorption isotherms of the solutes X~phe and X~trp, the Elution by Characteristic point (ECP) method was used. This method uses data points from the trailing edge of an individual component peak to generate isotherm data. The ECP method is known to be sufficiently accurate for chromatographic systems with a plate count greater than 100 (Guan et al. 1994). Assuming ideal chromatographic conditions, it is derived that (Conder and Young 1979):

$$(\frac{dx}{dt})_c = \frac{u_0}{1 + \phi \frac{dq}{dc}} \tag{2.7}$$

u_0 is the void velocity, ϕ is the phase ratio and q is the adsorbed concentration.

Given that $u_c=(dx/dt)_c$ is the adsorbate velocity corresponding to any concentration c, and that this velocity is constant throughout the column for the trailing edge of any peak, this equation can be rewritten in terms of time,

$$t_c = t_0(1+\phi\frac{dq}{dc}) \tag{2.8}$$

and in terms of volume,

$$V_c = V_0(1+\phi\frac{dq}{dc}) \tag{2.9}$$

Using equation 2.4, we have finally:

$$V_c = V_0 + V_{SP}\frac{dq}{dc} \tag{2.10}$$

Then q is obtained, by integrating $\frac{dq}{dc}$,

$$\frac{dq}{dc} = \frac{V_c - V_0}{V_{SP}} \rightarrow q = \int\frac{V_0(c)-V_0}{V_{SP}}dc \tag{2.11}$$

Assuming a single component isotherm, q can be obtained from a preparative single component chromatographic run. The procedure involve the following steps (Figure 2.4):

1) Pick a concentration, e.g. c_1, and find the corresponding V_{c1}.

2) V_{SP} is known: $V_{SP} = V_{ec} - V_m = V_{ec} - V_0$, where V_{ec} is the empty column volume.

3) Find $\frac{dq}{dc}$ since V_c, V_0, and V_{SP} are known.

4) Integrate $\frac{dq}{dc}$ to get q.

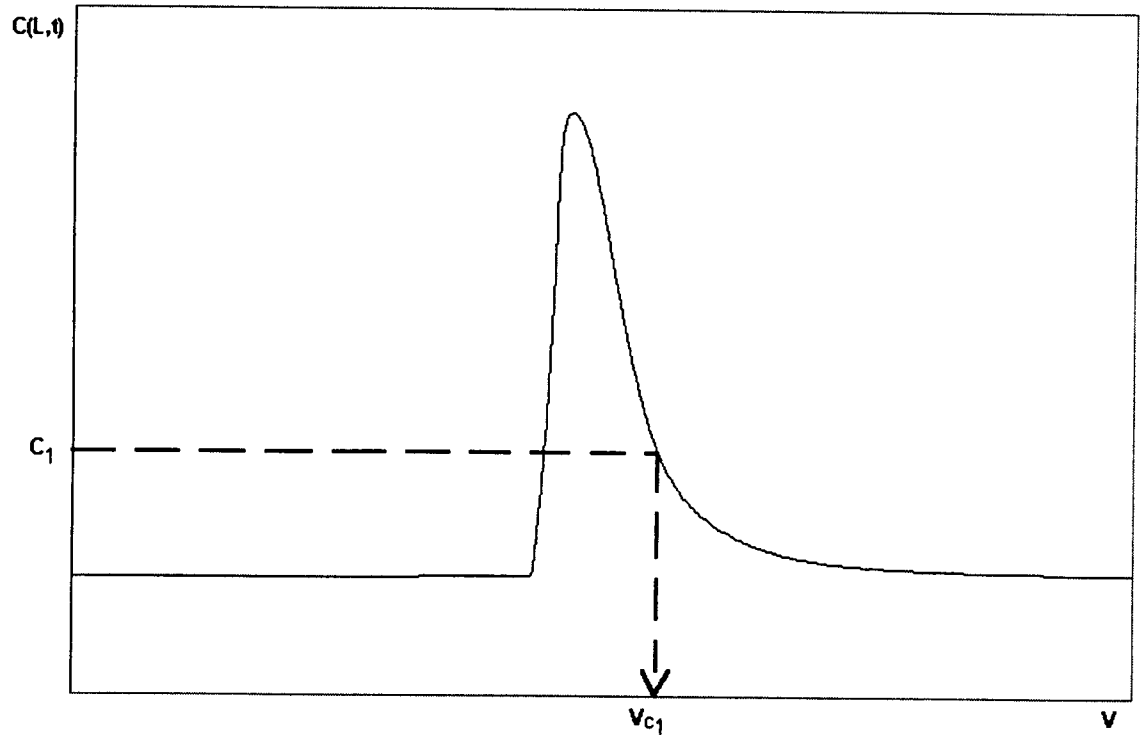


Figure 2.4: Chromatograph of a single component preparative run.

But we need to take into account the feed volume (V_{inj}). The feed volume is given by

$$V_{inj} = t_{inj} \cdot F. \tag{2.12}$$

$$q = \int \frac{V_0(c) - V_0 - \frac{V_{inj}}{2}}{V_{SP}} dc \tag{2.13}$$

This integration can be calculated numerically. A QBASIC program is given in appendix A. Isotherm results are given in sections 3.2.4 and 3.3.2.

2.4 Peptide solubility

The solubility of the dipeptides were low in both acetonitrile and methanol; peptide insolubility in organics is not uncommon (Menzeleev et al. 1994). Solubility can frequently be a problem in preparative purifications, due to the high loading conditions. This is a common problem in industry. As mentioned by (Porsch 1994), solubility can be crucial in preparative L.C.: "it determines the possible loading, performance, throughput and concentration of collected fractions". In general, insolubility should be avoided. Therefore, all the preparative conditions were chosen based on this limitation.

Depending on the organic solvent used, the solubility limits varied. It was found that the solubility of the peptides depended on the method used to purify it. For instance, X~phe seem to not dissolve in TFA. If, however, the peptides were already dissolved in the solvent, then there was a tendency to remain in solution despite adding TFA after, although the dissolution may not necessarily be permanent. Therefore various "solubilizing methods" were used. The methods are described in Table 2.2, and an example for making a 2mg/ml of X~phe and X~trp solution in 40/60 ACN/buffer solution is included (Table 2.3).

In the example in Table 2.3, only method B could solubilize 2mg/ml of X~phe and 2mg/ml of X~trp in 40/60 ACN/buffer. Sections 3.2.3 and 3.3.1 describe the results of the solubility tests.

No TFA	With TFA
Method A: Add solutes to mixture*	Method C: Add solutes to mixture; then add TFA
Method B: Add solutes to organic; then add buffer	Method D: Add solutes to organic; then add buffer; then add TFA
	Method E: Add solutes to mixture already containing TFA

*mixture=organic+(buffer or water)

Table 2.2: Methods used to try to solubilize the peptides in either MeOH or ACN, and H_2O or sodium phosphate buffer.

Method	To make a 2mg/ml solution in 40/60 ACN/buffer solution:
A	1. prepare a 40/60 ACN/buffer solution in a 4ml vial 2. add 8mg of P and T 3. stir until clear
B	1. prepare 1.6ml of ACN in a 4ml vial 2. add 8mg of P and T 3. stir for 1/2 hour at least (does not have to clear) 4. add 2.4 ml buffer 5. stir until clear
C	1. prepare 1.6ml of ACN 2. add 8mg of P and T 3. stir for at least 1/2 hour at least (does not have to clear) 4. add 2.4 ml buffer 5. add 4µl of TFA
D	1. prepare a 4ml, 40/60 ACN/buffer 2. add 8mg of P and T 3. stir for at least 1/2 hour at least (does not have to clear) 4. add 4µl of TFA 5. stir until clear
E	1. prepare a 4ml, 40/60 ACN/buffer with 4µl TFA solution 2. add 8mg of P and T 3. stir until clear

Table 2.3: Preparation of solutions using the methods A-E listed in Table 2.2.

Although TFA may reduce solubility, it is needed in the samples and throughout the column. Reversed phase without TFA results in long tailing and very weak retention. The effect becomes dramatic under preparative conditions: a preparative run where no TFA was present throughout gave mixed peaks eluting at nearly t_0 (Figure 2.5), but an identical run with TFA throughout gave peaks that were separated and eluted much later (Figure 2.6). TFA is commonly used in reversed-phase columns for its ability to narrow peaks and improve retention. TFA lowers pH (Table 2.4); at 0.1% TFA the pH lowers to 2.7 in a sodium buffer solution, and 2.2 in water solution. Ion pairing with the protein or peptide forming an increased hydrophobicity (decreasing solubility) is one possible reason for the improved retention and selectivity (Wheelwright 1991). However, TFA is also known to have secondary effects whose exact mechanisms on the column are not completely understood (Bennet et al. 1981).

% TFA in 10mM sodium phosphate buffer	pH
0.1	2.7
0.075	3.3
0.05	5.6
<0.03	6.9

Solvent mixture	pH
NaHPO4 buffer	7.0
NaHPO4 buffer + 0.1% TFA	2.7
H_2O (distilled)	6.5
H_2O (DD) + 0.1% TFA	2.2

Table 2.4: pH of solvents under various conditions, with and without TFA.

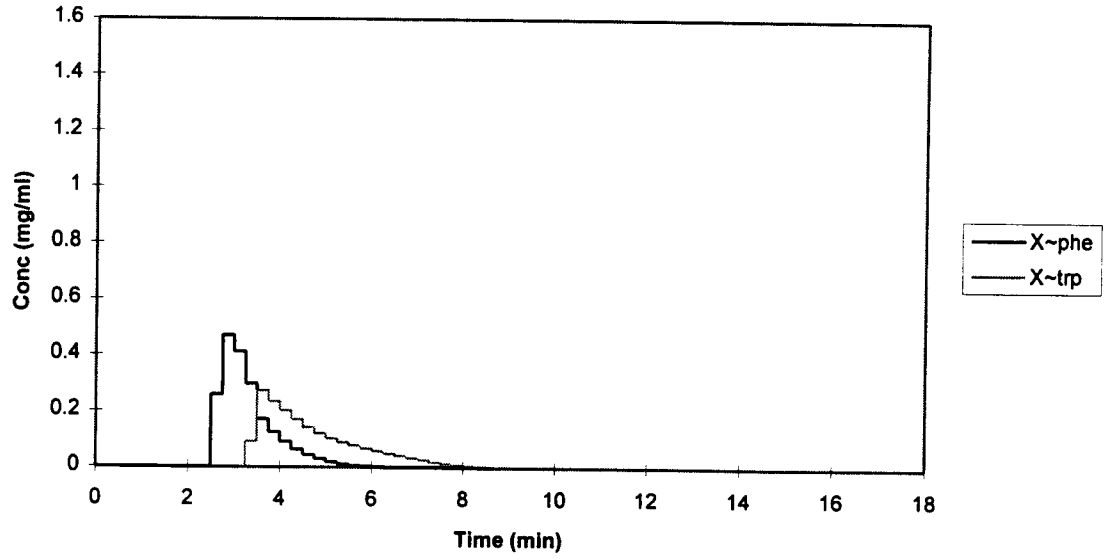


Figure 2.5: 10-40%ACN gradient in 20min. 10mM sodium phosphate buffer throughout (no TFA). Feed: 0.5 mg/ml X~phe and 0.4 mg/ml X~trp, 1 ml volume. Novapak C-18 (150 x 3.9mm I.D.), UV detection at 214nm.

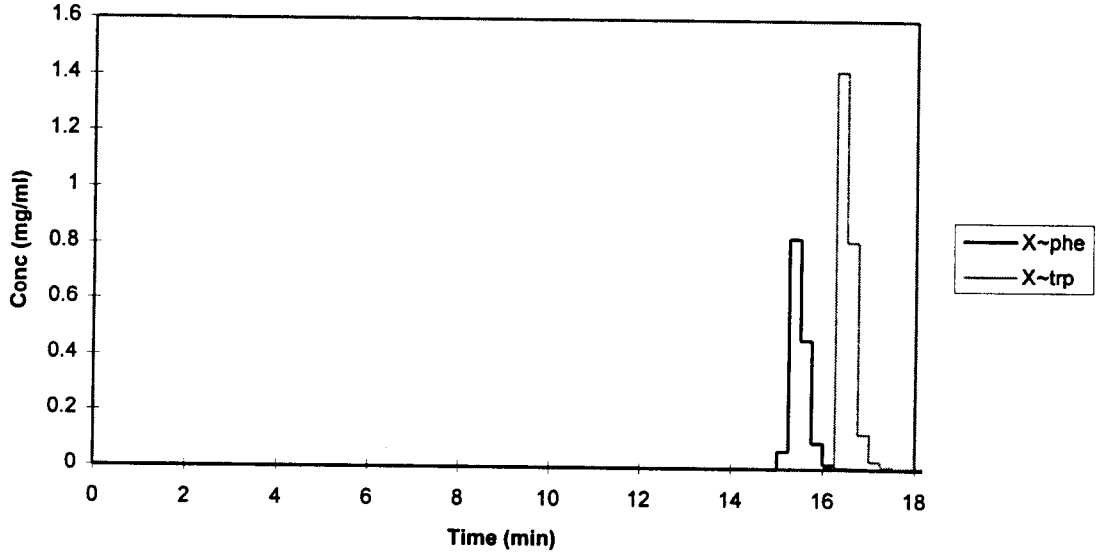


Figure 2.6: Feed has 0.4mg/ml X~phe and 0.5mg/ml X~trp, and TFA is present throughout. All other conditions same as Figure 2.5.

3 RESULTS AND DISCUSSION

3.1 Productivity and measure of error

Productivities are used to compare the effectiveness of preparative chromatographic separations. From equation 1.12 (section 1.6), the productivity term was defined by the cycle time and by V_{SP}, the volume of the stationary phase of the column. V_{SP} can be obtained from the empty column volume,

$$V_{SP} = (1 - \varepsilon_t) * V_{ec} \qquad (3.1)$$

where ε_t is the column total void fraction and V_{ec} is the empty column volume ($=L \cdot \pi r^2$). For the Novapak C-18 column (150 x 3.9mm), V_{ec}=1.79ml. Since the phase ratio is known (Section 2.2), we know ε_t:

$$\phi = \frac{1 - \varepsilon_t}{\varepsilon_t} \qquad (3.2)$$

therefore,

$$\varepsilon_t = \frac{1}{1 + \phi} = \frac{1}{1 + 0.5} = 0.67 \qquad (3.3)$$

and,

$$V_{SP} = (1 - 0.67) * 1.79ml = 0.59ml \qquad (3.4)$$

Productivity depends on the measurement of the mass collected. Therefore, in comparing productivities, it is important to have an idea of the extent of the error involved in measuring mass. Figures 3.1 and 3.2 give the error distributions for the

masurements of the mass balances for each peptides for the runs where the masses were accounted for. For the X~phe peptide, of the preparative chromatographic mass measurements described in the figure, 4 measurements are outside the 15% error range, meaning that within 15%, the data is representative of 91% (42/46=91%) of all the samples. For the X~trp peptide, 7 measurements have a greater error than 20%, therefore the measurements with less than 20% represent 87% (48/55=87%) of all the data. This means that any results obtained which vary within 15% from one another for X~phe and within 20% for X~trp are considered to be insignificant changes, while any results with a difference greater than 15% for X~phe and 20% for X~trp will be considered a significant change.

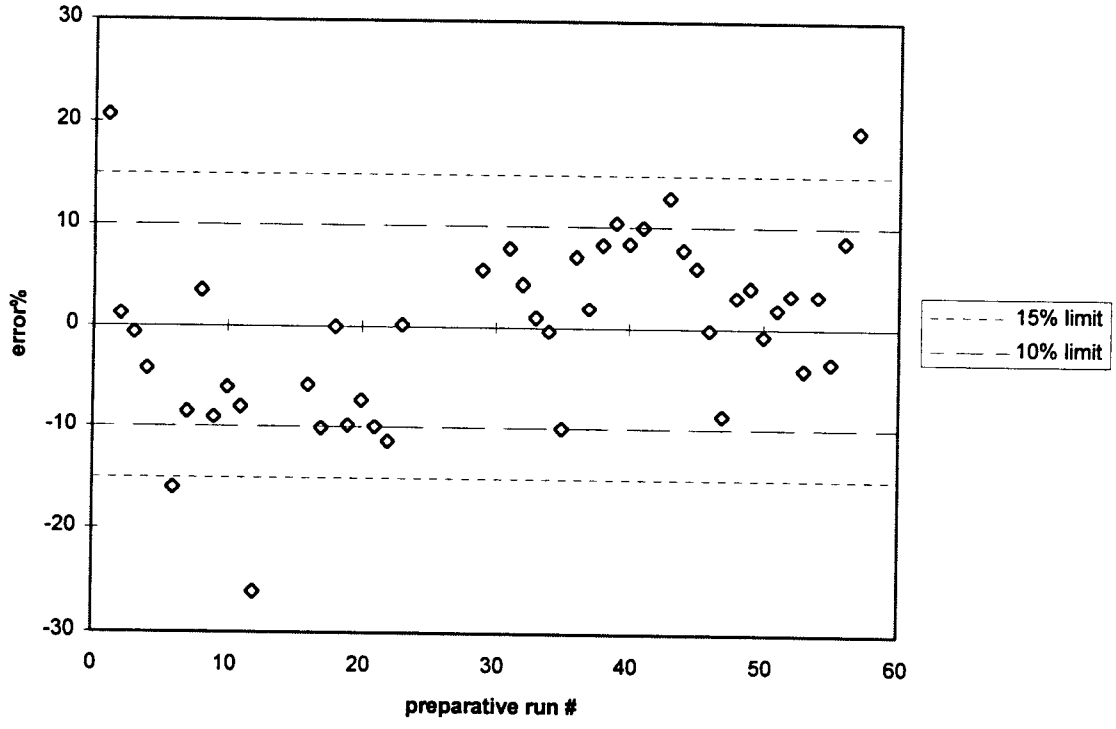


Figure 3.1: Error distribution of the X~phe peptide mass balances.

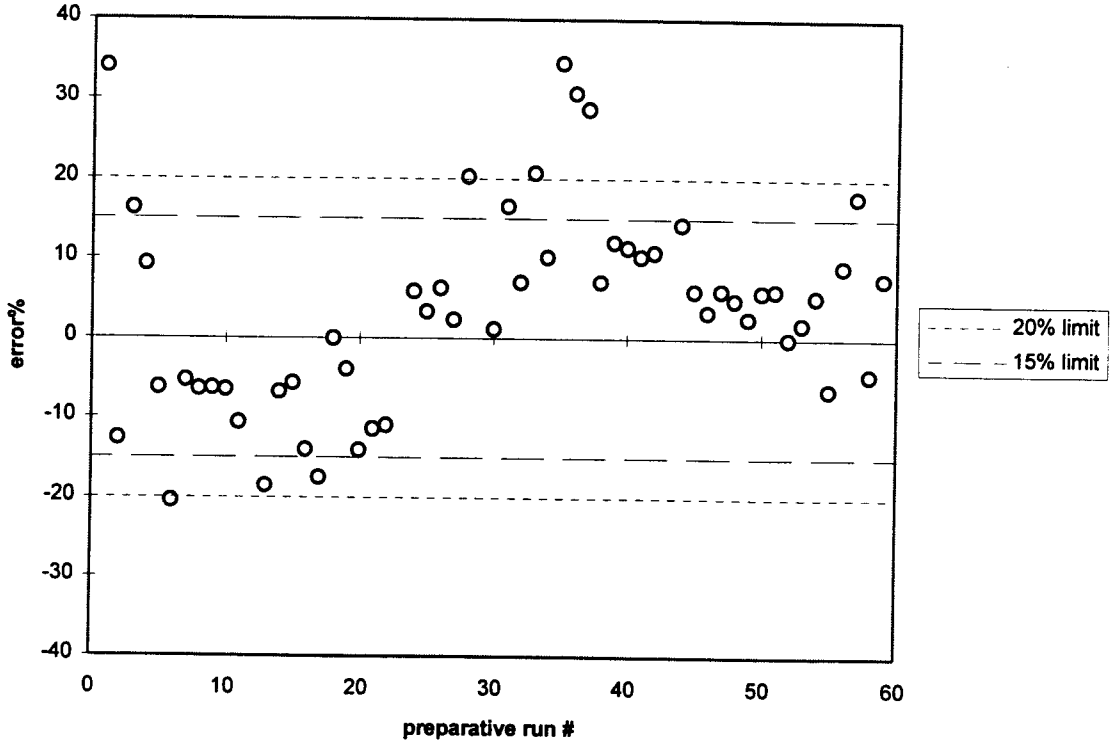


Figure 3.2: Error distribution on the X~trp peptide mass balances.

3.2 ACN conditions

3.2.1 Initial runs

Of the four peptides examined, X~phe and X~trp were considered interesting peptides to separate, in the sense of having a low separation factor yet eluting within a reasonable amount of time (Figure 3.3). X~phe and X~trp dipeptides have very similar retention. RP-HPLC is often used for very difficult separations. While slightly higher for preparative chromatography, in analytical chromatography a difficult separation is

considered to have a separation factor (α) of 1.1 or less. For instance, α=1.2 for X~phe and X~trp at 20% isocratic ACN (Table 3.1). X~ala elutes too fast (*k'*=0.554) while the X~val is not close enough to X~phe (α=4.0) or X~trp (α=4.9) to be a system we want to consider. Therefore X~phe and X~trp was used for preliminary preparative injections.

	X~ala	X~val	X~phe	X~trp
k' at 20% ACN	0.554	1.38	5.47	6.76
	X~phe -- X~trp	X~val -- X~phe	X~val -- X~trp	
α at 20% isocratic ACN	1.2	4.0	4.9	

Table 3.1: *k'* and α values for 20% isocratic ACN.

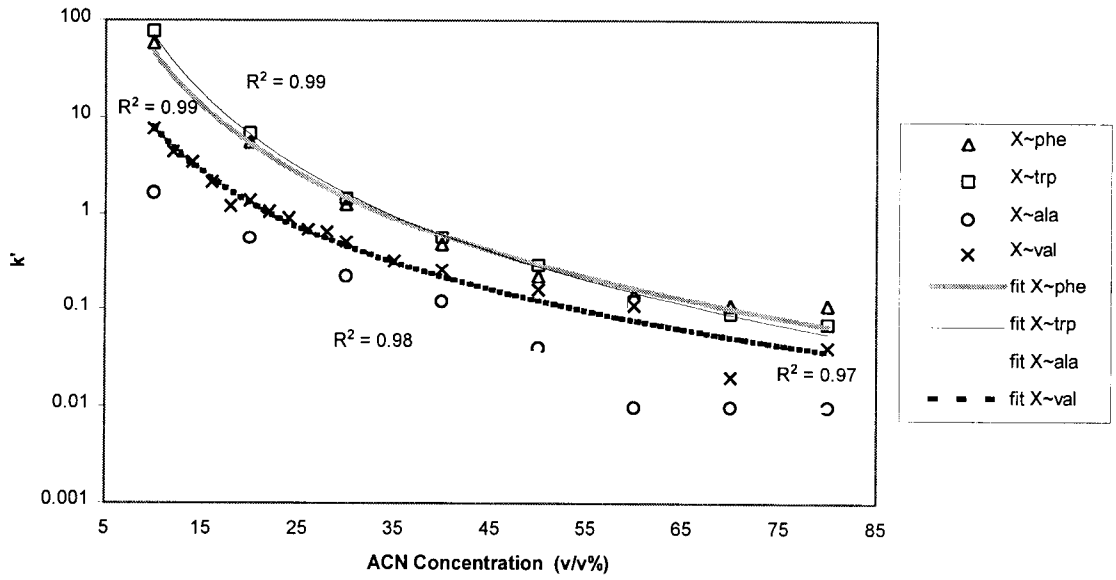


Figure 3.3: Adsorbate retention on Novapak C-18 column under Acetonitrile conditions.

A 20 minute ACN gradient from 10% to 40% was used for initial overloaded trials. This gradient was chosen because all solutes will have eluted by the time 40% ACN concentration is reached, and 20 minutes is a reasonable separation. A 0.5mg/ml feed concentration of X~phe and X~trp each in 0.5ml was tried. The result is shown in Figure 3.4. The peaks are mixed and relatively Gaussian, and resemble an isocratic elution, considering that the initial feed solution of 0.5 mg/ml diluted to about a peak of 0.15mg/ml. This indicates that additional mass can be added, since overloaded conditions were not reached (and higher loadings might improve the separation, due to nonlinear competitive effects). However increasing the concentration caused a "dip" in the phenylalanine peak, as shown in Figure 3.5. X~trp increased in concentration. The increase in concentration is probably due to the gradient compression effect under overloaded conditions. Increasing the volume instead of the mass (Figure 3.6) shows a similar dip but no concentration of the X~trp. Doubling both the X~trp and X~phe results in the X~phe being pushed forward as all binding sites seemed to be taken up. The dip however still remains.

A complication arose with these runs. The initial feed solutions were made in buffer solution containing no TFA, and so the initial column solvent conditions differed to the feed solvent composition. Under large injection volumes, this can affect peak shape. It was discovered later that X~phe is very insoluble in ACN/buffer/TFA, and therefore, the X~phe entered into conditions which were unfavorable in terms of solubility. This may have caused some of the X~phe to precipitate onto the SP at the

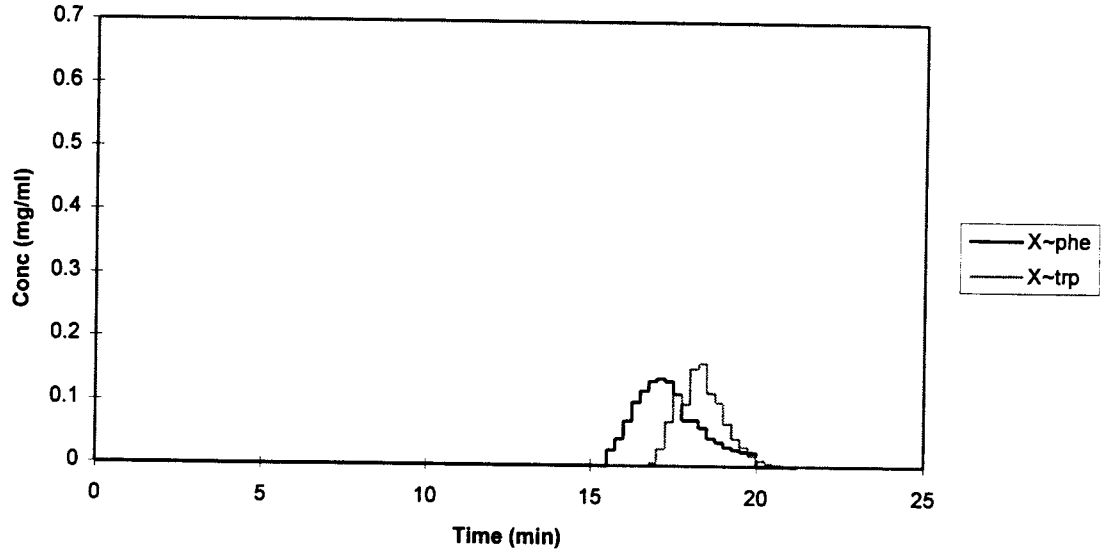


Figure 3.4: ACN gradient 10-40% in 20min. 0.5 mg/ml X~phe and 0.5mg/ml X~trp in 0.5ml feed volume. 0.1% TFA and buffer throughout. Flow rate 1ml/min. Novapak C-18 column (150 x 3.9 mm I.D.). UV detection at 214nm.

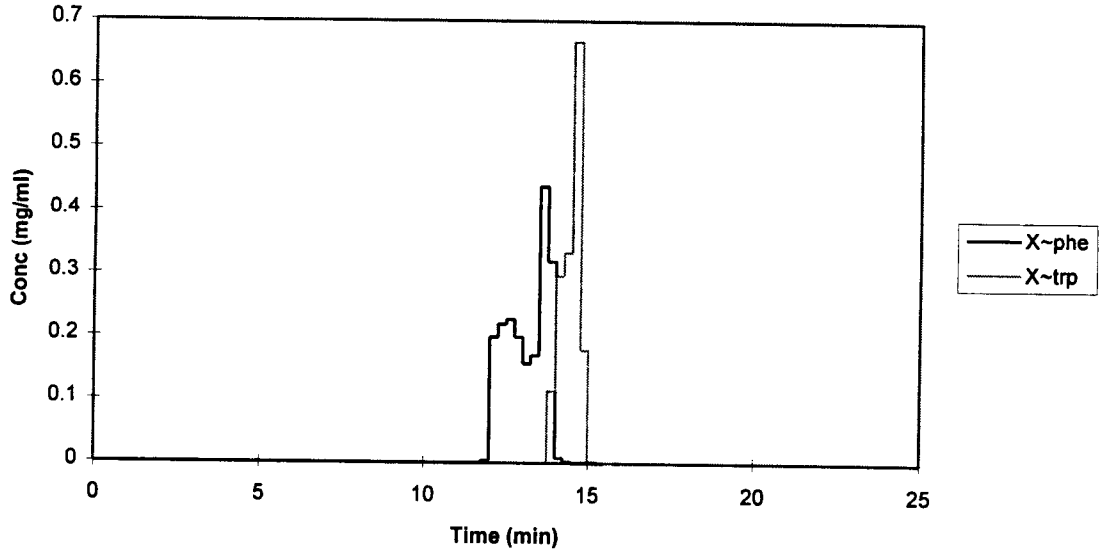


Figure 3.5: 1.0mg/ml X~phe and 1.0mg/ml X~trp, 0.5ml volume. All other conditions are identical to Figure 3.4.

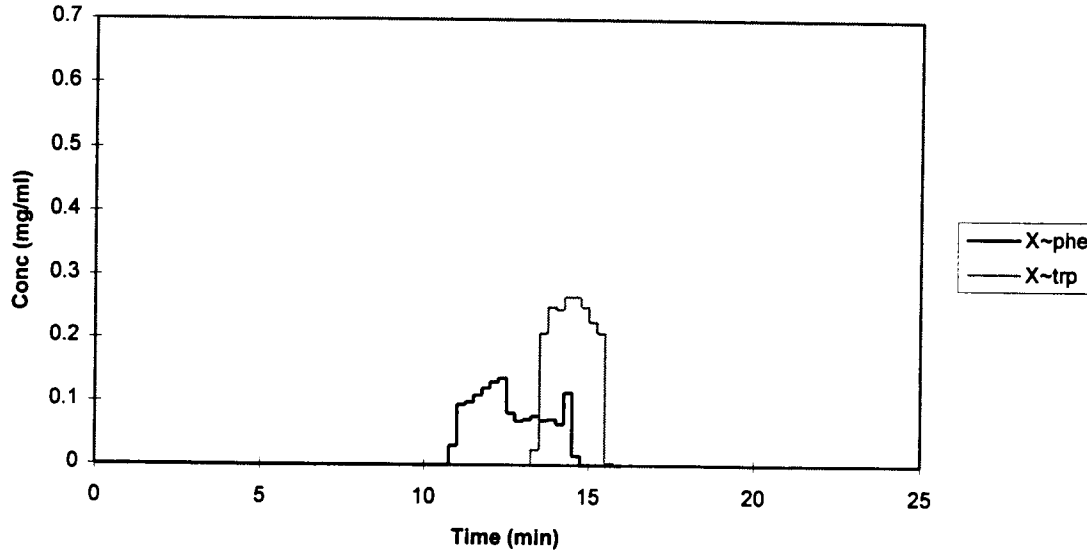


Figure 3.6: 0.5 mg/ml X~phe and X~trp, 1.0 ml volume. All other conditions are identical to Figure 3.4.

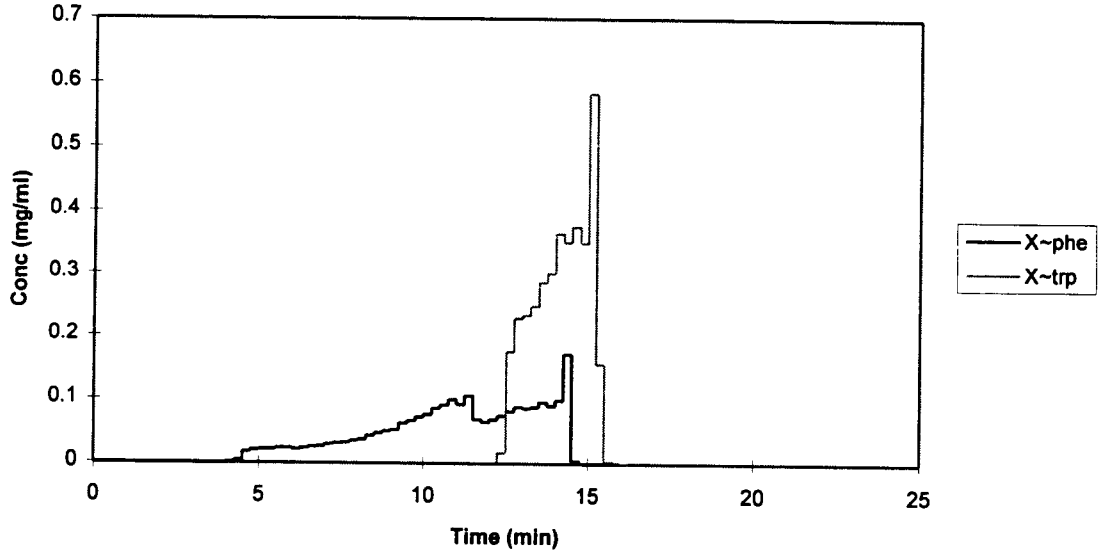


Figure 3.7: 1.0mg/ml X~phe and X~trp, 1.0 ml volume. All other conditions are identical to Figure 3.4

inlet of the column and caused some X~phe to elute ahead. The long band in Figure 3.7 is due to this high concentration causing the X~phe to elute almost unretained due to competition for binding sites. As the dipeptides elute, at a certain point, the X~phe resolubilizes either because the concentration decreased enough, or the insoluble X~phe became more soluble in the presence of X~trp and caused the X~trp to be pulled ahead. In Figure 3.5 the two dipeptides are well separated despite the dip. The presence of the dip in the X~phe peak suggests that X~phe binds at two different retentions. It is reasonable to think that it is due to the insolubility of X~phe. If the X~phe is more soluble in the presence of X~trp, for instance, some of the X~phe attempts to remain with the X~trp while the rest elutes quickly, possibly because this portion was pushed ahead by the higher organic concentration in the feed. Increasing the volume and/or the concentration further, causes the X~trp to be moved ahead noticeably, while the X~phe elutes almost unretained. It is as if the X~trp is being pulled by the X~phe. The X~phe, being in such unfavorable conditions, tries to elute as fast as possible.

The effect of the dip could not be examined any further. The conditions of the column changed (see section 3.2.4), and these dips could not be reproduced. Nevertheless, insolubility may be a cause of the dips. These results are interesting as they show that insolubility of solutes can be a great limitation when using HPLC. These runs were therefore discontinued, and attempts were made thereafter to obtain higher peptide solubilities.

3.2.2 Effect of feed conditions

At high injections of feed volume, the sample solvent and mobile phase should be identical to that of the initial column condition; otherwise the peaks deform and may split, and peak efficiency is reduced, affecting the separation (Porsch 1993). Nevertheless, a differing sample solvent condition tends to be the case in practice; to have as high a solubility as possible frequently requires the feed samples be in different conditions from that of the column mobile phase. If the organic modifier in the feed sample is less than that of the mobile phase, then enrichment can occur (Werkhoven et al. 1981). This is similar to using a stepwise elution. In stepwise elution, the feed components are injected in low modulator concentration, and after a short time, the modulator concentration is increased in a step. The lower isocratic condition causes the components to be strongly bound to the column. The step increase in modulator suddenly makes the peptides elute almost unretained, and band broadening is decreased, and peaks are sharpened.

A typical preparative trace is shown in Figure 3.8. At 214nm under high loadings, the X~phe and X~trp peaks saturate, while the presence of impurities are present. In 40/60/0.1 ACN/buffer/TFA a higher concentration could be obtained for the peptide mixture than in 10/90/0.1 ACN/buffer/TFA (see section 3.2.3). In addition, samples with no TFA were more soluble. Therefore, the possibility of using feed conditions that are different than initial starting column conditions was examined. A 0.5mg/ml of the dipeptide mixture in 40/60 ACN/buffer was injected into a 10-40% ACN gradient (Figure 3.9). Comparing to a 0.5mg/ml solution in 10/90 ACN/buffer

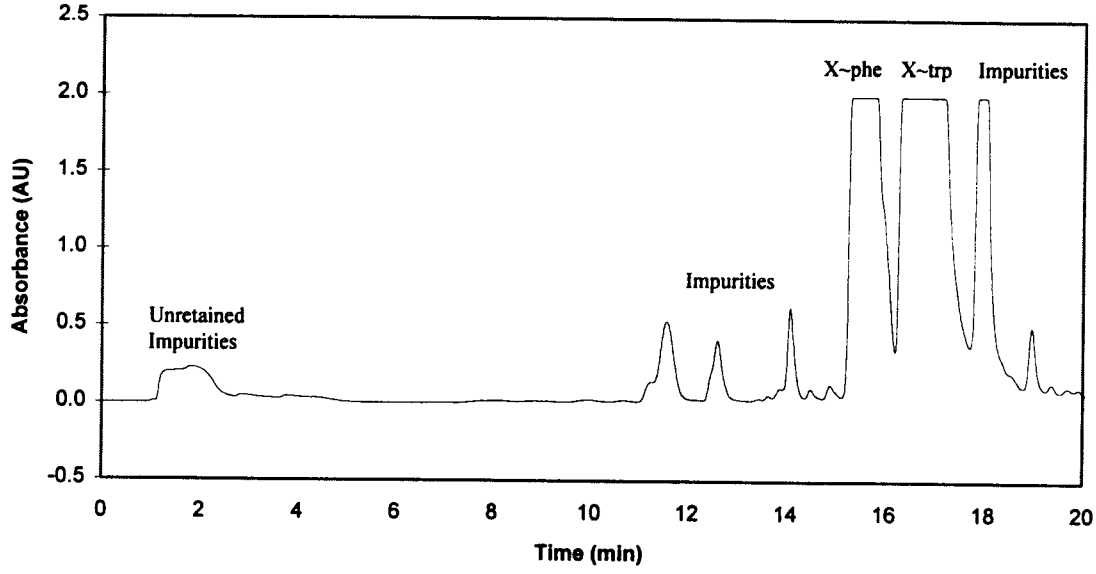


Figure 3.8: Trace of preparative chromatographic run, in which the column and the feed have identical conditions (10/90/0.1 ACN/buffer/TFA). The conditions are 10-40% ACN gradient in 20min in 10mM sodium phosphate buffer, 0.1% TFA throughout. 0.5mg/ml X~phe and 0.5mg/ml X~trp. 1ml feed volume. Flow rate 1ml/min. Novapak C-18 column (150 x 3.9mm I.D.). UV detection at 214nm. The plateau is due to the saturation of the UV detector.

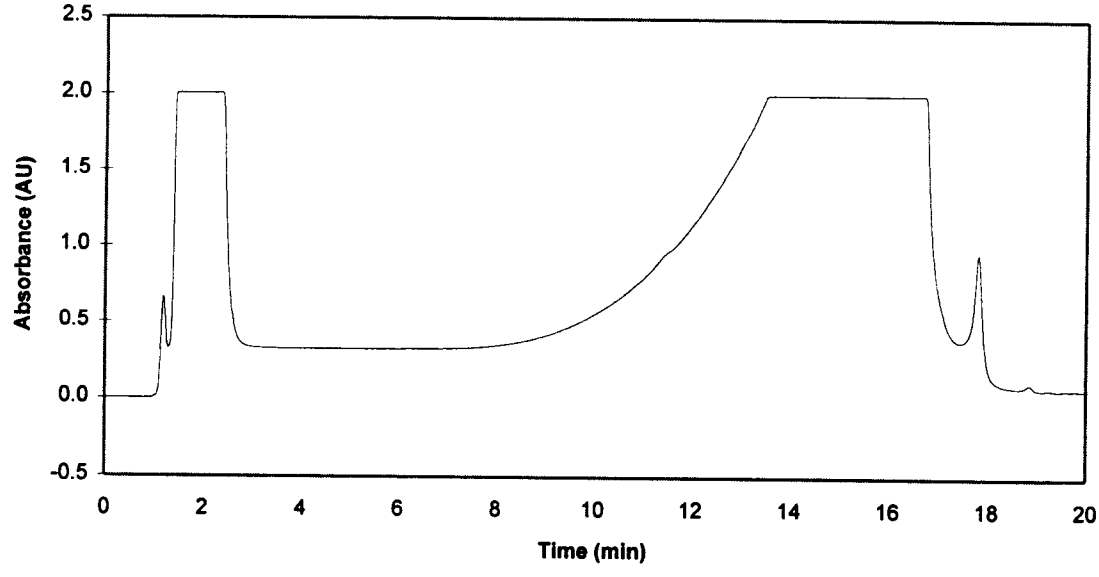


Figure 3.9: Trace of preparative chromatographic run, in which the feed is in 40/60ACN/buffer. All other conditions are identical to Figure 3.8.

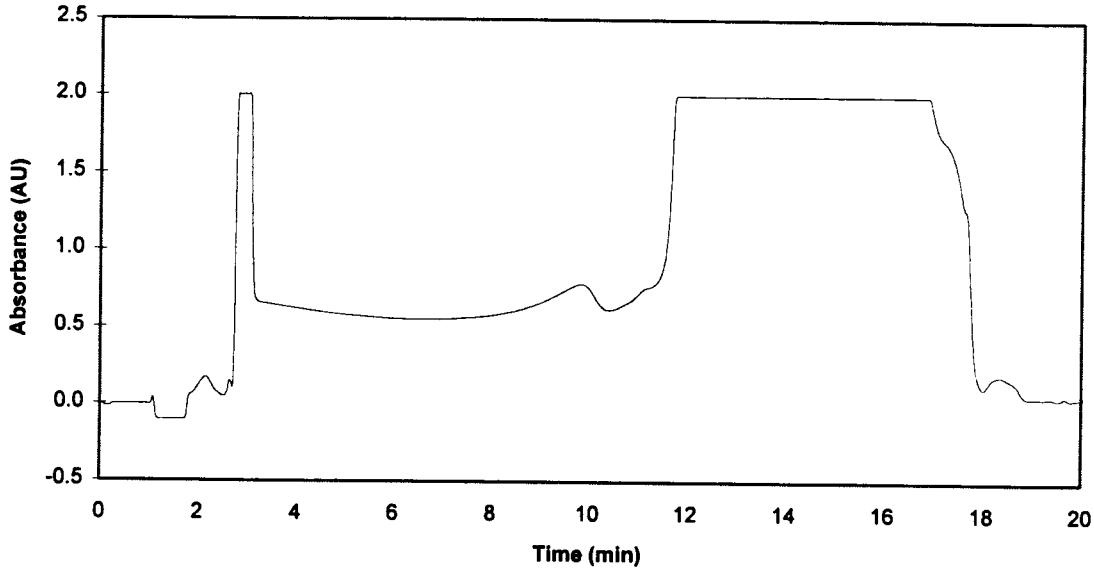


Figure 3.10: Trace of preparative chromatographic run, in which the column is pre-equilibrated in 10/90/0.1 ACN/buffer/TFA, but the feed is in 10/90 ACN/buffer (no TFA). All other conditions are identical to Figure 3.8.

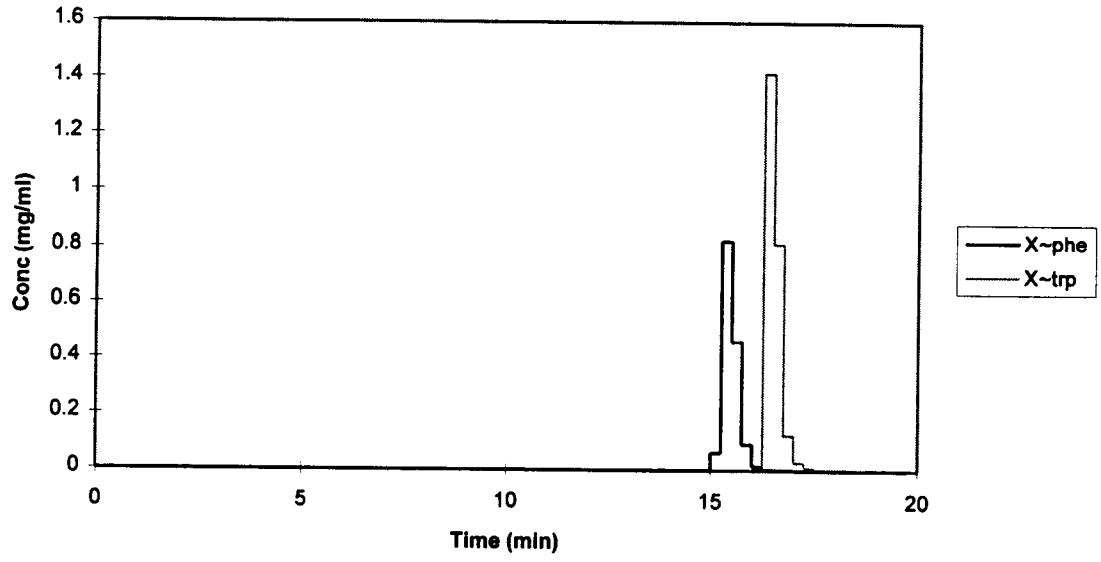


Figure 3.11: Preparative chromatographic run identical to Figure 3.8, with fraction collection. The column and the feed are in identical conditions.

with an identical gradient (Figure 3.8), the difference is remarkable. Having a higher organic feed condition caused part of the feeds to elute unretained, causing an unusually large "split" in the chromatographic peaks.

A similar effect occurs if TFA is removed from the feed (Figure 3.10) but keeping the organic equal to the column condition. A lack of TFA in the feed acts like having a greater organic concentration in the feed. Fractions were collected for these preparative runs. The chromatogram obtained from the fraction collection seems to indicate that X~phe is particularly sensitive to such a change in condition; most of the X~phe peak elutes at t_0 (compare Figures 3.11 and 3.12). A possible explanation is that

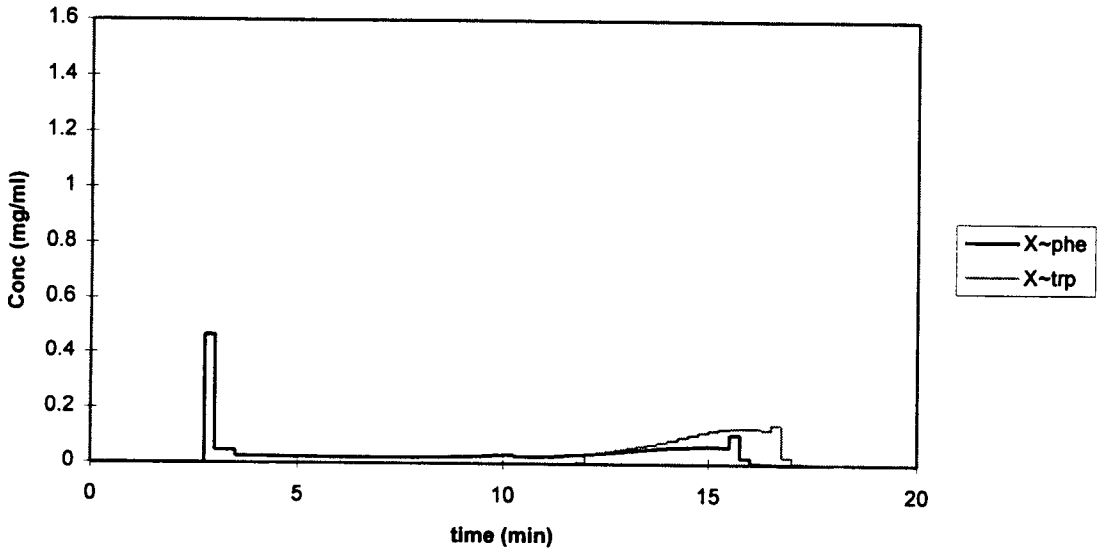


Figure 3.12: Preparative chromatographic run identical to Figure 3.10, with fraction collection. Column is pre-equilibrated with 10/90/0.1 ACN/buffer/TFA but the feed is in 10/90 ACN/buffer (no TFA).

a local "no-TFA" region moves through the column causing the relatively insoluble X~phe to want to remain in that region, and therefore move with it, ahead of other solutes not in that region, and a deformed peak results. Having both a 40% ACN feed that does not contain TFA results in an extreme condition where both peptide peaks split into two regions (Figure 3.13).

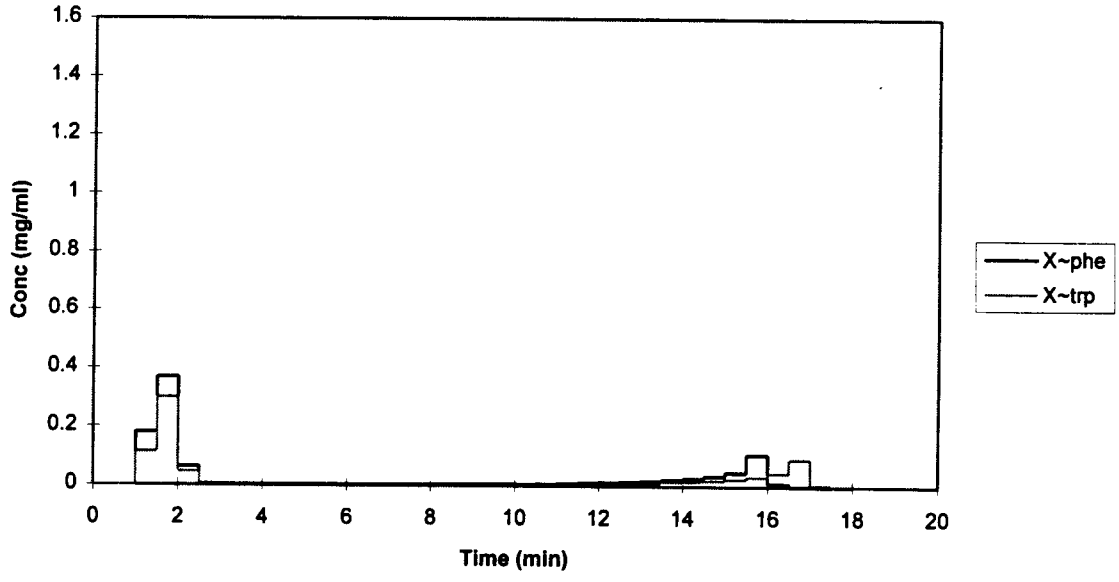


Figure 3.13: Preparative chromatographic run identical to Figure 3.12, except the feed is in 40/60 ACN/buffer (no TFA).

3.2.3 Peptide solubility

The initial runs have shown the necessity of having the initial column condition equal to the feed conditions, and therefore the need to maximize solubility at low

mobile phase modulator concentration. The X~phe and X~trp need to be in organic modifier concentrations of around 10-20% to have the same conditions as that of the column initial conditions. Solubility estimation of these molecules was done qualitatively; that is, the solution was considered soluble if no precipitate was observed.

	Feed Component	Concentration mg/ml	Solvent condition (method)	Solubility
(1)	X~phe	0.75	15/85 ACN/buffer (A)	S
	X~phe	0.75	15/85/0.1 ACN/buffer/TFA (E)	I
	X~phe	1.0	15/85 ACN/buffer (B)	S
(2)	X~trp	1.0	15/85 ACN/buffer (A)	S
	X~trp	1.0	15/85/0.1 ACN/buffer/TFA (D)	S
(3)	X~phe	0.75	15/85/0.1 ACN/buffer/TFA (D)	I
	X~phe + X~trp	0.75	15/85/0.1 ACN/buffer/TFA (D)	S
(4)	X~phe	0.5	15/85/0.1 ACN/buffer/TFA (E)	I
	X~phe	0.5	15/85/0.1 ACN/buffer/TFA (D)	S
(5)	X~phe + X~trp	0.5	10/90/0.1 ACN/buffer/TFA (E)	S
	X~phe + X~trp	0.5	10/90/0.1 ACN/H_2O (A)	I
(6)	X~phe + X~trp	1.5	40/60/0.1 ACN/buffer/TFA (C)	S
	X~phe + X~trp	1.5	40/60 ACN/H_2O (A)	I

Table 3.2: Solubility of feeds in ACN/buffer (S: soluble, I: insoluble).

In trying to solubilize these dipeptides, various methods (A, B, C, D and E) were used as described in section 2.4. The methods C, D and E correspond to samples that contained TFA.

Several observations were made about the X~phe and X~trp peptides (Table 3.2):

(1) There are some indications that suggest that X~phe by itself is insoluble in 0.1%TFA. For instance, at 15/85 ACN/buffer, 0.75 mg/ml could be obtained without TFA using method A, but not with TFA present using method E. Even 1mg/ml could be obtained with method B when no TFA is present.

(2) On the other hand, X~trp seemed unaffected by the TFA; 1mg/ml could be obtained in 15/85 ACN/buffer and 15/85/0.1 ACN/buffer/TFA. The X~trp was more soluble than X~phe for all conditions used in the runs. The limitation on solubility was therefore primarily due to X~phe.

(3) X~phe seemed to solubilized in the presence of X~trp, but not by itself. 0.75mg/ml of X~phe alone would not solubilize in 15/85/0.1 ACN/buffer/TFA, but would solubilize if X~trp was present.

(4) An interesting observation was that adding the X~phe in the organic solvent first seemed to improve its solubility; 0.5mg/ml of X~phe using method E would not solubilize, but would solubilize using method D.

(5) The buffer was also compared to water. 0.5mg/ml of X~phe and X~trp could be obtained in 10/90/0.1 ACN/buffer/TFA, but could not be obtained in 10/90 ACN/H_2O even though TFA was not even present in the latter case.

(6) At higher organic concentration of 40/60/0.1 ACN/H_2O, 1.5mg/ml X~phe and X~trp solution was insoluble. Up to 1.5mg/ml of the dipeptide mixtures could be obtained at 40/60/0.1 ACN/buffer/TFA.

To obtain an isotherm for X~phe, a high concentration is needed under a low organic solvent concentration. 0.5mg/ml in 15/85/0.1 ACN/buffer/TFA (method C or D) shown in Table 3.2 was used for the isotherm determination. It should be noted that adding TFA at the end (methods C or D) worked for 0.5mg/ml of X~phe for a limited time. Stirring the sample for a long period of time (e.g. 2 hours) would eventually cause the peptide to fall out of solution. Solubilizing the peptide initially in no TFA conditions apparently allowed it to enter into solution, and eventually the added TFA would cause

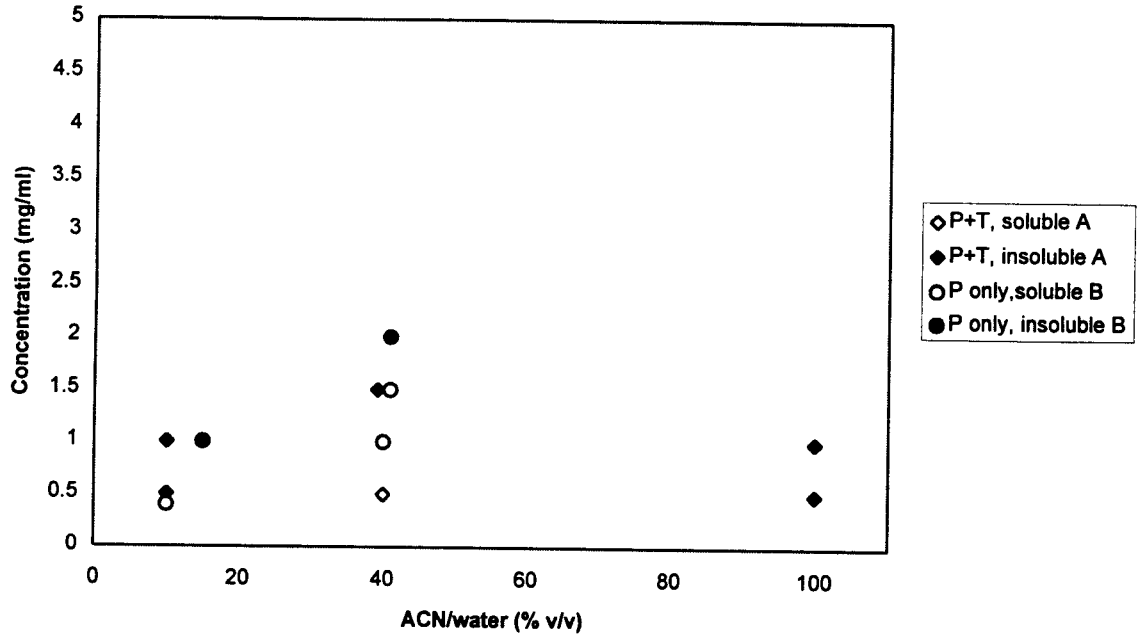


Figure 3.14: Solubility of X~phe (P) and X~trp (T) in ACN/water solutions, using methods A and B (without TFA)

it to precipitate. This "metastable" state can be used to advantage. In preparative chromatography, if the sample in question can remain soluble long enough to be used during the chromatographic purification, after the fractions are collected, the metastable peptides can either be immediately diluted or the conditions changed so as to prevent it from precipitating. This was done for some of the preparative runs (see section 3.2.6).

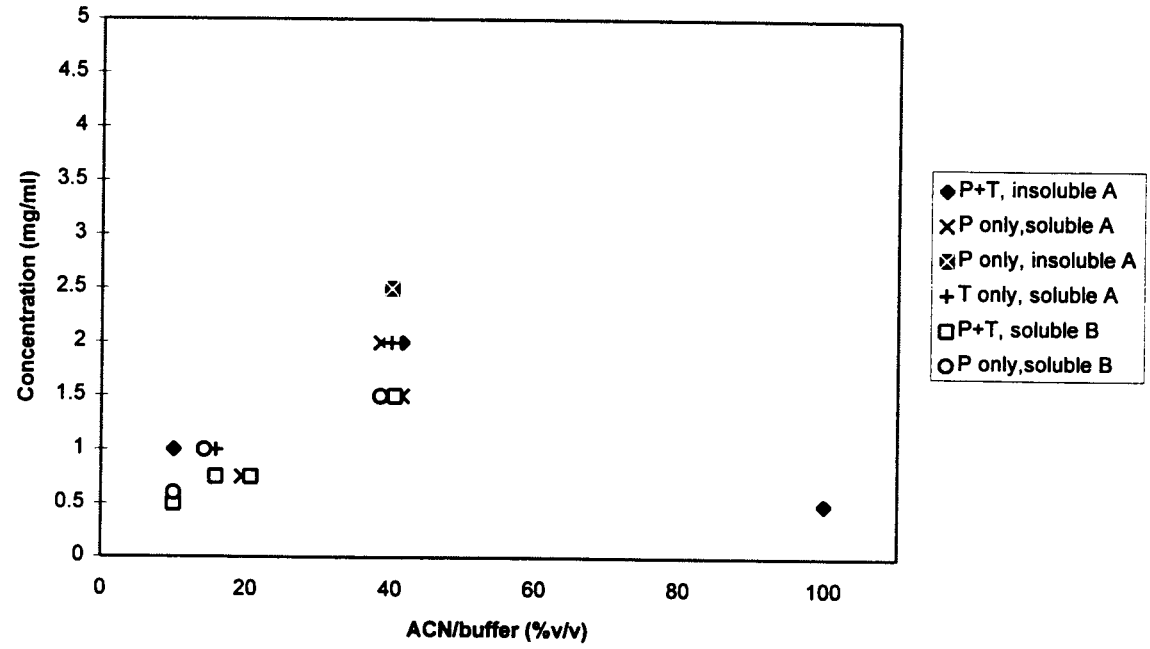
Up to 1mg/ml in 40/60 ACN/buffer had been used for the runs where the feed differed from the column conditions (see section 3.2.2). Since 0.75mg/ml of X~phe and 0.75mg/ml X~trp mixture solubilized at 10/90 ACN/buffer, these feed concentrations were used for the preparative runs (section 3.2.5 and 3.2.6).

A summary of all the solubilities done for ACN/buffer and ACN/H_2O is shown in Figures 3.14 and 3.15 (a and b).

3.2.4 Adsorbate retention and adsorption isotherms

After the preliminary runs were done, the column used under ACN/buffer conditions was tested again for the retention of X~phe and X~trp. The column was found to have deteriorated over time. Comparing Figure 3.17 with Figure 3.3 (section 3.2.4), retention time decreased. At 20% isocratic ACN/buffer, the capacity factors decreased by about 30% (Table 3.3).

a)



b)

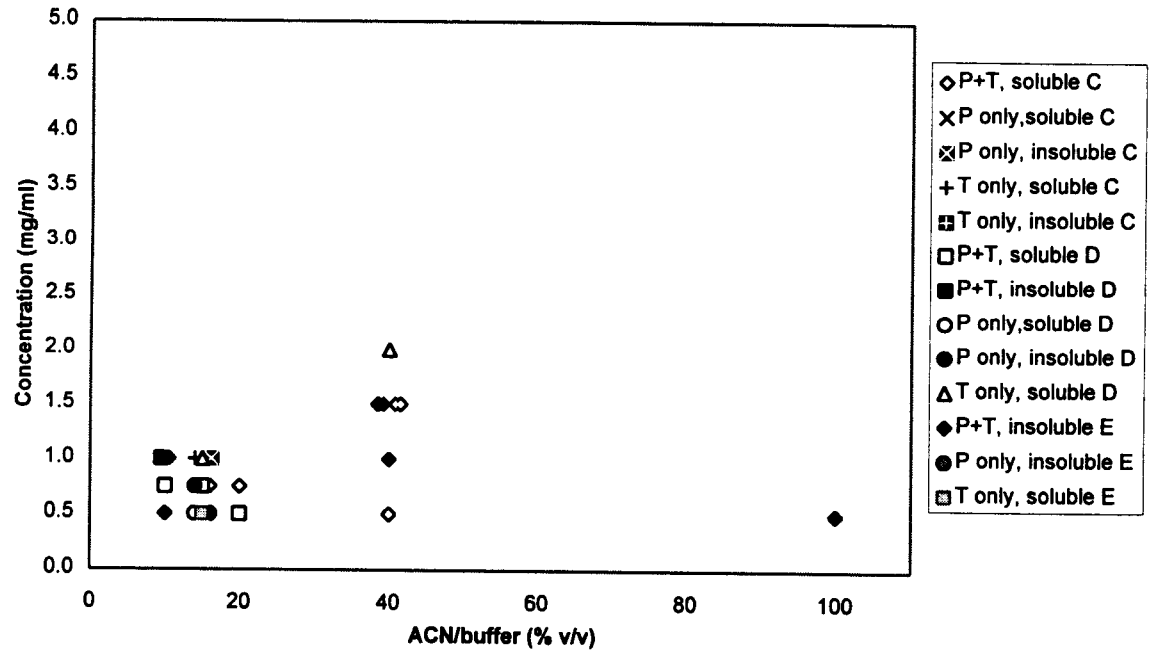


Figure 3.15: Solubility of X~phe (P) and X~trp (T) in ACN/buffer solutions, using different methods (A-E): (a) without TFA, (b) with 0.1% TFA

	X~phe	X~trp
k' of 6/27/95 (20-80 ACN-buffer)	5.5	6.8
k' of 9/13/96 (20-80 ACN-buffer)	4.2	5.2

Table 3.3: Retention factors *k'* in 20% isocratic ACN on Novapak column; comparison of retention times, and of their changes over time.

The retentions of the peptides do not vary greatly using H_2O as solvent or using buffer conditions (Figure 3.16 and Figure 3.17). Since a concentration of 0.75mg/ml could be obtained in 10/90/0.1 ACN/buffer/TFA, preparative runs were done under those conditions. Several observations can be made from figure 3.17: 1) The plot is non-linear. In reversed phase, the logarithmic adsorbate retention factor is commonly assumed to depend linearly on the organic modifier concentration, e.g. in the linear solvent strength (LSS) theory (Snyder 1980) . The LSS theory states that under reversed phase conditions, *k'* varies exponentially with time. This condition requires that *k'* be exponentially dependent on the modulator concentration and that the gradient be linear at the inlet of the column. The *k'* dependence on the modulator concentration means that

$$k' = Ae^{-BC_M} \qquad (3.1)$$

Taking the logarithm on both sides,

$$\ln k' = \ln A - BC_M \qquad (3.2)$$

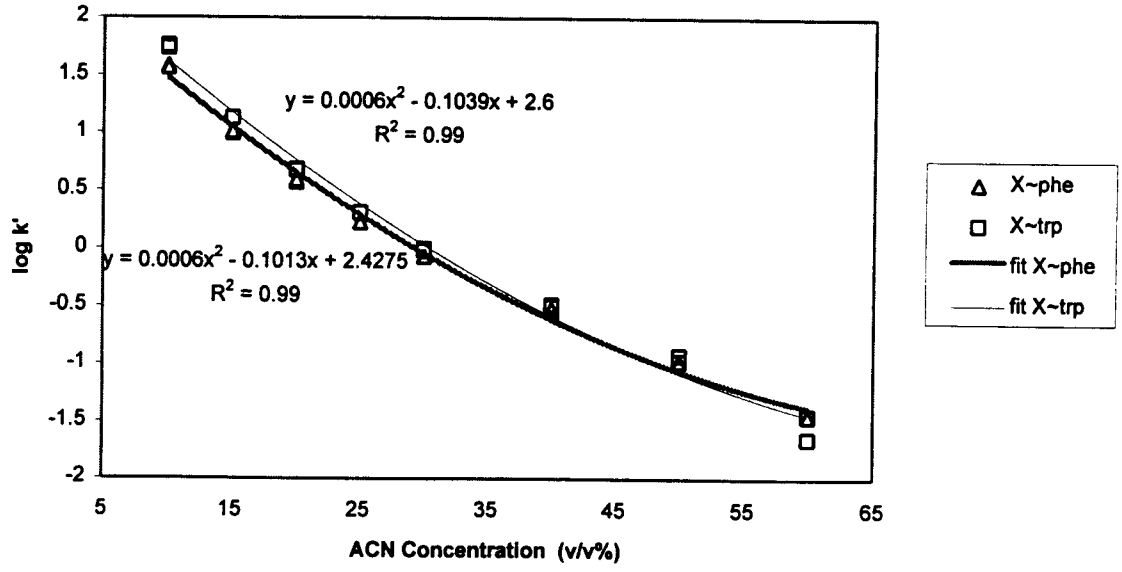


Figure 3.16: Adsorbate Retention factors in ACN/H2O.

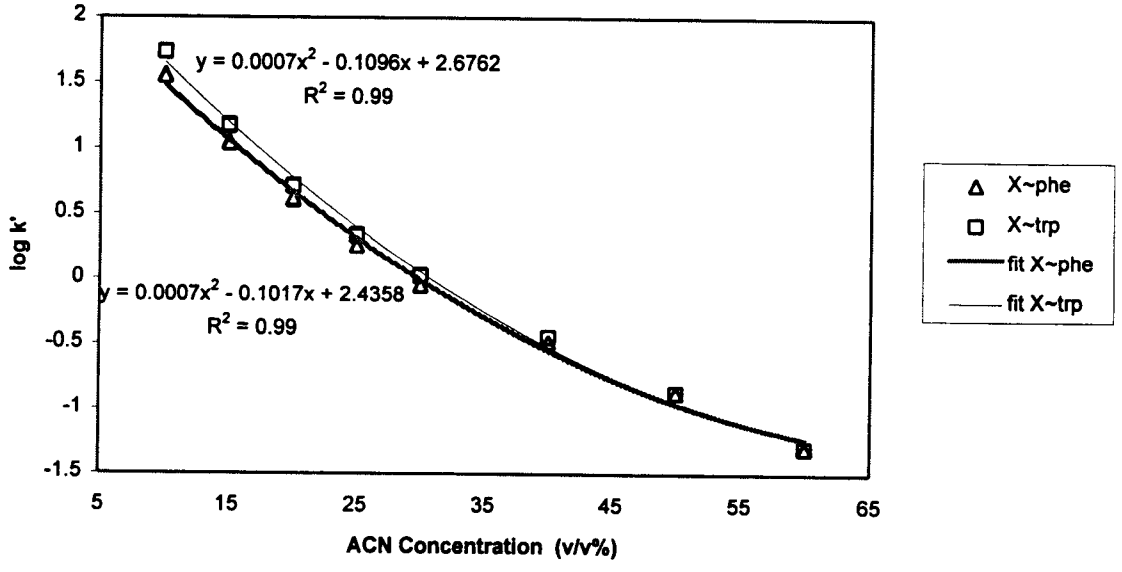


Figure 3.17: Adsorbate Retention factors in ACN/buffer.

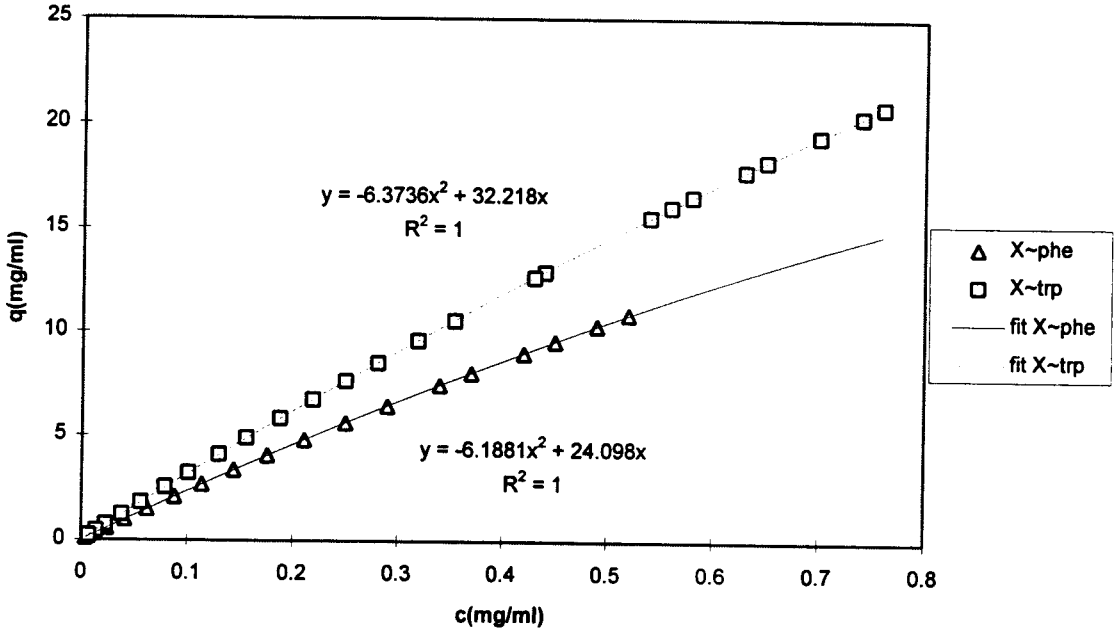


Figure 3.18: Isotherms of X~phe and X~trp obtained by using the "Elution by Characteristic Point" method. These were obtained from the isocratic runs on Figure 3.19 and 3.20.

We see that this relationship is linear. However, the experimental data for the system we are using for X~phe and X~trp are not linear (Figure 3.17); the data was fitted to a quadratic function $f(x)=Ax^2+Bx+C$. Therefore, the LSS theory does not hold. This has implications for the computer simulations of this system. The LSS theory allows one to predict the retention of the peptides given the gradient change and isocratic k'. However, according to (Snyder 1986), the non-linearity of $ln\ k'$ vs. C_M can be approximated as linear over a range of $1<k'<10$. Further work by Glajch et al. (1986), show that "non-linearity of log k' vs. ϕ plots can be treated by LSS theory as well as the

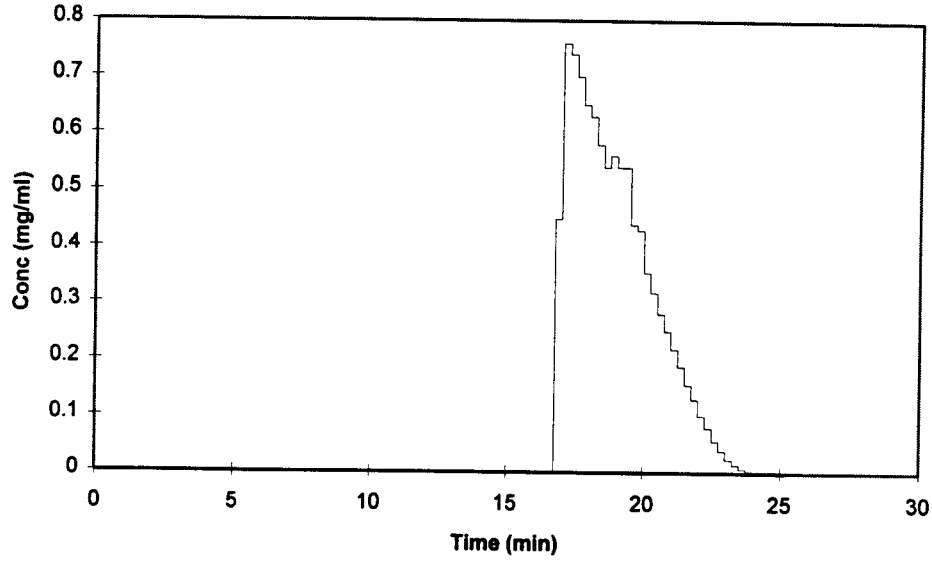


Figure 3.19: Isocratic run for isotherm determination. 15% isocratic ACN, buffer and 0.1% TFA throughout. 0.9mg/ml X~trp, 2.4ml volume. Novapak C-18 (150 x 3.9 mm I.D.). UV detection at 214nm.

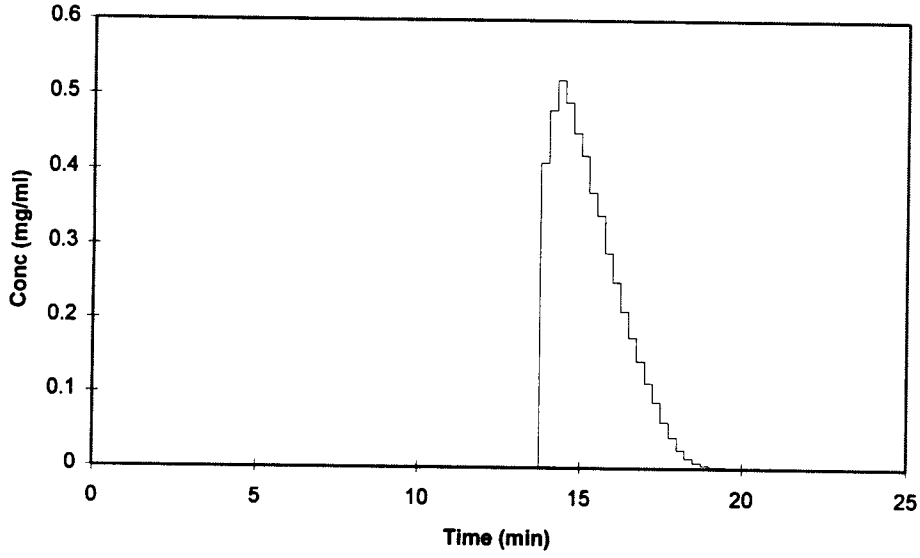


Figure 3.20: Isocratic run for isotherm determination. 0.5mg/ml X~phe in 2.4ml volume. All other conditions are identical to Figure 3.18.

case of linear plots". This is reasonable so long as only an approximation is required. However, for simulation purposes, an exact fit is required.

(2) The curves are very close: the plot shows that the X~phe and X~trp compounds are very closely related, and therefore very difficult to separate. $\alpha \leq 1.1$ is considered a difficult separation; for preparative separations the value is generally higher. At 15% ACN, α=14.8/11.3=1.3, but at 20% ACN, α=4.1/5.3=0.7.3) The curves converge: As the modulator concentration is increased, the curves become narrower and converge. That means the higher the organic concentration the more difficult the separation.

The above observations depict a realistic situation in peptide purifications. These limitations make purification extremely expensive. However, such a situation can be improved under conditions of high loading.

The adsorption isotherm were obtained using the ECP method (see section 2.3) for individual preparative runs of X~trp and X~phe in 15/85/0.1 ACN/buffer/TFA conditions (Figure 3.18). The highest concentration obtainable for the single component runs for X~trp was 0.9mg/ml (Figure 3.19) and 0.5mg/ml for X~phe (Figure 3.20). The adsorption isotherms show the beginnings of non-linearity.

A Langmuir equation did not fit the data well on Figure 3.18 so a quadratic fit was used. The quadratic gives a good fit because the isotherm is very shallow, and an approximation can be made on the Langmuir isotherm. The Langmuir isotherm is given by:

$$q = \frac{ac}{1+bc} = ac(1+bc)^{-1} \qquad (3.3)$$

Considering the binomial expansion, and since the isotherm curve is shallow, $bc<1$,

$$\frac{1}{1+bc} = 1 - bc + b^2c^2 - b^3c^3 + ... \approx 1 - bc \qquad (3.4)$$

$$q = ac(1-bc) = ac - bc^2 = quadratic \qquad (3.5)$$

Therefore a quadratic is a good representation of the isotherm behavior of these peptides and could be used for simulation purposes.

3.2.5 Isocratic elution

Using 0.75mg/ml of X~phe and 0.75mg/ml of X~trp in 20/80/0.1 ACN/buffer/TFA mixture an isocratic 20/80/0.1 ACN/buffer/TFA was done (Figure 3.21). It is known that $k' \approx 55$ (Figure 3.17, section 3.2.4) for the dipeptides in 10% isocratic ACN, and $k' \approx 5$ for peptides in 20% isocratic. Therefore a 20% isocratic ACN is a reasonable starting point; the resulting peaks are just beginning to mix (Figure 3.21). There seems to be very little peak tailing, and because of the short time the peptides have spent in the column, there has not been too much peak dilution. Any further increase in volume for instance from 1ml to 2.4ml only increases the mixing of the two compounds (Figure 3.22).

This isocratic run can be further optimized by improving the resolution of the peaks by lowering the organic concentration to 15%. However, lowering the concentration too much will result in too long a retention ($k' \approx 55$), which will decrease the productivity. At 15% ACN, the runs are very well separated under similar

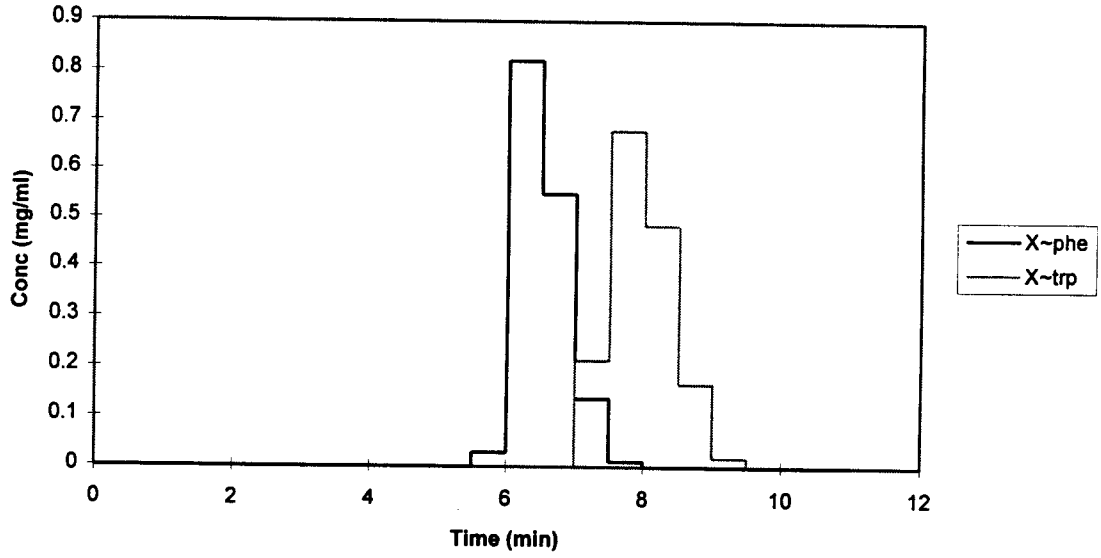


Figure 3.21: Isocratic 20/80/0.1 ACN/buffer/TFA. 0.75mg/ml X~phe and X~trp in 1 ml feed volume. Fractions were collected at 1/2 minute intervals. Novapak C-18 RP column (150 x 3.9mm I.D.). UV detection at 214nm.

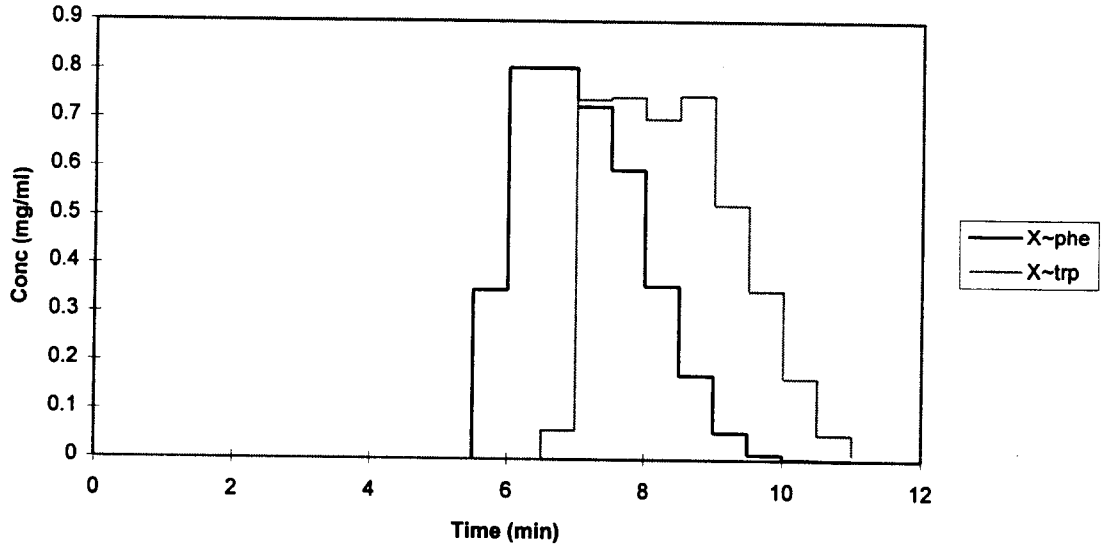


Figure 3.22: Identical to Figure 3.21 except for 2.4 ml feed volume.

conditions to the 20% ACN run Figure 3.23). The 0.5mg/ml feed concentration was used because it was made by diluting a 0.75mg/ml at 20/80/0.1 ACN/buffer/TFA. However, a higher concentration (0.75mg/ml) was eventually found to be possible at 15/85/0.1 ACN/buffer/TFA. Increasing the volume further to 2.4ml was possible (Figure 3.24) as well as 3ml (Figure 3.25). At 3ml volume the X~phe is beginning to significantly tail into the X~trp.

The increased mixing by volume overloading under 20% isocratic ACN resulted in no increase in the amount of peptides purified. The yields decreased dramatically (90% to 30% for X~phe and 86% to 14% for X~trp, for instance) and the amount purified remained about the same for X~phe and decreases for X~trp (Table 3.4). As expected, the decrease in the modulator to 15% isocratic ACN resulted in 100% yield, but an increase to 3ml reduces the yield to 94%. The peptides are being affected by the increase in the volume as the X~phe begins to tail into the X~trp peak. Nevertheless, a large amount is produced. Due to the solubility limitation (the X~phe peak is concentrated beyond its solubility limit), no further increase in volume was considered.

The productivities of these isocratic runs varied from 1.32 mg/ml to 6.6mg/ml. In calculating the productivity, the t_{cyc} given in equation 3.1 defines a cycle time. The cycle time refers to the time it takes for the column to have completed the whole purification cycle. This is the time which includes the preparative purification step (t_{pur}), plus the regeneration time (t_{reg}):

$$t_{cyc} = t_{pur} + t_{reg} \qquad (3.3)$$

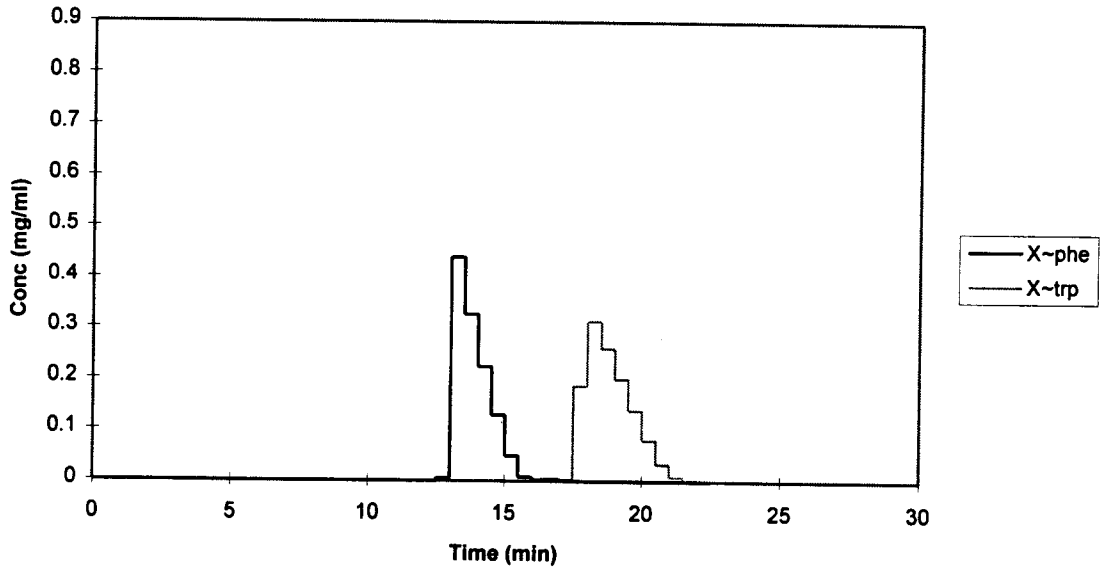


Figure 3.23: Isocratic 15/85/0.1 ACN/buffer/TFA. 0.5mg/ml X~phe and 0.5mg/ml X~trp in 1ml feed volume. All other conditions same as Figure 3.21.

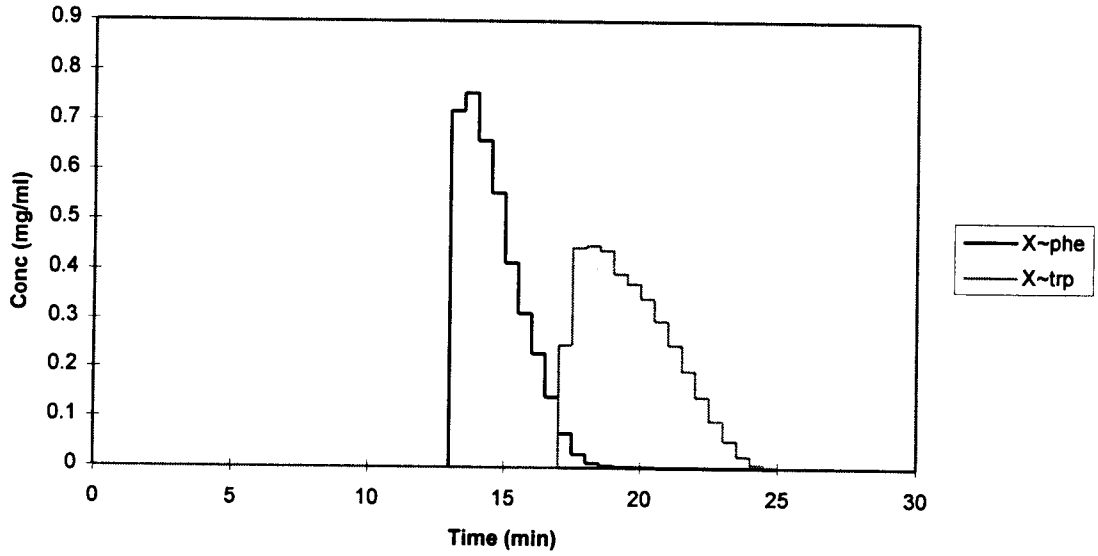


Figure 3.24: Isocratic 15/85/0.1 ACN/buffer/TFA. 0.75mg/ml X~phe and 0.75mg/ml X~trp in 2.4 ml feed volume. All other conditions same as Figure 3.23.

The regeneration time refers to increasing the organic modifier concentration to a high level (e.g., 80% ACN), to clean out the column by eluting all impurities that may be remaining. In gradient elution, a regeneration is performed after each preparative run. In an isocratic runs, it will be assumed that two runs can be performed before a regeneration step is needed. A regeneration time of 20 minutes was taken, meaning that for isocratic elution the $t_{cyc} = t_{pur} + 10$ min, while the gradient would be $t_{cyc} = t_{pur} + 20$ min.

The optimized 15/85/0.1 ACN/buffer/TFA isocratic gave a productivity of around 6.6mg/ml·hr for X~phe and up to 7.2mg/ml·hr for X~trp (Table 3.4). The

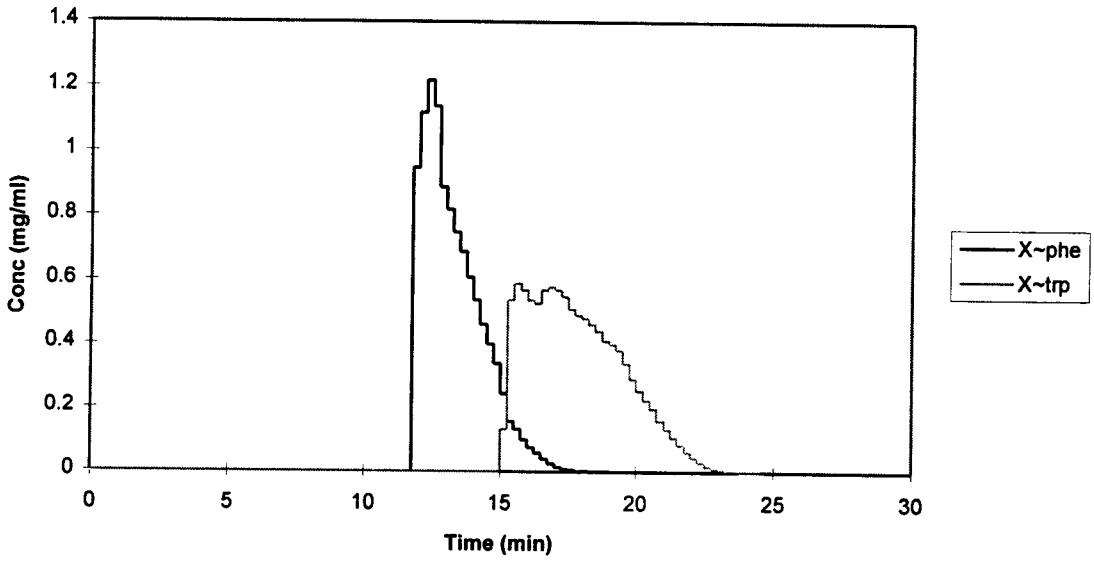


Figure 3.25: Isocratic 15/85/0.1 ACN/buffer/TFA. 0.75mg/ml X~phe and 0.75mg/ml X~trp in 3.0 ml feed volume. All other conditions same Figure 3.24.

Description	X~phe						X~trp					
	98% purity			95% purity			98% purity			95% purity		
	yield (%)	Enrich.	Product. mg/ml·hr	yield (%)	Enrich.	Product. mg/ml·hr	yield (%)	Enrich.	Product. mg/ml·hr	yield (%)	Enrich.	Product. mg/ml·hr
isocratic 20% 0.7mg/ml X~phe 0.7mg/ml X~trp 1ml volume	**90**	**0.65**	**3.54**	**86**	**0.65**	**3.54**	**90**	**0.37**	**3.48**	**86**	**0.37**	**3.48**
isocratic 20% 0.8mg/ml X~phe 0.8mg/ml X~trp 2.4ml volume	**30**	**0.77**	**2.7**	**14**	**0.88**	**4.56**	**51**	**0.19**	**1.32**	**14**	**0.19**	**1.32**
isocratic 15% 0.6mg/ml X~phe 0.6mg/ml X~trp 1.0ml volume	**100**	**0.24**	**1.92**	**100**	**0.24**	**1.92**	**100**	**0.22**	**1.92**	**100**	**0.22**	**1.92**
isocratic 15% 0.7mg/ml X~phe 0.7mg/ml X~trp 2.4ml volume	**97**	**0.64**	**5.52**	**93**	**0.64**	**5.52**	**97**	**0.33**	**5.22**	**100**	**0.33**	**5.52**
isocratic 15% 0.7mg/ml X~phe 0.7mg/ml X~trp 3ml volume	**94**	**0.98**	**6.6**	**78**	**0.98**	**7.2**	**94**	**0.36**	**6.6**	**94**	**0.39**	**6.6**

Table 3.4: Isocratic yields, enrichments (enrich.) and productivities (product.) of X~phe and X~trp, for 98% and 95% purity.

enrichment factor is the ratio between the average concentration of the collected fractions by the average concentration in the feed. The isocratic runs are not able to concentrate the feed (El Fallah and Guiochon 1991), thus the enrichment factor remains below 1. Although at the inlet of the column, the process of feed introduction serves to concentrate X~phe initially, it eventually becomes diluted below the average feed sample concentration. The generally poor enrichment factor for the X~trp is due to its tailing. Collecting all of the tail results in a low average concentration of the region collected.

3.2.6 Gradient Elution

Before considering gradient purification, a note should be made concerning gradient delay. In a chromatographic system, the solvents used to change the gradient (in this case the ACN and buffer) pass through two pumps and a gradient former. Then, before the gradient reaches the beginning of the column, it must first pass through tubing that connects the pump to the autosampler, then through tubing that connects the autosampler to the sample loop where the feed samples were injected. Therefore, there is a delay between the time the feed samples are injected into the column and the time the gradient is able to reach the column inlet. The peptides remain under isocratic conditions for the duration of the gradient delay. This delay must be taken into account when considering the gradient separations. In the isocratic case, because the organic modifier concentration remains constant at all time, there is no time delay.

Finding the gradient delay was done as follows. After 5 minutes of equilibration, a 0.5 minute pulse of 5% acetone was programmed from the solvent reservoir (Figure 3.26). Therefore, after 5 minutes of equilibration, the acetone pulse must travel through the tubing described above, and then through the column void volume until it is detected by UV. The time (t_P) that it takes for the pulse to emerge from the column and be detected by UV is the total of the time delay (t_{delay}), the time it spent in the column unretained (t_0=1.2 min), and the equilibration time before the pulse was injected (5 minutes).

$$t_P = t_{delay} + 1.2\,\text{min} + 5\,\text{min} \qquad (3.4)$$

In addition, the peak detected by UV is measured in terms of its center of mass. Therefore the center of mass of the pulse must be considered. A 0.5 minute pulse has a center of mass of 0.25 minutes. Therefore,

$$t_P = t_{delay} + 1.2\,\text{min} + 5\,\text{min} + 0.25\,\text{min} \qquad (3.5)$$

And finally, the time delay can be calculated,

$$t_{delay} = t_P - 5\,\text{min} - 1.2\,\text{min} - 0.25\,\text{min} \qquad (3.6)$$

The t_R is the time of the unretained pulse. t_R was 11.8 minutes, which gives a delay time of 5.4 minutes. Therefore, a delay of 5.4 minutes is expected for the modulator gradient to reach the adsorbates at the inlet of the column.

A gradient of 10 to 40% ACN, in 20 minutes (1.5%/min gradient steepness) was done using 0.5 mg/ml of X~phe and X~trp in 1ml volume (Figure 3.27). The enrichment factor was 1, meaning that the peaks did not dilute and instead remained at

their same concentration. The separation is also complete with 100% yield. Yields, enrichments and productivities are summarized at the end of this section (Table 3.5).

This suggests that a peak focusing effect due to the gradient is occurring. Under isocratic conditions, the elution of the components down the column causes the peak to broaden and therefore dilute. Since a gradient is being used the peaks did not dilute but remained at a relatively high concentration (identical to the feed in this case). To take advantage of this effect further, the concentration was increased to 0.75mg/ml and the volume to 2.4ml. The resulting preparative run was well separated (Figure 3.28). The

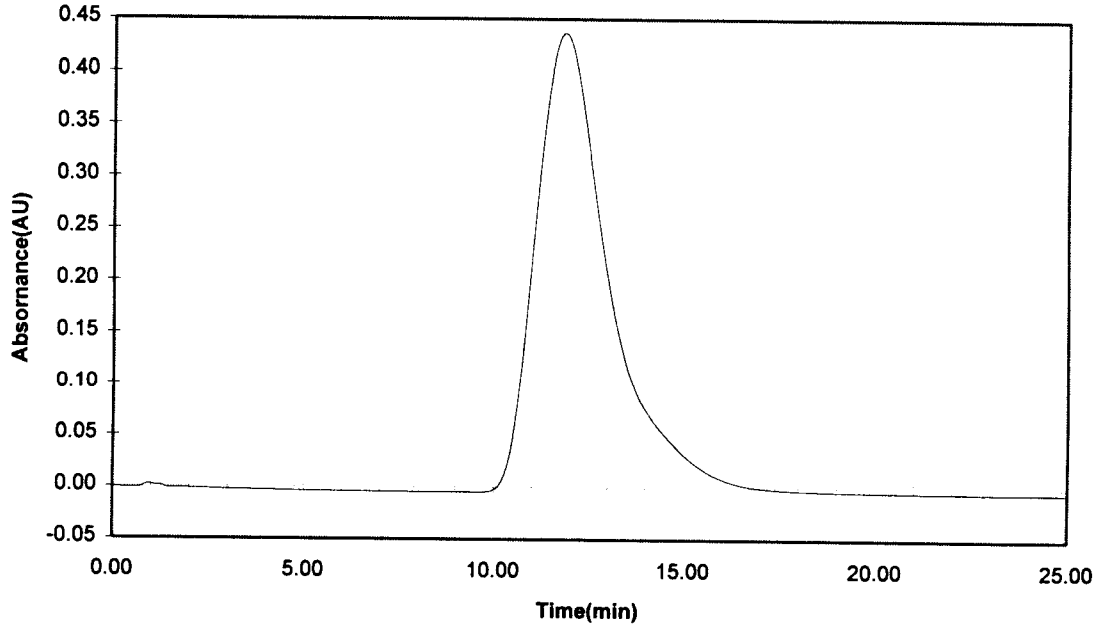


Figure 3.26: Measurement of the gradient delay time. A 0.5 minute pulse of 5% acetone was programmed at 5 minutes from the solvent reservoir to the detector. The Novapak C-18 column was pre-equilibrated to 50/50 ACN/H_2O at 1ml/min. Detection was done at 254nm.

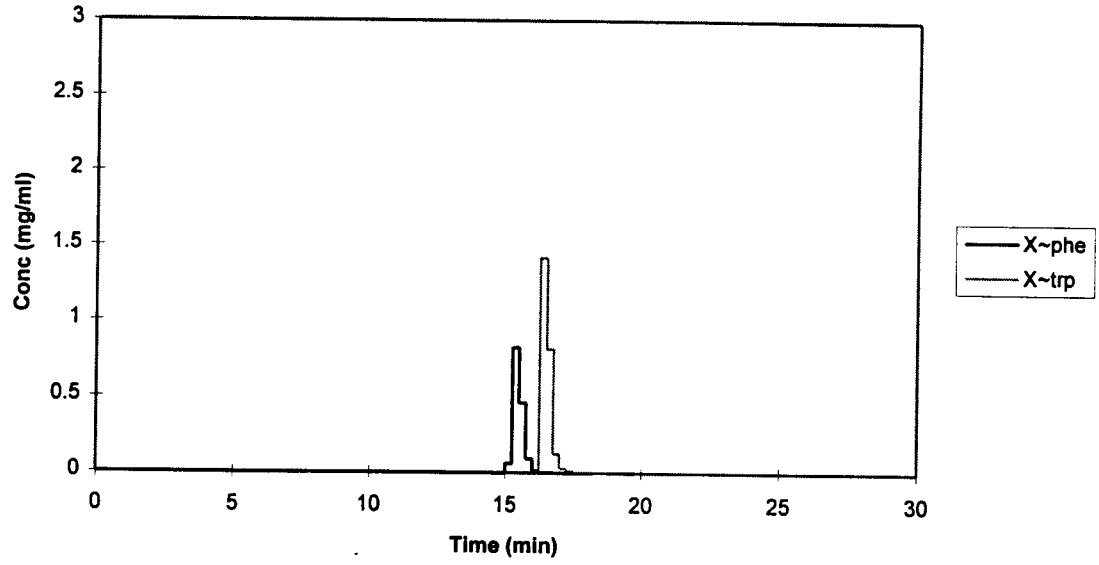


Figure 3.27: ACN gradient 10-40% in 20min, 0.4mg/ml X~phe and 0.5mg/ml X~trp in 1ml feed volume. buffer and 0.1% TFA throughout. Novapak C-18 RP column (150 x 3.9mm I.D.). Detected by 214nm UV.

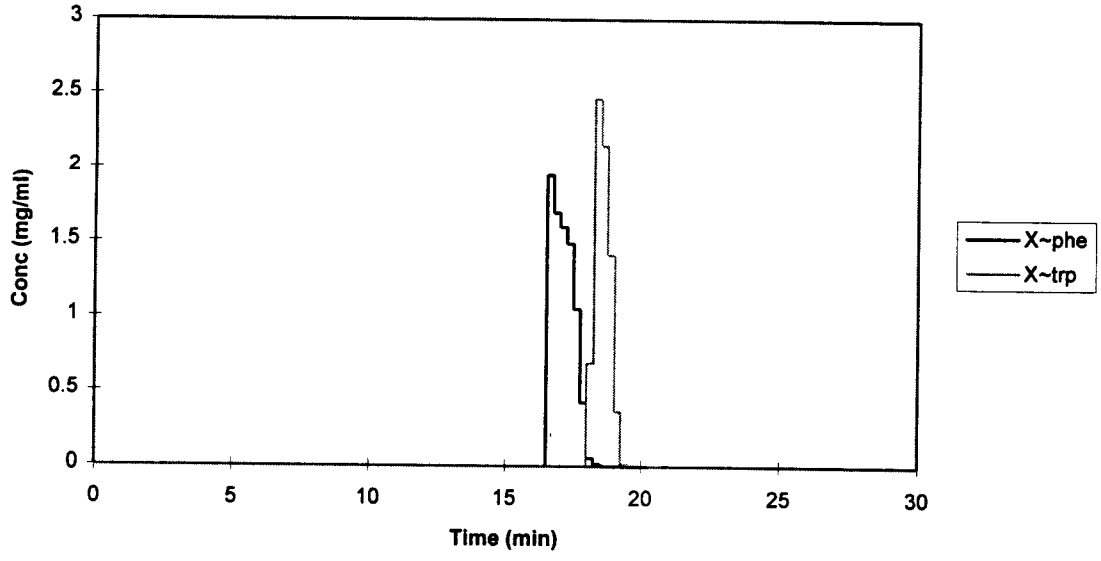


Figure 3.28: ACN gradient 10-40% in 20min, 0.75 mg/ml X~phe and 0.75mg/ml X~trp in 2.4 ml feed volume. All other conditions same as Figure 3.27.

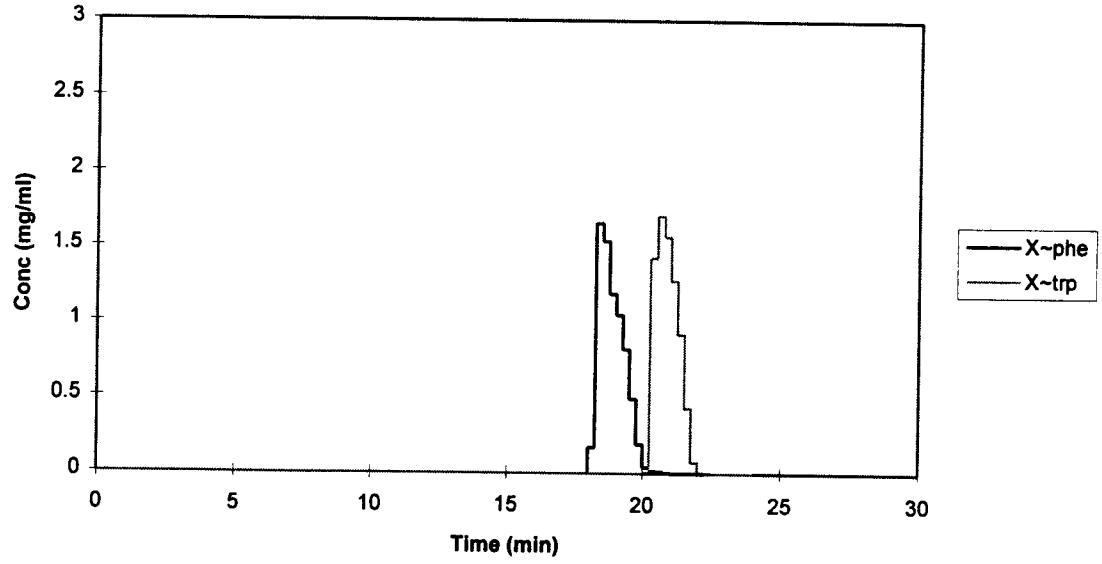


Figure 3.29: ACN gradient 10-30% in 20min, 0.75 mg/ml X~phe and 0.75mg/ml X~trp in 2.4 ml feed volume. All other conditions same as Figure 3.27.

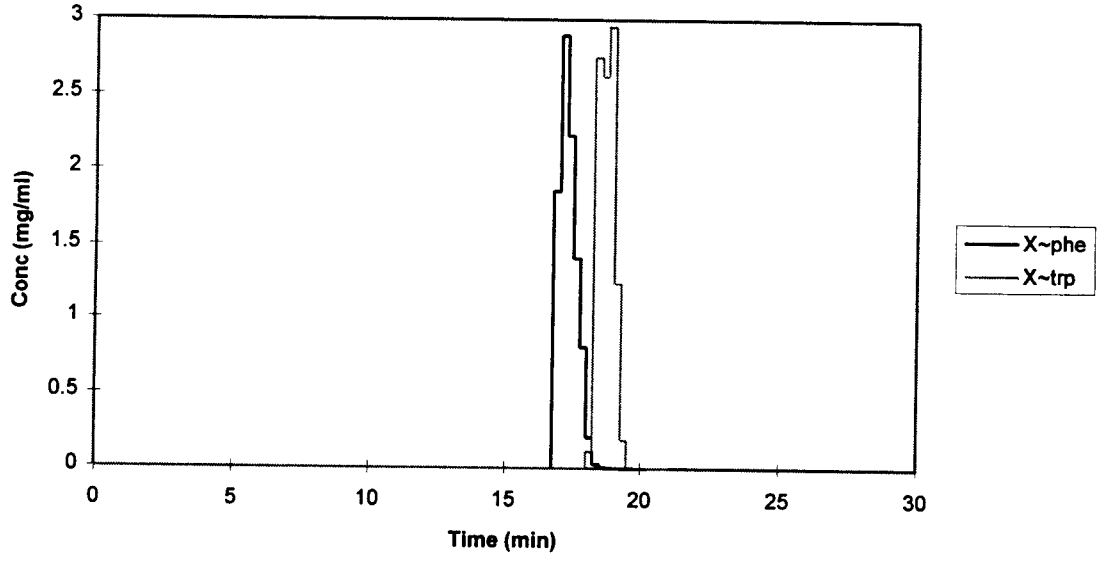


Figure 3.30: ACN gradient 10-40% in 20min, 0.75 mg/ml X~phe and 0.75mg/ml X~trp in 3.0 ml feed volume. All other conditions same as Figure 3.27.

difference in the retention time at the front of the first peak is due to the increased feed volume (from 1ml to 2.4ml) that the gradient must travel through before reaching the inlet of the column.

Sharp gradients improve the focusing effect. A steep slope of the gradient may therefore add to the focusing effect and cause the peaks to concentrate further. A 10 to 30% ACN gradient shows that shallow gradient is less concentrated but better separated (Figure 3.29). The reason is that the gradient linearization of the isotherm is not as pronounced at 30% ACN. Nevertheless the yields remain identical, since all the peptides can be recovered.

Increasing the volume even more to 3ml brings the run to the limits of the peptide's solubility (Figure 3.30). Any further increase in volume is risking the precipitation of the X~phe peptide onto the column. Already, X~phe is being concentrated to 2.9mg/ml, which is not possible in ACN/buffer conditions. In fact, the concentrated X~phe fractions for this run crystallized a few minutes after they were collected. This suggests that the column SP was able to stabilize the X~phe, and prevent it from precipitating until after it came out of the column. The separation and yields are complete (100%). Some time after the collection of fractions, for the highly concentrated fractions, the peptides tended to crystallize. Apparently, the column was able to stabilize the peptides such that the fractions could concentrate up to 2mg/ml when inside the column, but began falling out of solution once out of the column. Once the separated peaks emerged, the fractions were therefore resolubilized in neutral pH (by adding buffer) and prevented from precipitating.

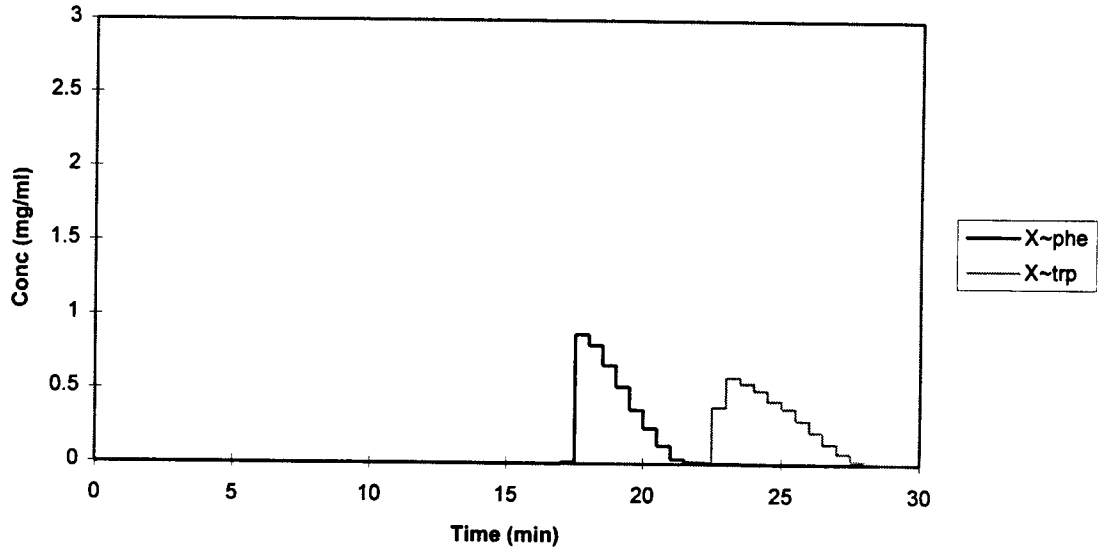


Figure 3.31: ACN 1/2min step gradient 10-15%, 0.75mg/ml X~phe and 0.75mg/ml X~trp, 2.4 ml volume. All other conditions same as Figure 3.27.

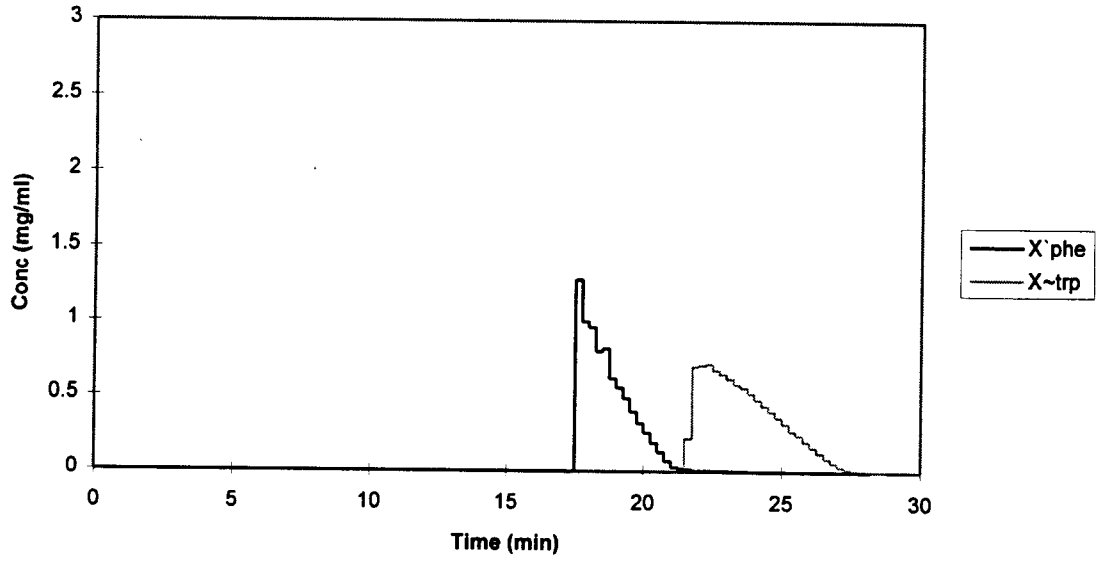


Figure 3.32: Step gradient 10-15% in 0.5 minutes, 0.72mg/ml X~phe and 0.7mg/ml X~trp. 3.0 ml volume. All other conditions same Figure 3.29.

Description	X~phe						X~trp					
	98% purity			95% purity			98% purity			95% purity		
	yield (%)	Enrich.	Product. mg/ml·hr	yield (%)	Enrich.	Product. mg/ml·hr	yield (%)	Enrich.	Product. mg/ml·hr	yield (%)	Enrich.	Product. mg/ml·hr
gradient 10-40% in 20 min 0.4mg/ml X~phe 0.5 mg/ml X~trp 1 ml volume	**100**	**1**	**1.02**	**100**	**1**	**1.02**	**100**	**0.64**	**1.62**	**100**	**0.64**	**1.62**
gradient 10-40% in 20min 0.7mg/ml X~phe 0.7mg/ml X~trp 2.4ml volume	**99**	**1.9**	**5.34**	**100**	**1.9**	**5.34**	**99**	**2**	**4.62**	**100**	**2**	**4.62**
gradient 10-40% in 20 min 0.7mg/ml X~phe 0.72mg/ml X~trp 3ml volume	**99**	**2.1**	**6.06**	**100**	**2.1**	**6.06**	**99**	**2.3**	**6.36**	**100**	**2.3**	**6.36**
gradient 10-30% in 20 min 0.75mg/ml X~phe 0.75mg/ml X~trp 2.4ml volume	**100**	**1.2**	**4.26**	**100**	**1.2**	**4.26**	**100**	**1.2**	**4.26**	**100**	**1.2**	**4.26**
step gradient 10-15% in 0.5 min 0.72mg/ml X~phe 0.7mg/ml X~trp 2.4ml volume	**100**	**0.51**	**3.72**	**100**	**0.46**	**3.72**	**100**	**0.46**	**3.72**	**100**	**0.46**	**3.72**
step gradient 10-15% in 0.5 min 0.7mg/ml X~phe 0.7mg/ml X~trp 3ml volume	**99**	**0.69**	**4.14**	**100**	**0.65**	**4.14**	**99**	**0.45**	**4.56**	**100**	**0.45**	**4.56**

Table 3.5: Gradient enrichments (enrich.) and productivities (product.) of X~phe and X~trp, for 98% and 95% purity.

Even though the 15% isocratic run was at the limit of overlapping in 2.4ml feed volume, increasing the feed volume to 3ml improved productivity and enrichment, suggesting a displacement effect. A stepwise elution allows the peptides to retain for a longer period at the inlet of the column; once the modulator front reaches the inlet of the column, the peptides are force to elute without much retention, and less band broadening results. Applying this to the 15% isocratic preparative run (Figure 3.24), the separation can be improved. A 10-15% stepwise elution in half a minute at the beginning of the elution run improved the separation of the two peaks (Figure 3.31). In contrast to the isocratic 15% run. The initial low 10% ACN condition at the inlet of the column may cause the X~phe and X~trp to compete for sites, with the X~phe being pushed ahead until the modulator front forced both peptides to elute with much less retention. The productivity and enrichments of the step elutions, however, are lower then for the isocratic runs (Table 3.6). This is probably due to the gradient delay. The gradient delay adds 5.4 minutes more to the half minute step. The peptides are therefore affected by the step increase in modulator after 5.9 minutes and not 0.5 minutes, as it takes that long for the gradient to reach the inlet of the column. Thus the peptides may already start to advance allowing bandspreading effects to occur, and the modulator front will not have as strong an impact. The gradient delay may also be the reason for the large difference in the breakthrough time of the 2.4ml feed volume stepwise elution separation (Figure 3.31) compared to the 15% ACN isocratic elution (Figure 3.24). Nevertheless, the separation being better, the feed volume was again increased to 3ml (Figure 3.32). The increase in volume gave similar enrichment to the 15% isocratic run,

but the productivity was still lower. The isocratic elution therefore turned out to be better than the stepwise elution runs (Table 3.6).

Table 3.6 compares the preparative runs for isocratic, gradient and stepwise elution preparative runs. Although the isocratic runs seem to have greater or comparable productivity to the three other modes of operation, the linear gradient shows a clear enrichment of its peptides. For instance the 10-40% gradient at 2.4ml volume gives an enrichment of 1.9. However, interestingly, the 3ml volume only gives an enrichment of

Description	X~phe 98% Enrich.	Product. mg/ml·hr	95% Enrich.	Product. mg/ml·hr	X~trp 98% Enrich.	Product. mg/ml·hr	95% Enrich.	Product. mg/ml·hr
isocratic 15% 0.7mg/ml X~phe 0.8mg/ml X~trp 3ml volume	**0.98**	**6.6**	**0.98**	**7.2**	**0.36**	**6.6**	**0.39**	**6.6**
step gradient 10-15% in 0.5 min 0.7mg/ml X~phe 0.7mg/ml X~trp 3ml volume	**0.69**	**4.14**	**0.65**	**4.14**	**0.45**	**4.56**	**0.45**	**4.56**
gradient 10-40% in 20min 0.7mg/ml X~phe 0.7mg/ml X~trp 2.4ml volume	**1.9**	**5.34**	**1.9**	**5.34**	**2**	**4.62**	**2**	**4.62**
gradient 10-40% in 20 min 0.7mg/ml X~phe 0.72mg/ml X~trp 3ml volume	**2.1**	**6.06**	**2.1**	**6.06**	**2.3**	**6.36**	**2.3**	**6.36**

Table 3.6: Comparisons of the maximum productivities obtained using isocratic, step gradient and linear gradient elution.

2.1. The increase in enrichment is small relative to the increase in the volume. This is a sign of the self interference effect. At such high loading, gradient linearization is no longer valid, and the peak focusing effect is overcome by non-linear peptide interactions. That is, adsorbate behavior occurs in the non-linear region of their SCI. Although ideally one would want to use the focusing effect to achieve tremendously high enrichments, in this case, because of the solubility problems of the X~phe, the self-interference of the peptides was beneficial to this separation. Nevertheless, it is clear that the isocratic runs reached their limits of productivity; the gradients, were it not for the solubility limitation, would have been able to give even higher productivities. In addition, these productivities are much greater than the productivities normally obtained in literature discussed in the introduction (Table 1.3, section 1.7).

3.3 Methanol conditions

3.3.1 Peptide solubility

Having obtained an optimized gradient elution condition for ACN/buffer conditions, it is worth considering if productivity will improve under MeOH conditions, as changing the mobile phase modulator changes peptides solubilities and retentions. In an attempt to obtain as high a concentration of peptides as possible under methanol conditions, preliminary trials indicated that the peptides preferred MeOH to ACN: at 100% ACN, not even a 0.5mg/ml of X~phe and X~trp mixture could be solubilized, but

at 100% MeOH, 2mg/ml of that mixture could be solubilized. Also, water was used rather than buffer because in the presence of TFA, 1mg/ml of dipeptide mixtures could be solubilized in 40/60/0.1 MeOH/H_2O/TFA, but 1mg/ml could not be solubilized in 40/60/0.1 MeOH/buffer/TFA.

The elution strength of ACN is greater than that for MeOH; a 10% ACN isocratic is roughly equivalent to around 20% MeOH isocratic. This is because ACN is more hydrophobic than MeOH. A peptide mixture in 20/80/0.1 MeOH/H_2O/TFA is needed for preparative runs under MeOH conditions, as it is the lowest organic concentration necessary. At 20/80/0.1 MeOH/H_2O/TFA only 0.5mg/ml mixture X~phe

	Feed Component	Concentration mg/ml	Solvent Condition (method)	Solubility
(1)	X~phe and X~trp	0.5	100%ACN (A)	I
	X~phe and X~trp	2	100% MeOH (A)	S
(2)	X~phe and X~trp	1	40/60 MeOH/buffer (C)	I
	X~phe and X~trp	1	40/60/0.1 MeOH/H_2O/TFA (C)	S
(3)	X~phe and X~trp	0.5	20/80/0.1 MeOH/H_2O/TFA (C)	S
	X~phe and X~trp	1	40/60/0.1 MeOH/H_2O/TFA (C)	S
(4)	X~phe	0.6	30/70/0.1 MeOH/H_2O/TFA	I
	X~phe	0.5	30/70/0.1 MeOH/H_2O/TFA	S
	X~trp	3	30/70/0.1 MeOH/H_2O/TFA	S

Table 3.7: Solubilities in MeOH with comparison to ACN (S: soluble, I: insoluble).

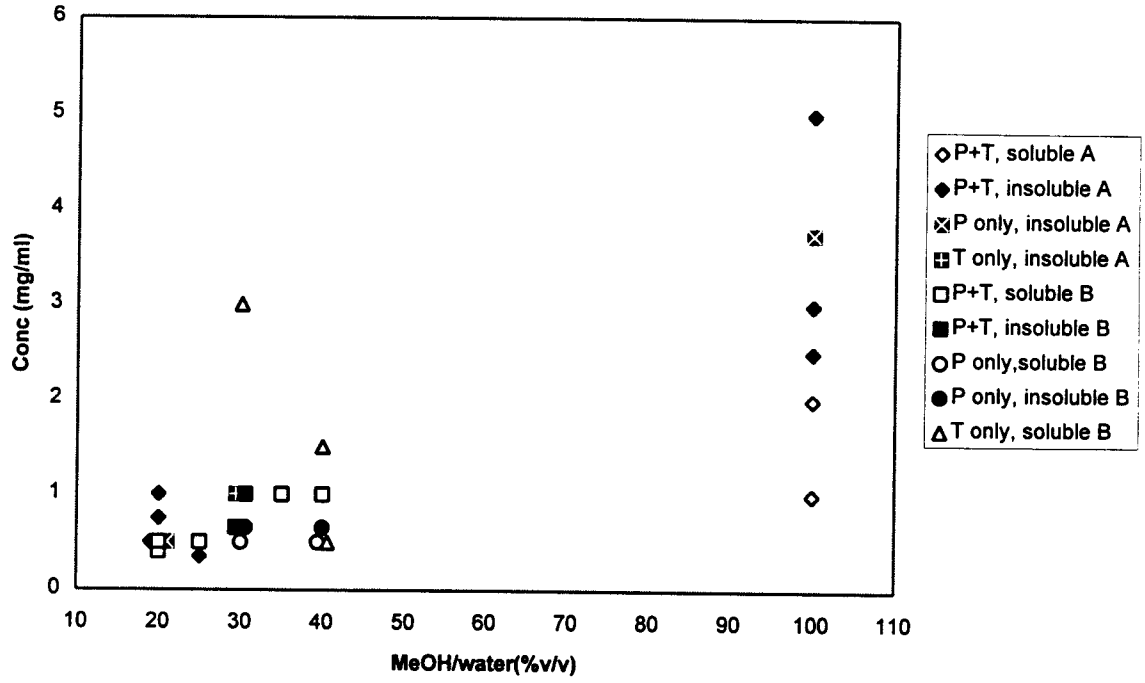


Figure 3.33: Solubility of X~phe and X~trp in MeOH/H_2O using methods A and B.

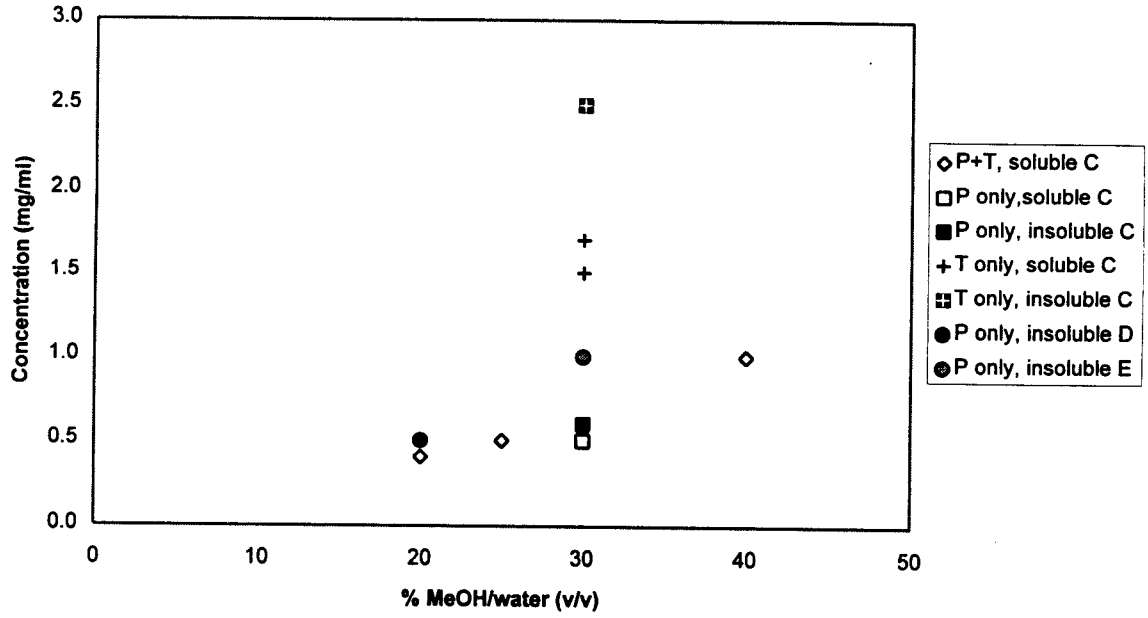


Figure 3.34: Solubility of X~phe and X~trp in MeOH-H_2O using methods C,D and E.

and X~trp could be solubilized. However, at 40/60/0.1 MeOH/H_2O/TFA, a 1.0mg/ml mixture of X~phe and X~trp could be solubilized. Therefore, the effects of using a feed condition at 40/60/0.1 MeOH/H_2O were examined (section 3.3.2).

Isotherms for the peptides under ACN conditions were done at 15% isocratic, which is roughly equivalent to a 30% isocratic MeOH run. Therefore, isotherms in 30/70/0.1 MeOH/H_2O/TFA were done. At these conditions, 0.5mg/ml of X~phe could be solubilized temporarily, but it would eventually fall out of solution after about an hour of stirring. This was probably due to the metastable state caused by the TFA as in the ACN case, as was discussed in section 3.2.3. The X~trp did not have these solubility problems when solubilized by itself, and a concentration of 3mg/ml could be obtained. Other solubility tests on the MeOH/H_2O solvents are shown in Figure 3.33 and 3.34.

3.3.2 Selectivity reversal and adsorption isotherms

Under MeOH/H_2O conditions, adsorbate retention factors were obtained for X~phe and X~trp on the Novapak C18 column. Preparative runs were done over two different time periods. Over these two periods, the peptide retentions on the Novapak column decreased, and this must be taken into account when comparing runs (Figure 3.35). The retention factors were linear (correlation factor not shown) under MeOH conditions as opposed to the ACN case (Section 3.2.1). Between 35 to 45% MeOH, the adsorbate retention lines of each peptides cross. This is known as "selectivity reversal".

Selectivity reversal as a function of MP composition implies that the order of retention of the adsorbates changes as the MP composition varies. For instance the lesser retained component at low concentrations of organic solvent becomes the more strongly retained component at high organic solvent concentrations. The trends of the retentions of the component species can better be understood if we consider the separation factor

$$\alpha = \frac{k'_T}{k'_P} \qquad (3.5)$$

Retention factors of two peptides can converge, diverge, remain parallel, or have a selectivity reversal. Under acetonitrile conditions, no selectivity reversal was present; instead the lines converged. Convergence means that the k' of each components become closer (α becomes smaller) with increasing modulator concentration. Divergence occurs when the separation of the adsorbates increases with modulator concentration. Parallel retention means that the peptides elute in equal proportion throughout the modulator levels. With selectivity reversal, at modulator concentrations less than the selectivity cross point the k' converge ($\alpha < 1$), and at higher modulator concentrations the k' diverge ($\alpha > 1$)(Figure 3.36).

The X~phe dipeptide could only be dissolved to a concentration 0.5mg/ml in 30/70/0.1 MeOH/H_2O/TFA conditions, and would precipitate at higher levels. The adsorption isotherm obtained using the ECP method (see section 2.3) shows that due to the low concentration of X~phe, only the linear portion of its isotherm could be obtained (Figure 3.37). The non-linearity can be seen with the X~trp isotherm (Figure 3.38). A quadratic equation was able to fit the X~trp isotherm very well. The k'

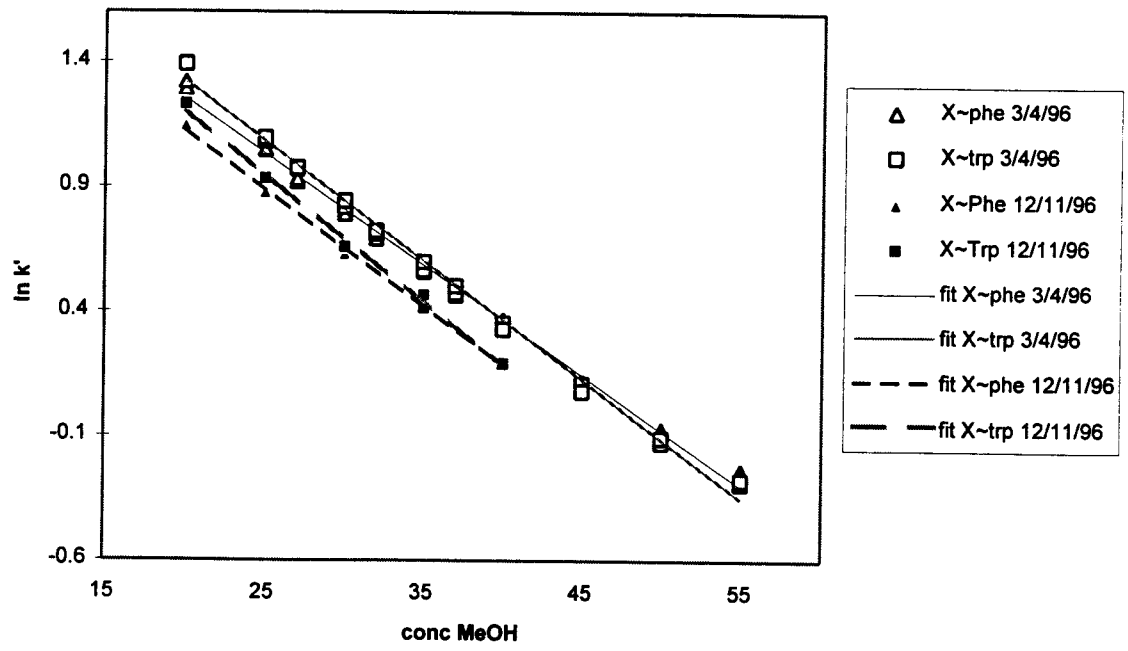
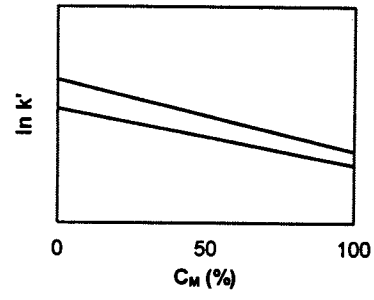
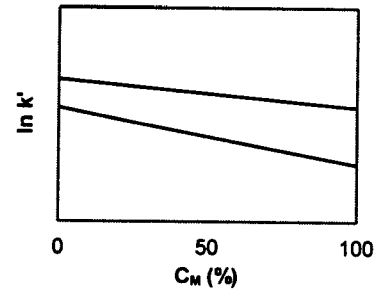


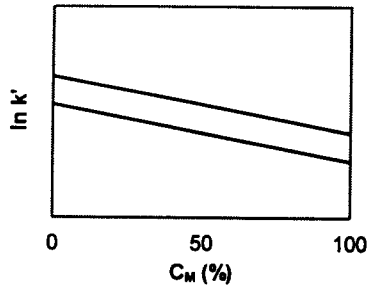
Figure 3.35: Adsorbate retention of X~phe and X~trp on a Novapak C-18 column under methanol/water conditions, at two different time periods (3/4/96 and 12/11/96).



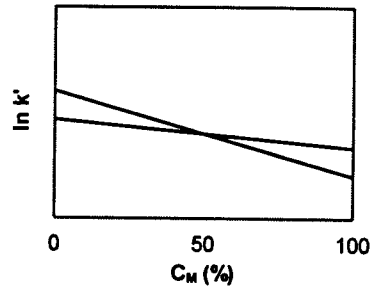
Convergent (α decreases with C_M)



Divergent (α increases with C_M)



Parallel (α constant)



Selectivity reversal
(α>1 before intersection, α<1 after intersection)

Figure 3.36: Separation factor variation with changing MP concentration due to the presence of selectivity reversal. (C_M: modulator concentration).

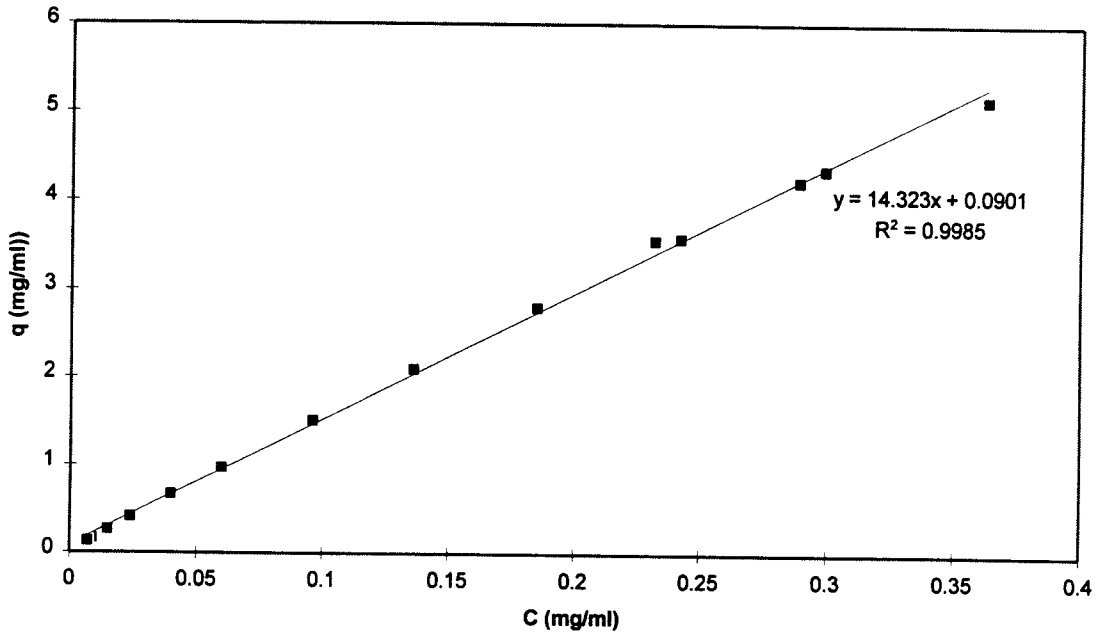


Figure 3.37: Isotherm for X~phe on a Novapak C-18 RP column (150 x 3.9mm I.D.). The non-linear portion could not be reached because of the X~phe would precipitate at higher than 0.5mg/ml concentration.

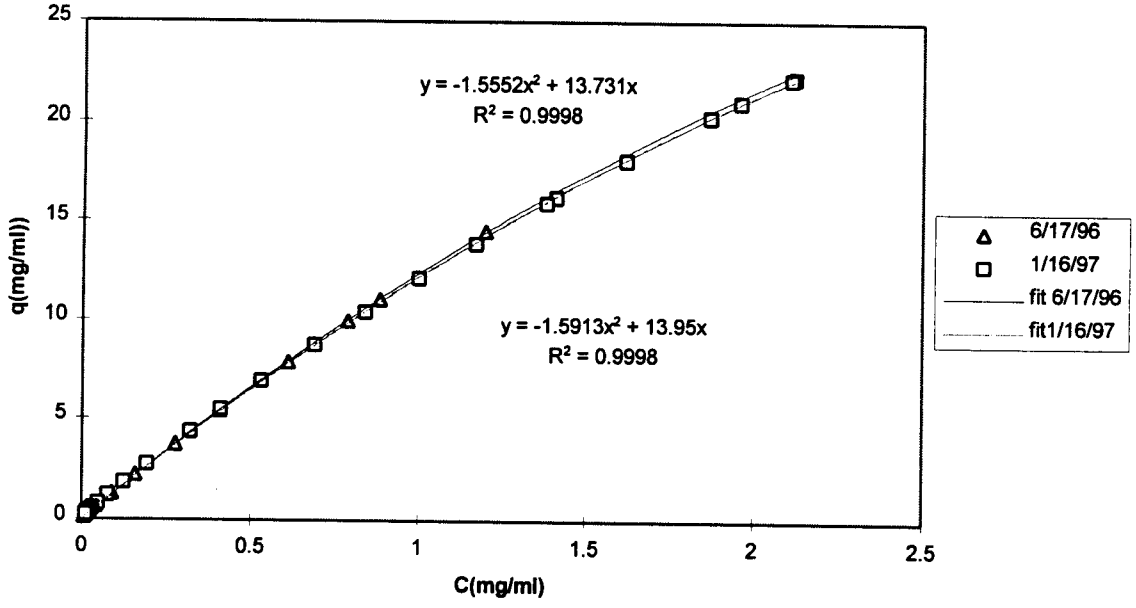


Figure 3.38: Isotherm for X~trp on a Novapak C-18 RP column (150 x 3.9mm I.D.). This was done at two different times to see if any changes occurred over time.

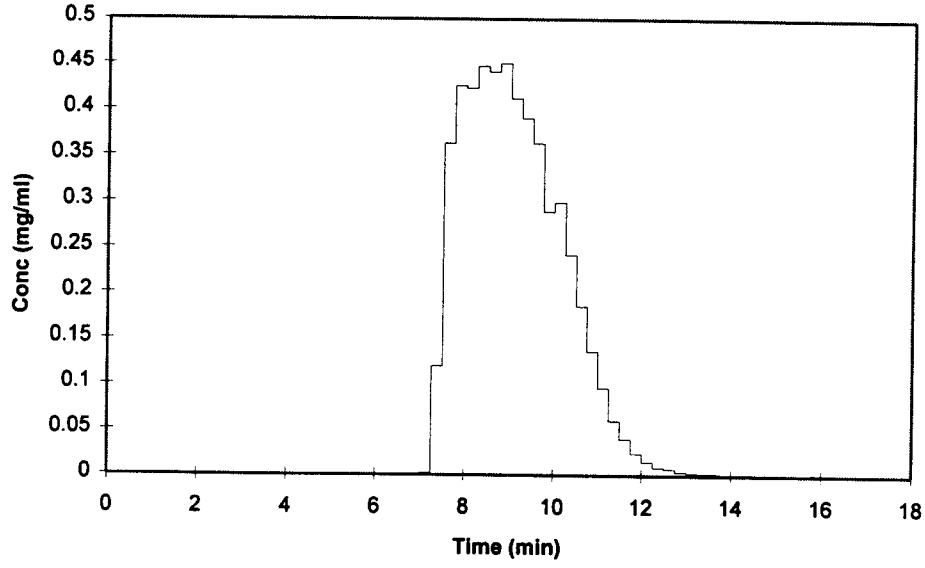


Figure 3.39: Isocratic 30/70/0.1 MeOH/H_2O/TFA run for isotherm determination for X~phe. 0.5mg/ml X~phe in 2.4ml volume. Novapak C-18 (150 x 3.9mm I.D.), UV detection at 214nm.

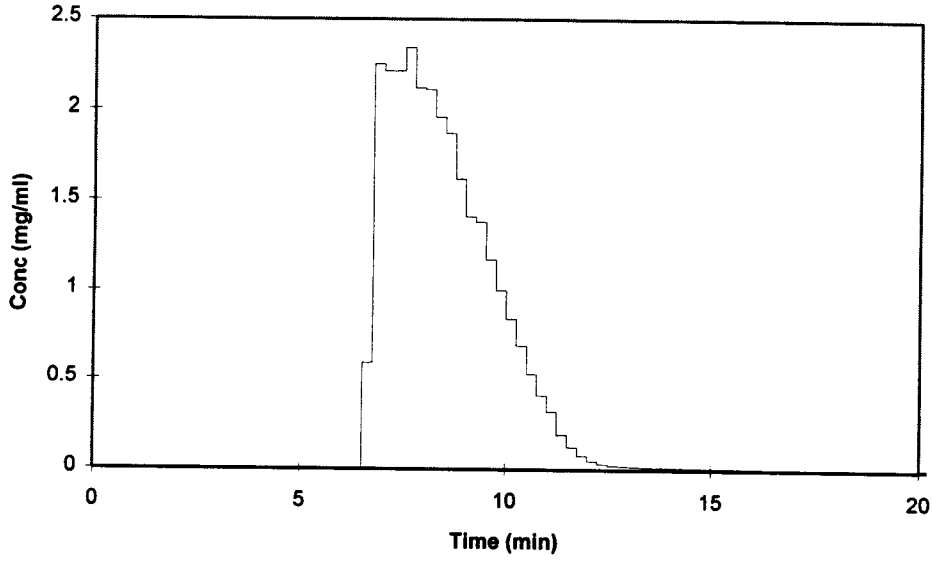


Figure 3.40: Isocratic run for isotherm determination for X~trp. 3.0mg/ml X~trp in 2.4ml volume. All other conditions same as 3.37.

dramatically changed between a the periods 3/4/96 and 12/11/96 (Figure 3.35); the column had been left in 0.1% TFA during storage, which had an effect on the column. Thereafter, the column conditions remained very stable as can be seen from the shapes of the isotherms: the X~trp isotherm was measured at a 6 month time interval and did not change over that time (Figure 3.36). The peptides are fairly strongly retained under the point of intersection of the selectivity reversal. The single component runs used to obtain the isotherms by the ECP calculations are shown in Figures 3.39 and 3.40.

3.3.3 Initial runs

X~phe and X~trp are poorly soluble under ACN conditions. Using 40/60 MeOH/H_2O conditions, however, 1mg/ml concentrations of both peptides could be solubilized. The consequences of having a high level of MeOH in the feed were therefore examined. Using feed conditions that are different than the column did not work for the ACN case. Nevertheless, since the solvent strength of the MeOH is different to that of the ACN, the effect of the feed was examined.

The ACN experiments have shown so far that a 20/80 ACN/buffer gives a reasonable capacity factor for X~phe and X~trp in the sense that they retain long enough to separate and yet elute close enough to remain a difficult separation ($\alpha \simeq 1.1$). A 20% ACN is approximately equivalent to 30% MeOH. Therefore, as a first estimate, 30% isocratic MeOH was used under similar volume and concentrations as the previous ACN runs that were done in 20% initial conditions. This is depicted in Figure 3.41.

The chromatogram shows complete mixing. However, despite the 40% MeOH feed, the peptides are retained, and the long band eluting at t_0 observed in the ACN case does not occur. According to adsorbate retention plot (corresponding to 3/4/96, Figure 3.35), at 30% isocratic methanol, the k' of the two peptides are extremely close ($\alpha \simeq 1.1$), and as a result the peaks overlap at these high concentrations.

To allow the adsorbates to retain longer and increase the k' so as to reduce mixing, the organic concentration is decreased. Figure 3.42 shows a run at a 25% isocratic methanol under similar conditions as the 30% run. This run shows the beginnings of a separation, but the mixing is still substantial. Decreasing the organic will further improve the separation. However, at 25% methanol, the k' are already

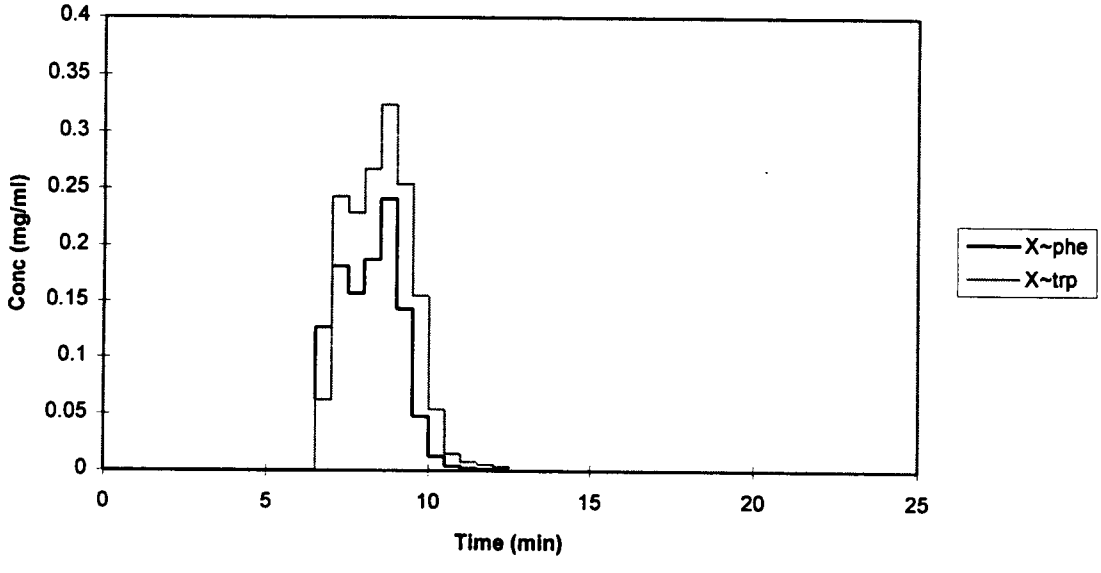


Figure 3.41: Isocratic 30/70/0.1 MeOH/H_2O/TFA, 1mg/ml X~phe and 1mg/ml X~trp, in 0.5ml feed volume, with 40/60/0.1 MeOH/H_2O/TFA feed. Novapak C-18 column (150x3.9mm I.D.), UV detection at 214nm.

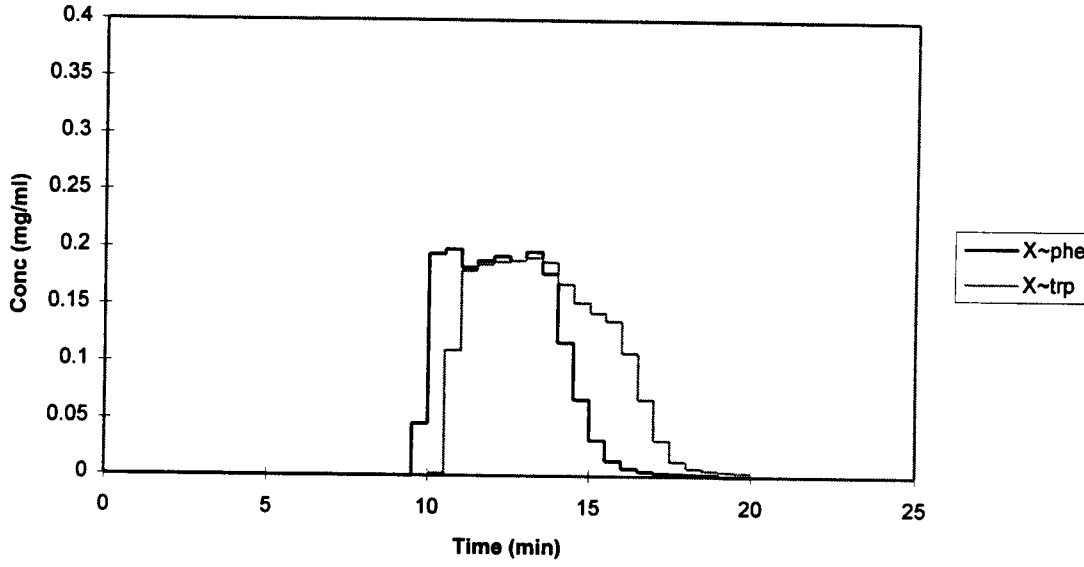


Figure 3.42: Isocratic 25/75/0.1 MeOH/H_2O/TFA, 1mg/ml X~phe and X~trp in 1ml feed volume. All other conditions identical to Figure 3.41.

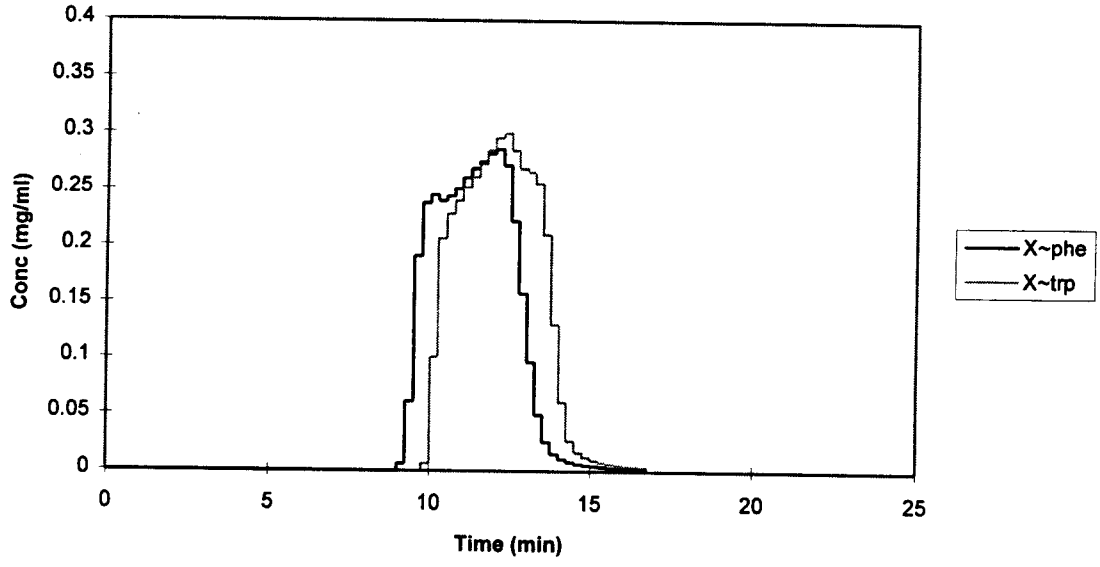


Figure 3.43: Linear gradient 25-40% MeOH in 25min, 1mg/ml X~phe and 1mg/ml X~trp in 1ml feed volume. All other conditions identical to Figure 3.39.

greater than 10 (k'=11 for X~phe and k'=12 for X~trp). Decreasing the methanol further may substantially increase the elution time. Therefore a gradient might turn out to produce a better separation at this point.

In choosing a gradient, however, selectivity reversal must be taken into account. Selectivity reversal can cause the more retaining feed component peak to envelop the less retaining peak as a result of the reversal in retention at higher modulator levels, worsening the separation. Therefore, the gradient should be adjusted so that all the peptides will have eluted before the point of intersection of the selectivity reversal is reached. The point of intersection of selectivity under analytical conditions is around 40% methanol according to Figure 3.35. At 40%, the k' of both compounds are around 2. The peaks in the 25% isocratic were all eluted by 20 minutes. The peaks are completely mixed in a linear gradient from 25 to 40% methanol in at least 20 minutes with 1mg/ml of dipeptides in 1ml feed volume, although the peaks are slightly more concentrated than before, as expected from gradient elution (Figure 3.43). There is the presence of a "shallow region" at the front of the two peaks. These odd shapes present may be due to the large feed volume injection causing the 40% feed to briefly pull the peptides ahead at the inlet of the column. Reducing the volume injected to 0.5ml minimizes the impact of the 40% feed, and brings back the more familiar fronting peaks generally found in preparative chromatography (Figure 3.44). However, there is noticeable X~phe peak tailing into the X~trp, and the separation remains poor. The tailing is probably due to selectivity reversal or the solubility of X~phe.

The poor separation may be improved by using curved gradients. The HPLC

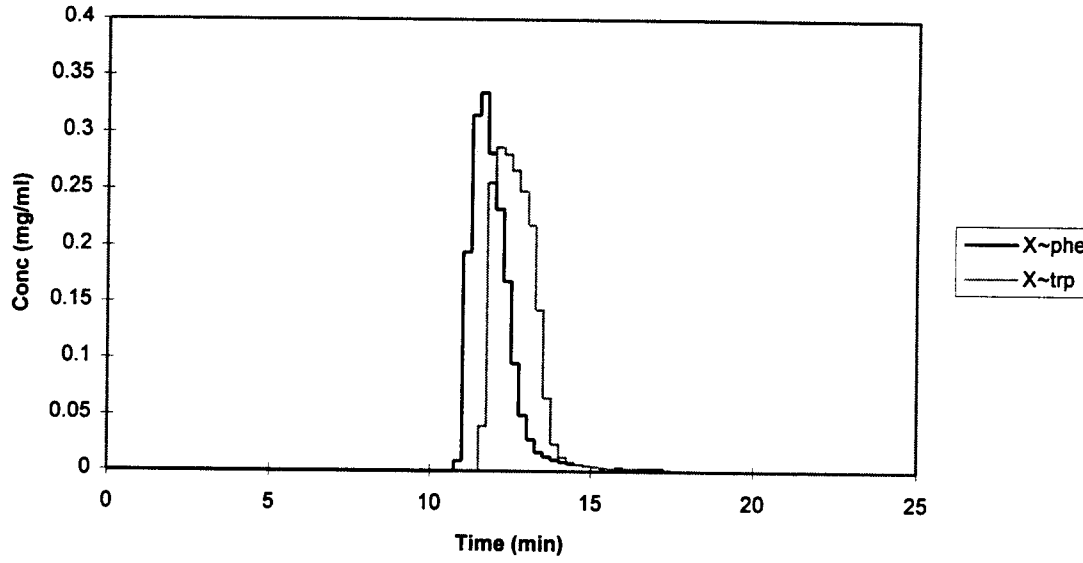


Figure 3.44: Linear gradient 25-40% MeOH in 20min, 1mg/ml X~phe and 1mg/ml X~trp in 0.5ml feed volume. All other conditions identical to Figure 3.41.

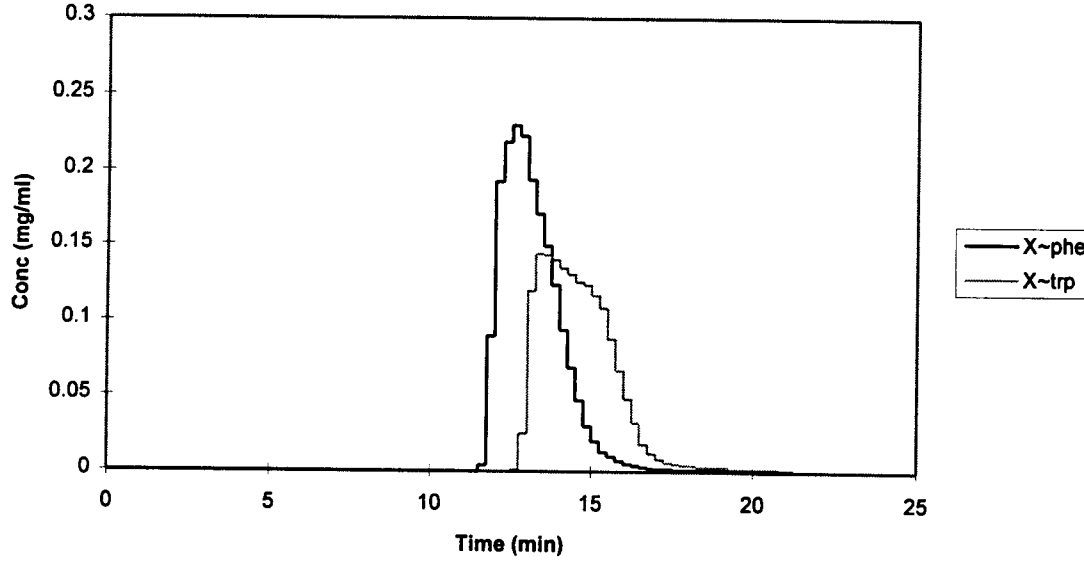


Figure 3.45: Curved 9 gradient 25-40% MeOH in 20min, 1mg/ml X~phe and 1 mg/ml X~trp in 0.5 ml feed volume. All other conditions identical to Figure 3.41.

instrument has settings available to perform curved gradients. A "curve 8" gradient remains fairly constant until a sharp increase in gradient occurs at about one third of the time given for the gradient. A curve "9" gradient begins curving sharply at about half way through the gradient time.

The tailing in Figure 3.43 occurs between 13 to 17 minutes. The gradient should therefore begin around that time. A 25-40% gradient in 20 minutes using curve 9 (Figure 3.45) has a sharp gradient increase occur after 15 minutes. The result is a slight improvement in the separation, probably due to a focusing effect on the X~phe peak. However, the gradient did not reduce the tailing of X~phe and X~trp peaks. The curved gradient improved the separation by having the peptides initially bind at a lower modulator concentration, where they have the biggest k' difference until after around 15 minutes where they are eluted with little binding because of the sharp curve 9 gradient. Increasing the time of the gradient to 30 minutes allows the peptides to remain in low modulator concentration for a longer period, and the separation improved (Figure 3.46). However,due to the increase in time, the peptides elute almost 10 minutes later. To try to gain time without losing separation resolution, a less curved gradient was tried. The curve 8 gradient improved retention time only slightly (Figure 3.47). The shallow region described earlier is present in the X~trp peptide. The 40% feed probably caused the feeds to move ahead at the inlet of the column, preventing the focusing effect from having a full impact on X~trp.

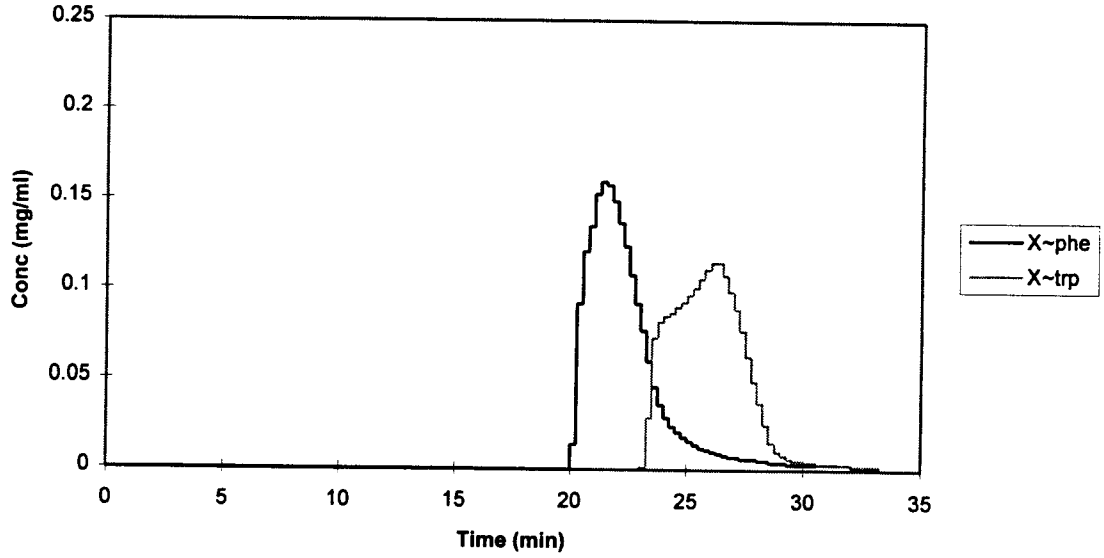


Figure 3.46: Curved 9 gradient 25-40% MeOH in 30min, 1mg/ml X~phe and 1 mg/ml X~trp in 0.5 ml feed volume. All other conditions identical to Figure 3.41.

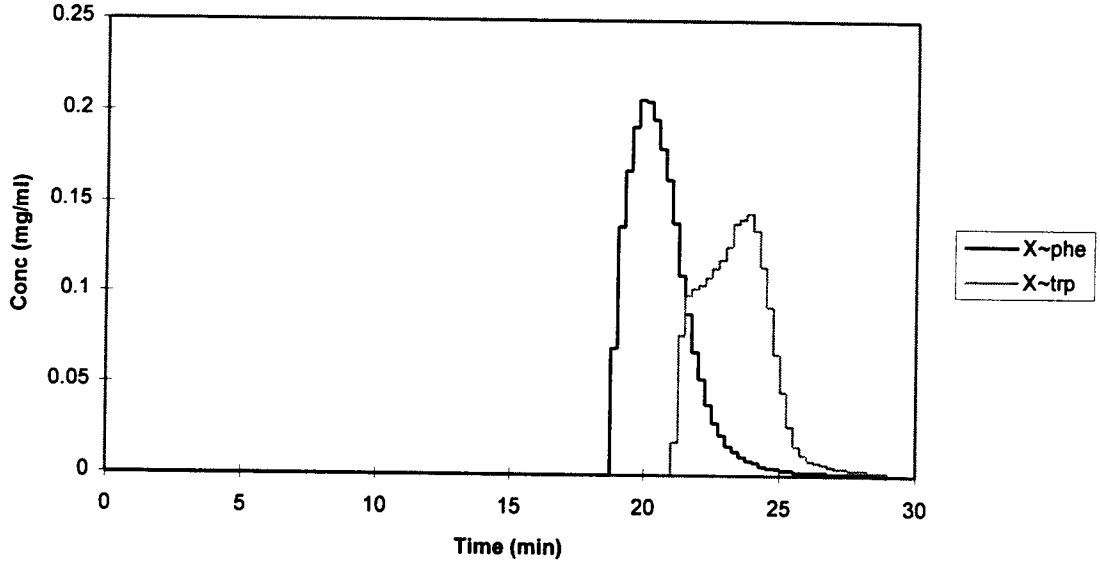


Figure 3.47: Curved 8 gradient 25-40% MeOH in 30min, 1mg/ml X~phe and 1mg/ml X~trp in 0.5 ml feed volume. All other conditions are identical to Figure 3.41.

Because the elution time was high for the gradient runs, an isocratic 20% elution was attempted. This run did not have a large increase in the retention time compared to the gradients so far, yet the peaks seemed just as separated (Figure 3.48). The tailing however is longer, probably because of bandspreading. The front of the X~trp has a shallow region just as before. Doing a 20-40% MeOH curve 9 gradient in 20 minutes is able to concentrate the X~trp (Figure 3.47). In order to attempt to reduce the tailing, a gradient from 20-30% in 25 minutes, and kept constant at 30% thereafter was done (Figure 3.50). This gradient would allow the separation to occur below the point of crossing of the selectivity reversal. The tailing remained throughout the different gradients and isocratic elutions attempted. This suggests that the tailing is caused more by the insolubility of the X~phe peptide rather than by the selectivity reversal.

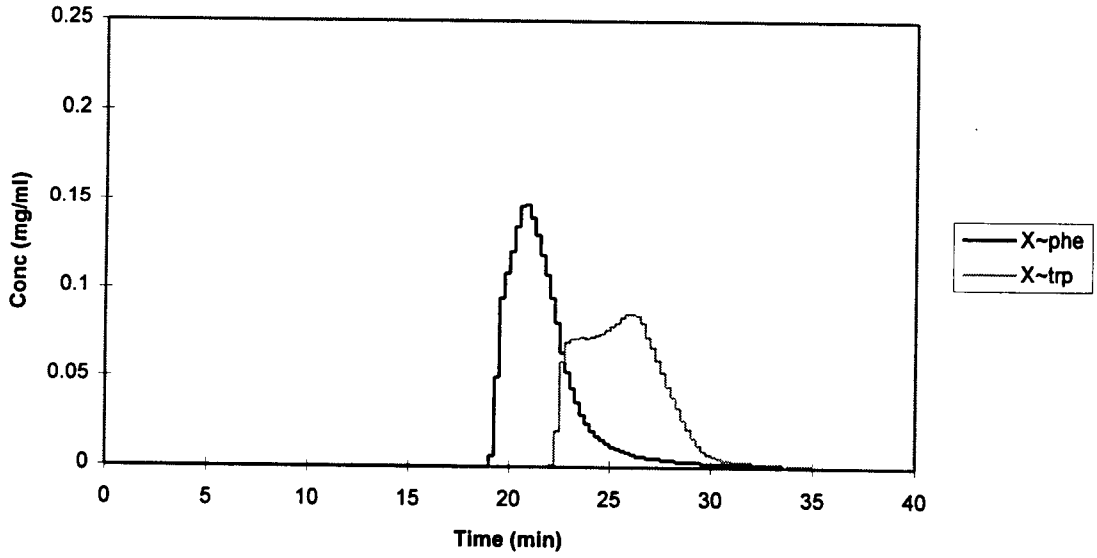


Figure 3.48: Isocratic 20% MeOH. 1mg/ml X~phe and 1mg/ml X~trp in 0.5 ml feed volume. All other conditions identical to Figure 3.41.

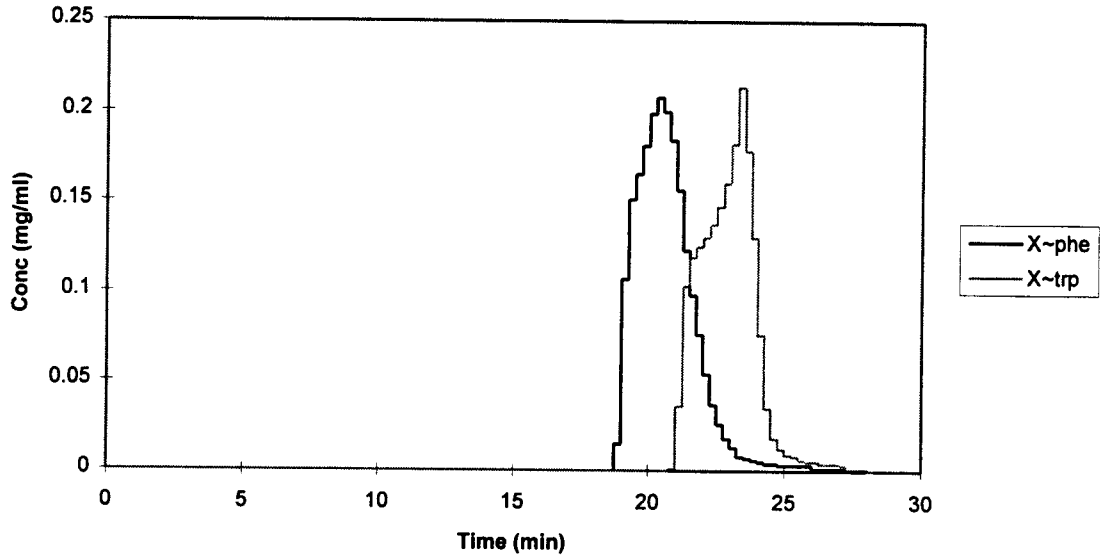


Figure 3.49: Curved 9 gradient 20-40% MeOH in 20min, 1mg/ml X~phe and 1 mg/ml X~trp in 1 ml feed volume. All other conditions identical to Figure 3.41.

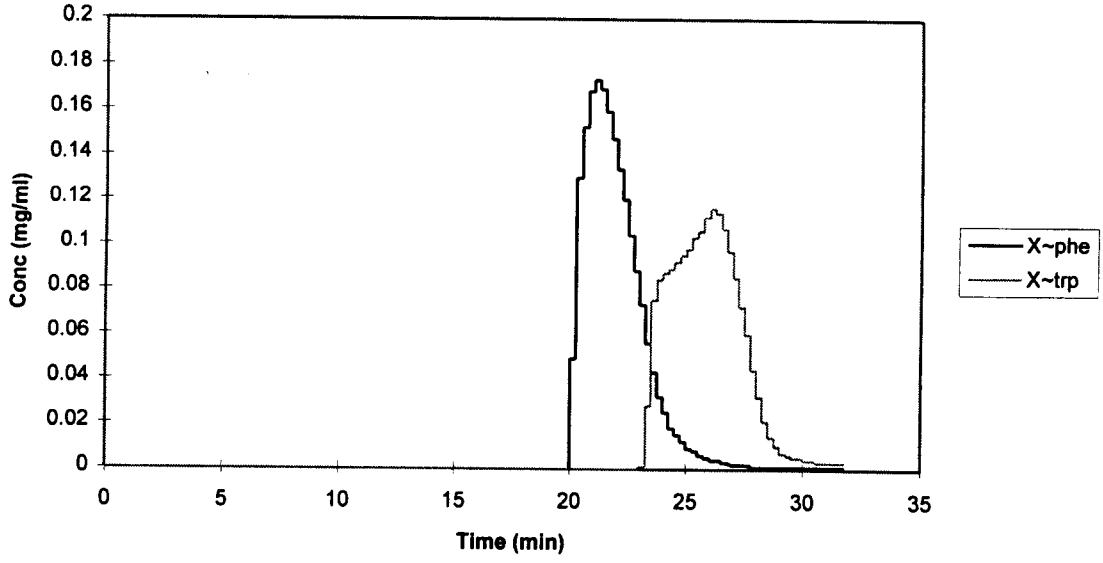


Figure 3.50: Curved 9 gradient 20-30% MeOH in 25 min, 1mg/ml X~phe and 1 mg/ml X~trp in 0.5 ml feed volume. All other conditions identical to Figure 3.41.

Description	X~Phe 98% Yield (%)	Enrich.	Product. mg/ml·hr	95% Yield (%)	Enrich.	Product. mg/ml·hr	X~trp 98% Yield (%)	Enrich.	Product. mg/ml·hr	95% Yield (%)	Enrich.	Product. mg/ml·hr
20% isocratic 1mg/ml X~phe 1mg/ml X~trp 40% feed, 0.5 ml	72	0.22	1.1	80	0.21	1.2	0	0	0	0	0	0
25-40% gradient 30min, curve 9 1.0mg/ml X~phe 1.0mg/ml X~trp 40% feed, 0.5ml feed	82	0.26	0.9	82	0.26	0.9	0	0	0	0	0	0
25-40% gradient 30min, curve 8 1.0mg/ml X~phe 1.0mg/ml X~trp 40% feed, 0.5ml feed	77	0.32	1.0	82	0.31	1.08	0	0	0	0	0	0
20-40% gradient 20min, curve 9 1.0mg/ml X~phe 1.0mg/ml X~trp 40% feed, 1.0ml feed	76	0.31	0.96	76	0.31	0.96	0	0	0	0	0	0
20-30-30% gradient 25min, curve 9 1.0mg/ml X~phe 1.0mg/ml X~trp 40% feed, 0.5ml feed	89	0.23	1.0	89	0.23	1.0	0	0	0	74	0.10	0.036

Table 3.8: Yields enrichments and productivities of selected preparative runs having 40% feed.

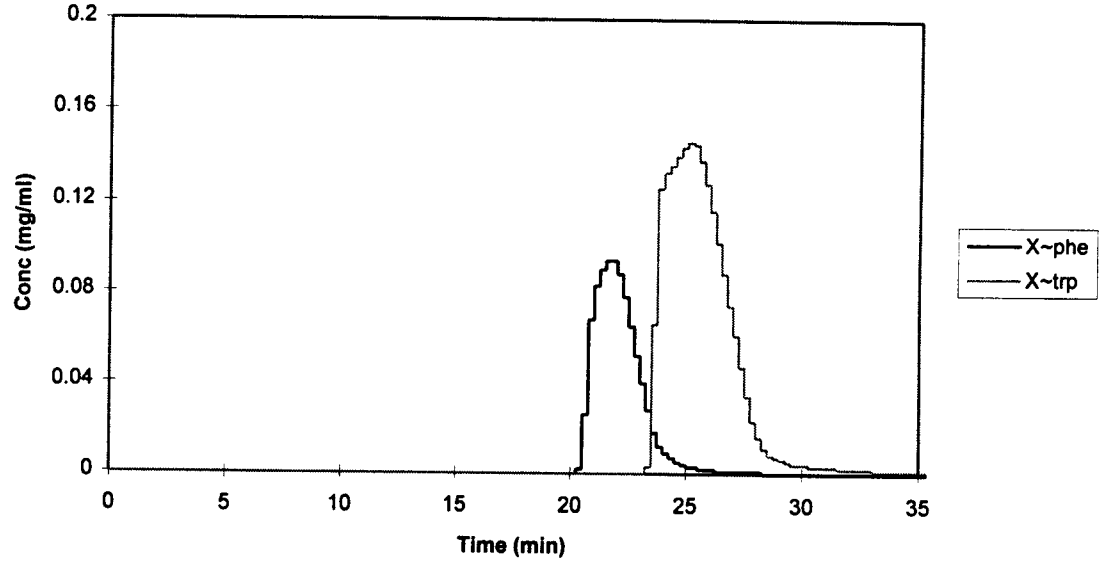


Figure 3.51: Linear MeOH gradient 20-30% in 25 min, then constant at 30% 0.4mg/ml X~phe and 1mg/ml X~trp in 35% feed, 0.5ml feed volume. All other conditions identical to Figure 3.41.

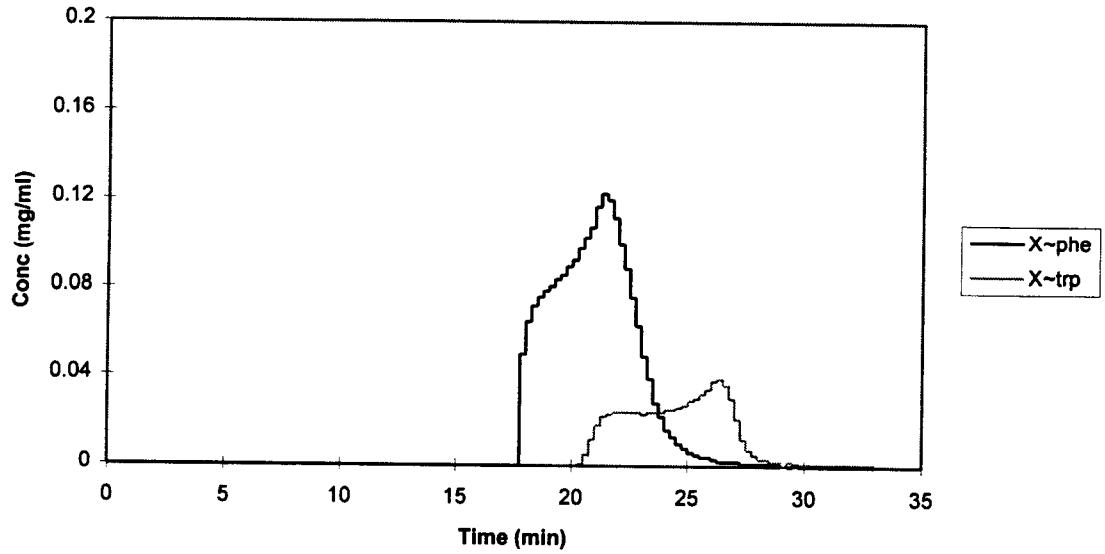


Figure 3.52: Linear MeOH gradient 20-30% in 25 min, then constant at 30% 1mg/ml X~phe and 0.4mg/ml X~trp in 35% feed, 0.5ml feed volume. All other conditions identical to Figure 3.41.

The selectivity reversal places a greater limit on the productivities of the runs since gradients sharper than 40% MeOH could not be used. The productivities of these runs ranged about 0.01-0.02 mg/ml-hr. Interestingly, the isocratic gave a better productivity (Table 3.8). This may be due to the 40% feed preventing the focusing effect from taking full effect. The enrichments were slightly better for the sharp 25-40% MeOH gradients as well as the curve 8 gradient. Only the 20-30% gradient allowed the X~trp to be separated.

The effect of the shallow region on the front of the peaks that has been observed throughout can better be observed when decreasing the concentration of one feed over the other. Taking a 1mg/ml X~trp and 0.4mg/ml X~phe feed, the shallow region in the front of the X~trp peak can be observed clearly (Figure 3.51). This effect can be seen (Figure 3.52) by both peaks if a low X~trp concentration is assumed (1mg/ml X~phe and 0.4mg/ml X~trp).

3.3.4 Isocratic elution

The poor separation due to the feed has shown once again how important it is to keep the feed conditions identical to the initial column conditions. Although 40% MeOH feed is better able to solubilize the peptides than 20% MeOH, it gives low productivities, and the X~trp could not be isolated. The following runs were done at a later time and, as discussed previously, the retention times decreased slightly (See section 3.3.2).

The maximum concentration that could be obtained under 20% MeOH was around 0.5mg/ml. Under identical feed and inlet column conditions, a 20% isocratic MeOH run showed two well-separated peaks except for some tailing of X~phe tailing into X~trp (Figure 3.53). Just as in the cases with 40% feeds, the probable cause of the tailing is the solubility of X~phe. The 0.5mg/ml feed concentration of the dipeptide mixtures is at the limit of X~phe solubility. The X~phe may have precipitated and resolubilized during the run which would have caused it to elute at the same time as the X~trp tail.

A 25% isocratic run showed mixing between the feed bands (Figure 3.54). The isocratic 20% was separated enough to allow an increase in the feed volume injected. Increasing the volume to 2.4ml showed that a large amount of X~phe is held back by the X~trp, as X~phe tails into X~trp (Figure 3.55). This separation could be used to obtain X~phe but because of the tailing of X~phe into the X~trp, the latter cannot be isolated at the desired 95 or 98% purity. The productivities show that to use the isocratic elutions to purify the X~trp peptide requires using the lower feed volume of 0.5ml (Table 3.9).

Because the column changed retention over time, the effect of the 40% feed was re-examined for comparison purposes with the productivities of the preparative isocratic 20% run. Using a 40% MeOH feed with 1mg/ml peptide mixture, the shallow region at the front of the peaks can be seen for both peaks (Figure 3.56). If the TFA is removed from the peak, the shallow region is accentuated for the X~phe case, and the X~trp peak forms a plateau and is about to form a double peak (Figure 3.57). The lack of TFA once

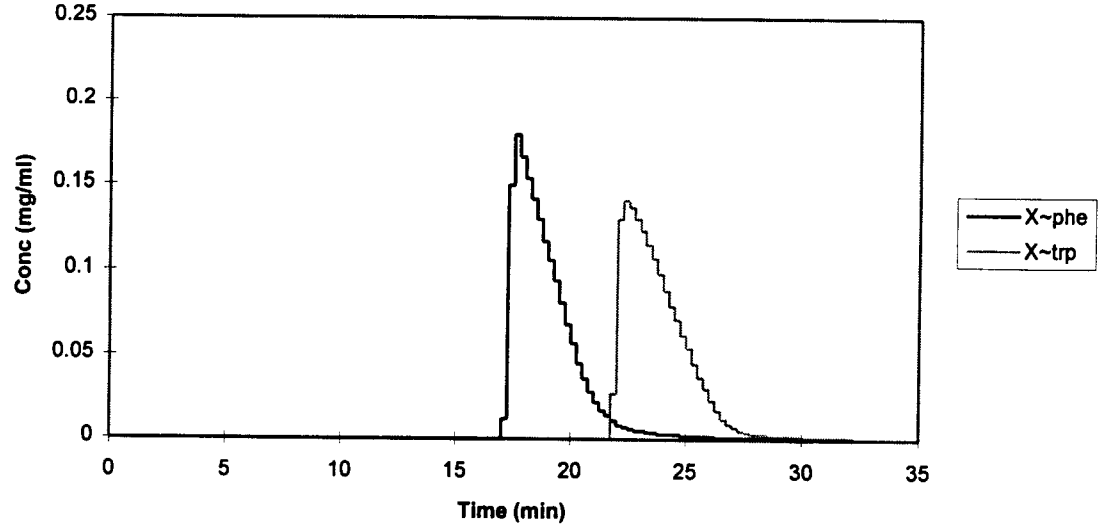


Figure 3.53: Isocratic 20/80/0.1 MeOH/H_2O/TFA. 0.4mg/ml X~phe and 0.4mg/ml X~trp in 1ml feed volume. H_2O and TFA throughout, Novapak C-18 column (150 x 3.9mm I.D.), UV detection at 214nm.

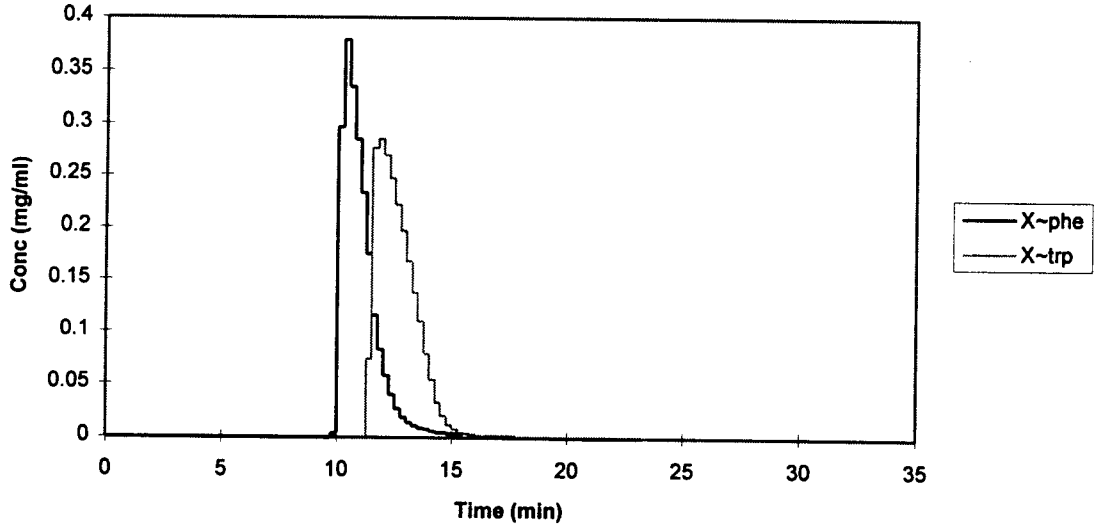


Figure 3.54: Isocratic 25/75/0.1 MeOH/H_2O/TFA. 0.5mg/ml X~phe and 0.5mg/ml X~trp in 1ml feed volume. All other conditions identical to Figure 3.53.

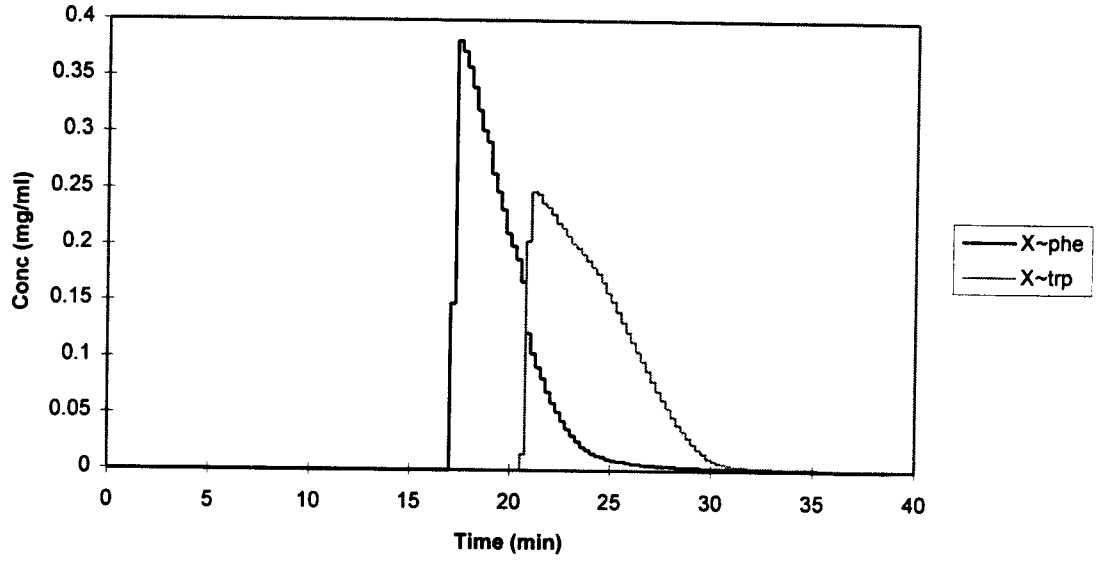


Figure 3.55: Isocratic 20/80/0.1 MeOH/H_2O/TFA. 0.5mg/ml X~phe and 0.5mg/ml X~trp in 2.4ml feed volume. All other conditions identical to Figure 3.53.

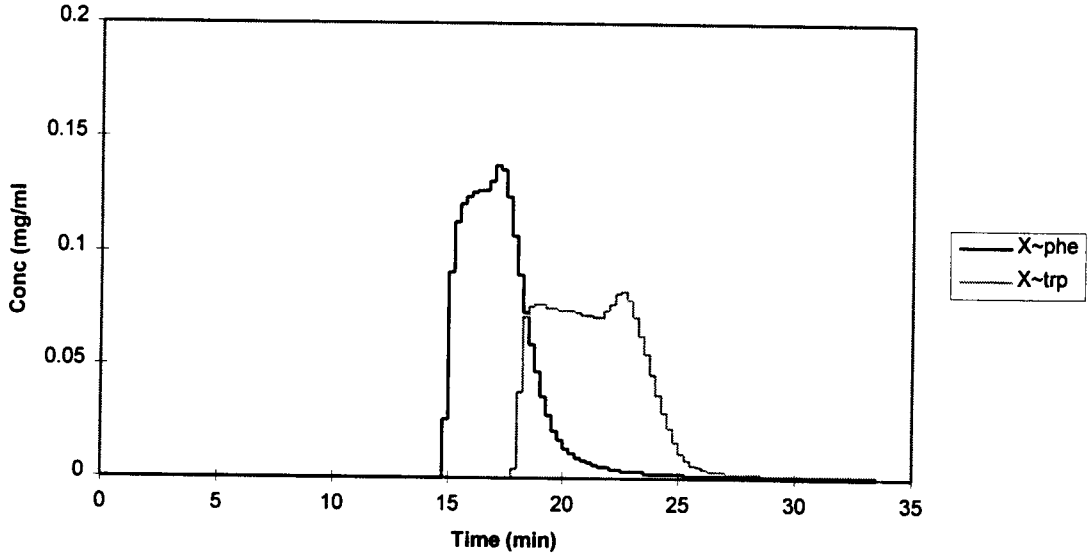


Figure 3.56: MeOH. Isocratic 20% 1mg/ml X~phe and 1mg/ml X~trp, 0.5 ml feed volume. 40/60/0.1 MeOH/H_2O/TFA. All other conditions identical to Figure 3.53.

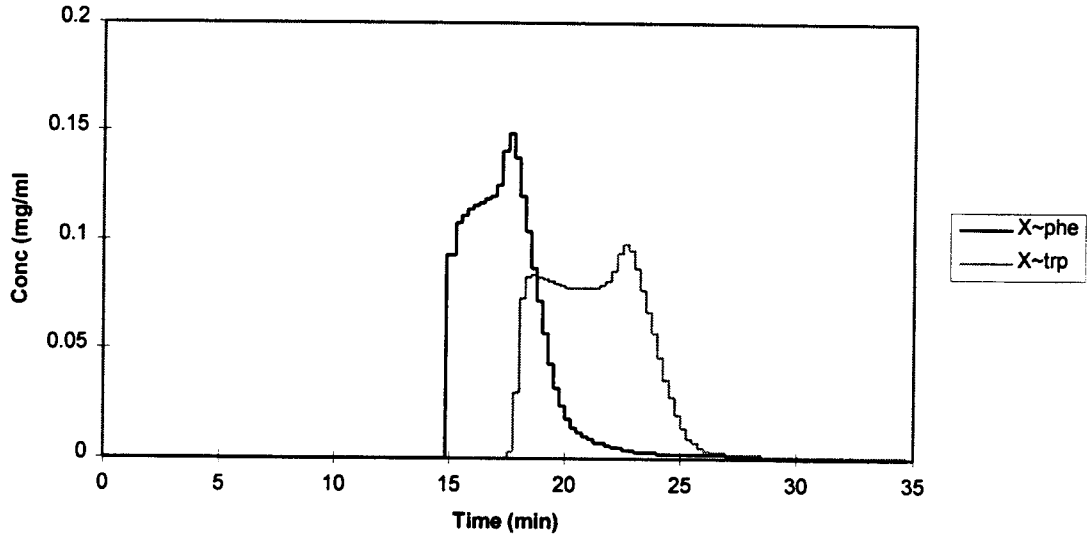


Figure 3.57: Isocratic 20% MeOH. 1mg/ml X~phe and 1mg/ml X~trp, 0.5ml feed volume. No TFA present in the feed. All other conditions identical to Figure 3.39.

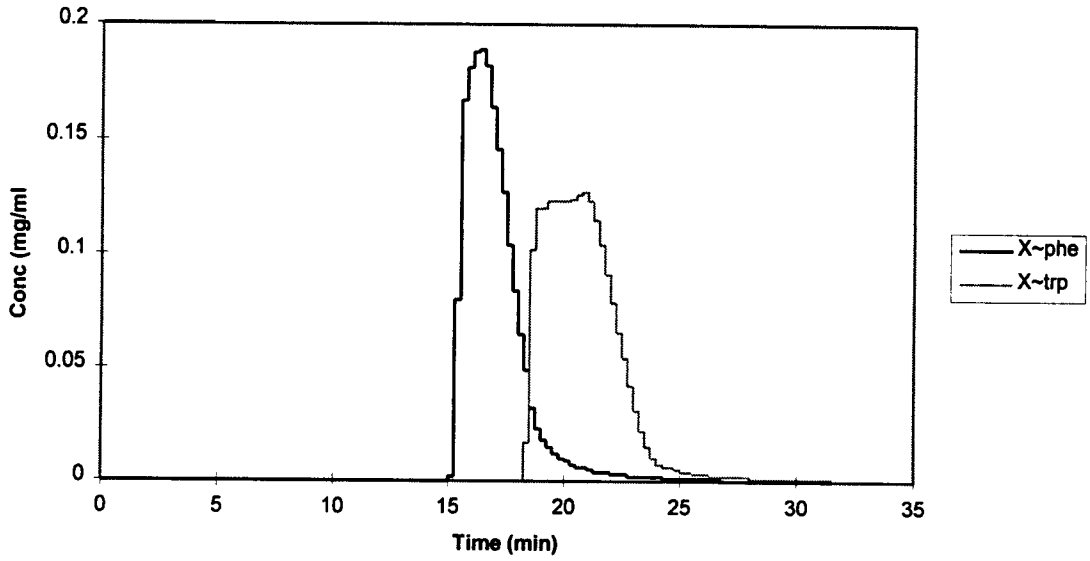


Figure 3.58: Isocratic 20% MeOH. 1mg/ml X~phe and 1mg/ml X~trp, 0.5ml feed volume, 35% feed, no TFA in feed. All other conditions identical to Figure 3.39

again adds to the 40% feed effect. Decreasing the MeOH level slightly in the feed to 35% resulted in the shallow region at the front of the X~phe to disappear, as well as the plateau of the X~trp peak (Figure 3.58). This run gave a slightly higher productivity than the 1ml run with the 0.5mg/ml mixture with equal feed and inlet column conditions (Table 3.9), despite the lower yield of the 35% feed run. Nevertheless the best productivity was achieved by the isocratic 20% at 2.4ml feed volume.

3.3.5 Gradient elution

Isocratic 20% at a 2.4ml volume gave the highest productivity. The X~trp however could only be purified at lower feed volume (0.5ml volume). A 20-45% curve 8 gradient in 20 minutes showed a similar separation as to the isocratic run (Figure 3.59). Although the concentrations were not greater than in the feed, the initial attempts seem slightly better than the isocratic 20% run. The mixing, however, is similar to that in the isocratic run. Increasing the gradient time to 30 minutes brought about a slightly better separation, but the peaks were diluted (Figure 3.60). Tailing remained despite the low concentration being used in the feed; however, the separation was better due to the absence of the 40% feed.

Using a linear gradient, and increase in volume to 2.4ml volume resulted in X~phe mixing with X~trp. The X~trp peak shape deformed, and the X~trp peak did not concentrate (Figure 3.61). This phenomenon may be explained by the selectivity

Description	X~Phe 98% Yield (%)	Enrich.	Product. mg/ml·hr	95% Yield (%)	Enrich.	Product. mg/ml·hr	X~trp 98% Yield (%)	Enrich.	Product. mg/ml·hr	95% Yield (%)	Enrich.	Product. mg/ml·hr
isocratic 20%, 0.4 mg/ml X~phe 0.4mg/ml X~trp 1.0 ml volume	**99**	**0.2**	**1.32**	**99**	**0.2**	**1.32**	**0**	**0**	**0**	**99**	**0.16**	**1.08**
isocratic 25%, 0.5mg/ml X~phe 0.5mg/ml X~trp 1.0ml volume	**71**	**0.47**	**1.86**	**79**	**0.45**	**2.04**	**0**	**0**	**0**	**0**	**0**	**0**
isocratic 20%, 0.5mg/ml X~phe 0.5mg/ml X~trp 2.4ml volume	**81**	**0.22**	**3.36**	**84**	**0.22**	**3.48**	**0**	**0**	**0**	**0**	**0**	**0**
isocratic 20%, 1mg/ml X~phe 1mg/ml X~trp 0.5ml volume 35% feed (no TFA)	**90**	**0.27**	**1.6**	**90**	**0.27**	**1.6**	**0**	**0**	**0**	**64**	**0.083**	**1.2**

Table 3.9: Yields enrichments and productivities of isocratic preparative runs in MeOH.

reversal effect. The points of the intersection of the selectivity reversal can vary according to the concentration of the solute and that of the organic. At low adsorbate concentrations, the retention plots are a reasonable approximation of the peptides' binding affinity. However, at much higher loadings, the adsorption isotherms become non-linear, meaning that the binding of the adsorbates becomes non-linear. Their retentions are then strongly dependent on the multicomponent binding of other adsorbates as well as the binding of the modulator. The point of intersection of the selectivity reversal plots may as a result change according concentration of adsorbates are present. For instance, a low concentration of 0.1mg/ml was used in the adsorbate retention graphs (Figure 3.35). At 0.5 mg/ml initial conditions, the selectivity reversal point may be lower or higher than that of the adsorbate retention using 0.1mg/ml. In Figure 3.61, if we assume that because of high concentration conditions (due in part to the large feed injection volume), the point of intersection of the selectivity reversal occurs at a lower level, then in high gradient concentration, this would allow some of the X~trp to catch up to the X~phe, thereby diluting its original peak. Some of the X~phe on the other hand will loose ground and the tailing of the X~phe occurs.

Lowering the gradient to 30% MeOH, which is lower than the point of intersection of selectivity reversal, results in a good separation (Figure 3.62). In fact the productivities are highest for these runs.

The point of intersection of the selectivity reversal was around 40%. A gradient of 30-50% MeOH was done to try to capture the effect of selectivity reversal. Such a gradient would cause the X~phe to initially elute first, until the modulator level is

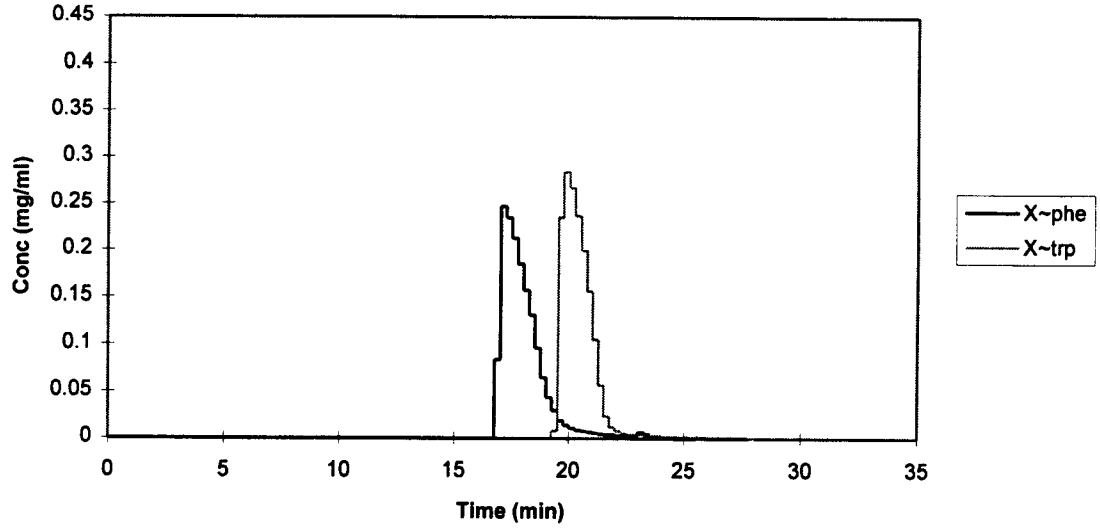


Figure 3.59: Curved no. 8 MeOH gradient 20 to 45% in 20min, 0.4mg/ml X~phe and 0.4mg/ml X~trp in 1ml feed volume. H_2O and TFA throughout, Novapak C-18 column, UV detection at 214nm.

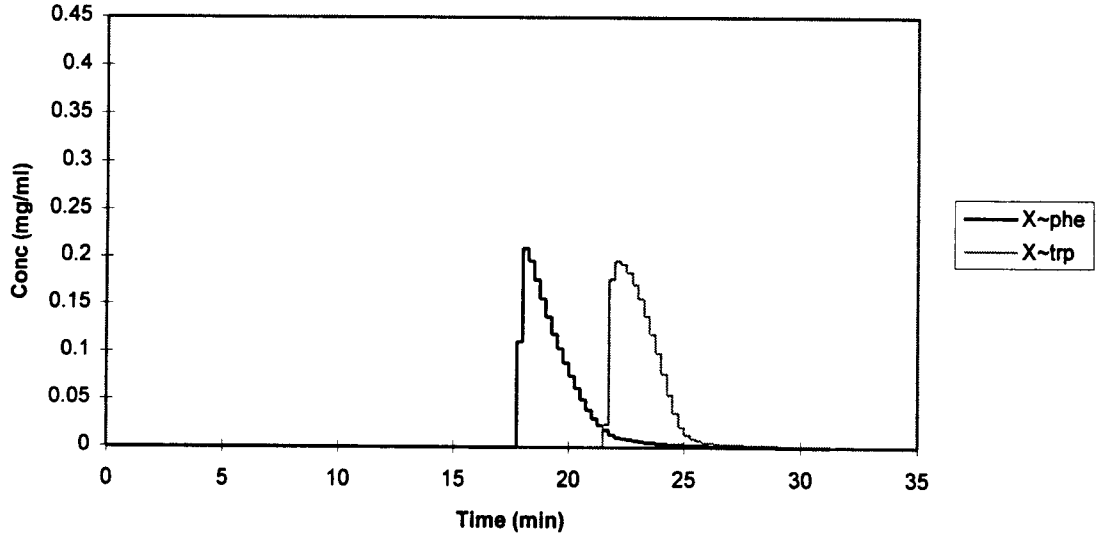


Figure 3.60: Curved no. 8 MeOH gradient 20-45 in 30min, 0.4mg/ml X~phe and 0.4mg/ml X~trp 1ml feed volume. All other conditions identical to Figure 3.59.

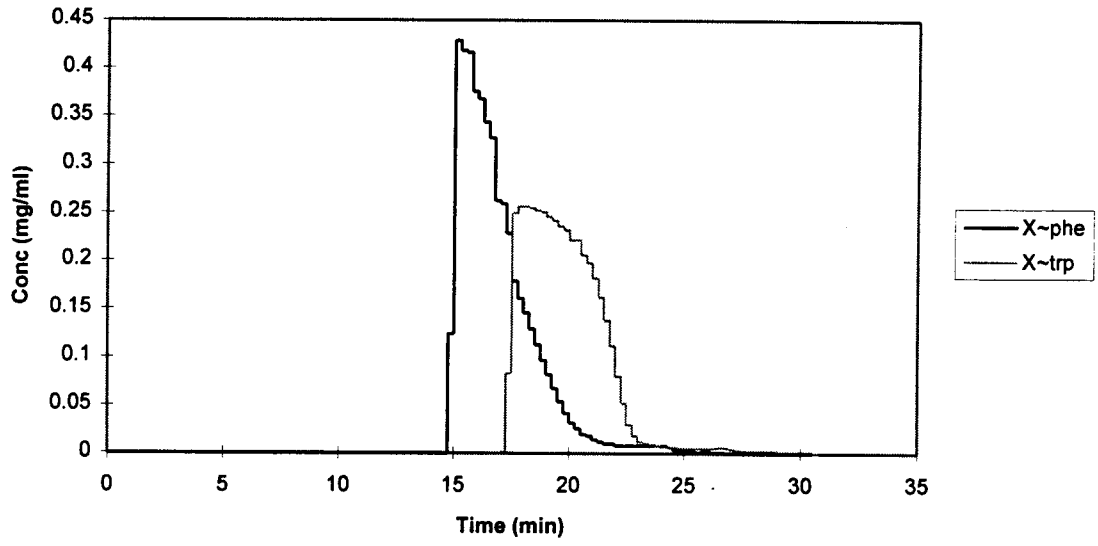


Figure 3.61: Linear MeOH gradient 20-45% in 20min, 0.4mg/ml X~phe and 0.4 mg/ml X~trp in 2.4ml feed volume. All other conditions identical to Figure 3.59.

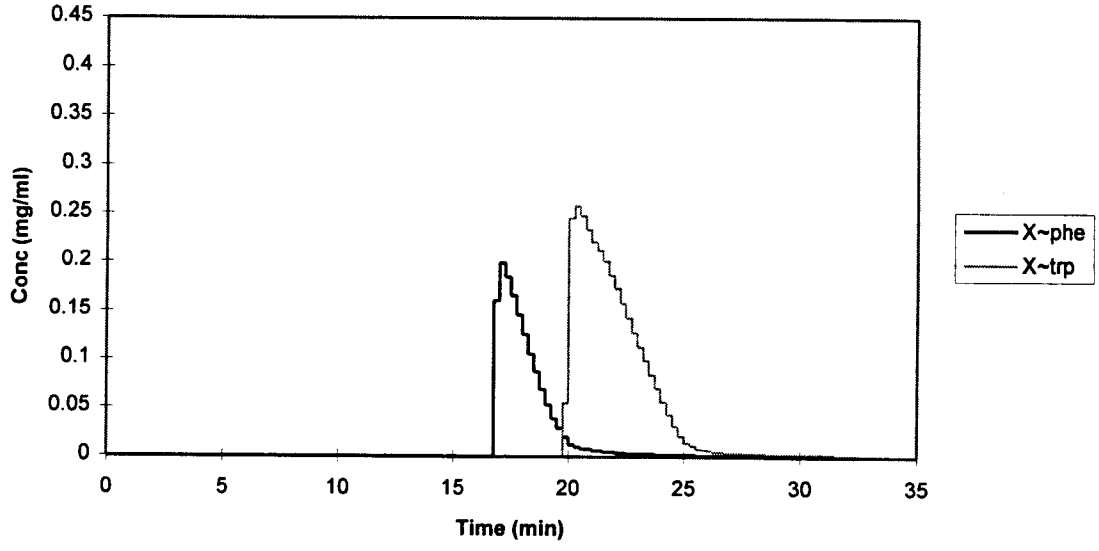


Figure 3.62: Linear MeOH gradient 20-30-30% in 25min, 0.4mg/ml X~phe and 0.8mg/ml X~trp in 1ml feed volume. All other conditions identical to Figure 3.59.

reached such that X~trp starts to elute faster, causing X~phe peak to be found on both ends of the X~trp peak. However, this reversal effect was not observed (Figure 3.63).

The isocratic runs gave better productivities in all cases for the purification of X~phe (Table 3.10). However, for the purification of X~trp, the 20-30% ACN linear gradient gave productivities that were better than the best isocratic run. However, no enrichment occurred for all the runs. The selectivity reversal had a limiting effect on the use of the gradient. Nevertheless, X~trp could be purified better with the gradient than with the isocratic.

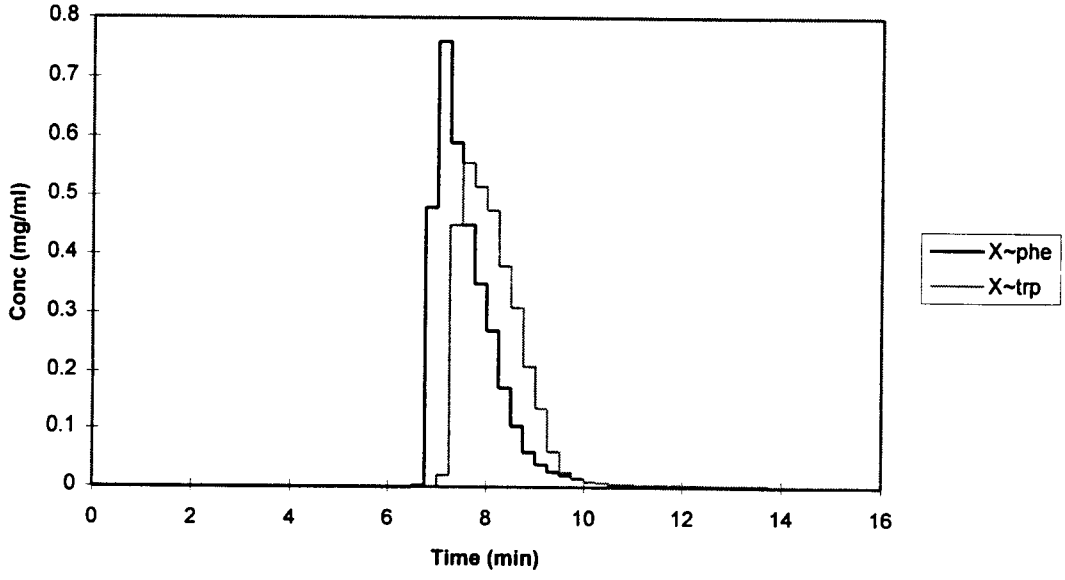


Figure 3.63: Linear MeOH gradient 30-50% in 10min, 0.8 mg/ml X~phe and X~trp in 1 ml volume. All other conditions identical to Figure 3.59.

Description	X~phe 98% yield (%)	Enrich.	Product. mg/ml·hr	95% yield (%)	Enrich.	Product. mg/ml·hr	X~trp 98% yield (%)	Enrich.	Product. mg/ml·hr	95% yield (%)	Enrich.	Product. mg/ml·hr
isocratic 20%, 0.4 mg/ml X~phe 0.4mg/ml X~trp 1.0 ml volume	99	0.2	1.32	99	0.2	1.32	0	0	0	99	0.16	1.08
isocratic 20%, 0.5mg/ml X~phe 0.5mg/ml X~trp 2.4ml volume	81	0.22	3.36	84	0.22	3.48	0	0	0	0	0	0
gradient 20-45 in 30min (c8), 0.4 mg/ml X~phe 0.4 mg/ml X~trp 1.0 ml volume	94	0.24	0.96	94	0.24	0.96	0	0	0	93	0.2	0.84
gradient 20-45 in 30min (c8), 0.4 mg/ml X~phe 0.4 mg/ml X~trp 2.4 ml volume	73	0.30	2.4	73	0.30	2.4	0	0	0	0	0	0
gradient 20-30-30% in 25min, 0.4mg/ml X~phe 0.8mg/ml X~trp 1ml volume	91	0.29	0.9	93	0.27	0.9	0	0	0	100	0.13	1.68

Table 3.10: Enrichments (enrich.) and productivities (product.) of X~phe and X~trp, for 98% and 95% purity.

4 CONCLUSIONS

The preparative purification of the chemotactic peptides X~phe and X~trp was a difficult process. Using ACN conditions, there were many factors that made this a difficult separation: solubility, convergence of adsorbate retentions, low separation factors, and non-linear adsorbate retentions. The MeOH conditions had an added complication, that of selectivity reversal. The importance of having the same feed conditions as starting column conditions cannot be over-emphasized, and has been shown with the various 40% feed preparative runs.

Under MeOH conditions, the gradient proved not to be beneficial, and was unable to concentrate the peaks. However, the X~trp could be isolated better than under isocratic conditions. In addition, a better understanding of selectivity reversal was achieved.

Nevertheless, using an ACN gradient, the peptides could be enriched to twice its concentration and productivities of 6mg/ml*hr were obtained for both peptides (see Table 3.7, section 3.26). Although the isocratic run could even produced almost 7mg/ml*hr, the fractions were not enriched. Furthermore, the cycle time added to the isocratic runs was less than that added to the gradient runs, thereby giving a higher productivity value for the isocratic runs.

If it were not for the limit of solubility of X~phe, the volume or concentration injected could be increased further, and simulations show that the competitive effects would allow much greater enrichments and concentrations, without much increase in

mixing. This is shown by a simulation using a 5ml feel volume (Figure 4.1).

Using experimental parameters, the simulation showed that the peaks could be concentrated to much higher levels, especially the X~phe peak. The simulation considered a Langmuirian feed-feed competitive interactions. A split of the second peak

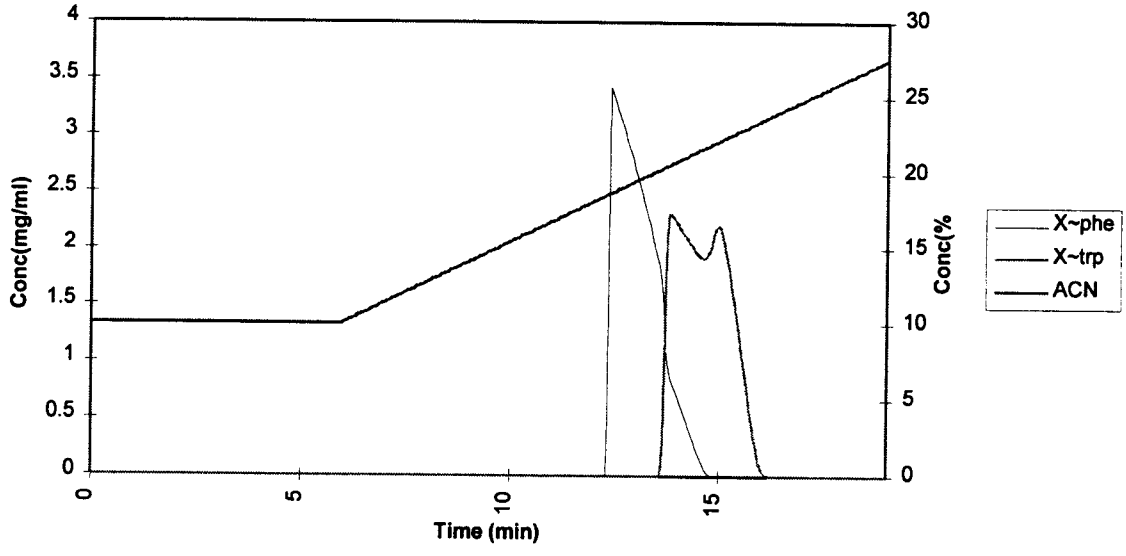


Figure 4.1: Simulation of 10-40% ACN gradient in 20min with 0.71mg/ml X~phe and 0.68mg/ml X~trp in 5ml feed volume (Velayudhan 1997).

and the tailing of X~phe tails into the X~trp peak were present. This reduces the yield of both X~phe and X~trp. However, it is commonly known that despite the lower yields, the productivity can be much higher in nonlinear preparative chromatography due to the concentration of the fractions (Guiochon 1986).

In Table 3.8 (section 3.2.6), the 10-40% gradient at 2.4ml volume gives an

enrichment of 1.9. However, interestingly, the 3.0ml volume only gives an enrichment of 2.1. The increase in enrichment is small relative to the increase in the volume. This is probably due to self interference adsorbate behavior occurs in the non-linear region of their SCI. At such high loading, gradient linearization is no longer valid, and the peak focusing effect is overcome by non-linear peptide interactions. Although ideally one would want to use the focusing effect to achieve tremendously high enrichments, in this case, the self-interference of the peptides was beneficial to this separation, because it prevented X~phe from falling out of solution.

These preparative runs have shown the potential of overloaded gradient elution as a method for preparative separations. Additional work could be done on ternary mixtures to purify the middle compounds, assuming the other two mixtures are impurities. The high loadings in this study was limited by solubility. Studies using high feed concentrations where no solubility limits exist, such as amino acid purifications could be examined.

There will always be purification challenges when dealing with biomolecules because of their inherent biological complexity such as solubility, denaturation, etc. However, the potential for non-linear gradient elution offers chromatographers an additional tool to overcome these challenges.

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APPENDICES

APPENDIX A

Qbasic program for isotherm calculations

```
' Isotherm Calculations
DIM SHARED t(200), c(200), q(200) AS DOUBLE
DIM SHARED n AS INTEGER
INPUT "filename where data is stored :"; isodata$
isofile$ = "f:/home/kimbi/research/traces/isotherm/isomeoh/" + isodata$
PRINT isofile$
'first store data in arrays t and c
OPEN isofile$ FOR INPUT AS #1
n = 0
DO WHILE NOT EOF(1)
  n = n + 1
  INPUT #1, t(n)
  INPUT #1, c(n)
  PRINT t(n), c(n)
LOOP
CLOSE #1
'next, calculate isotherms
INPUT "Feed volume being injected: "; Vinj
sum = 0
Vo = 1.19    'dead volume
Vs = .602    'solid volume
PRINT "C", "q"
P = n
FOR i = 1 TO P
   sum = c(n) + sum
   q(n) = sum * .25 + c(n) * (t(n) - Vo - Vinj)
   q(n) = q(n) / Vs
   PRINT c(n), q(n)
   n = n - 1
NEXT i
'finally store in file
INPUT "filename where q values can be stored :"; isodata$
isofile$ = "f:/home/kimbi/research/traces/isotherm/isomeoh/" + isodata$
OPEN isofile$ FOR OUTPUT AS #2
  FOR i = 1 TO P
  PRINT #2, c(i), ",", q(i)
  NEXT i
END
```

APPENDIX B

Plate count

Plate count is a measure of the efficiency of the column. It is used in the simulations for the determination of the plate height (H). From an analytical chromatogram of a sample of dipeptides run under isocratic elution, the plate count may be determined using the equations listed in Table B.1.

	Equation	description of variables
1	$N = 5.54(\frac{t_R}{w_h})^2$	w_h is the width at half height t_R is the retention time
2	$N = 16(\frac{t_R}{w_b})^2$	w_b is the width of the intersection of the tangents to the inflection points of the curves t_R is the retention time
3	$N = \frac{41.7(\frac{t_R}{A+B})^2}{\frac{B}{A}+1.25}$	These are for asymmetrical peaks. A and B represent the width for each side at 10% of the total height. t_R is the retention time
4	$N = (\frac{t_R}{\sigma_t})^2$	Using the rate theory, the variables can be obtained numerically. $t_R = \mu_1 = \frac{\int_0^\infty tcdt}{\int_0^\infty cdt}$ $\sigma_t^2 = \mu_2' = \frac{\int_0^\infty (t-t_R)^2 cdt}{\int_0^\infty cdt}$

Table B.1: Summary of equations used for plate count determination

The plate counts of the Novapak C-18 (150 x 3.9mm I.D.) are given in Table B.2. There is some asymmetry in the peaks, which causes the numbers in the last column to be somewhat lower than the other entries. Nevertheless these results show that this is an efficient column.

	$N = 5.54(\frac{t_R}{w_h})^2$	$N = 16(\frac{t_R}{w_b})^2$	$N = \frac{41.7(\frac{t_R}{A+B})^2}{\frac{B}{A}+1.25}$
Trial 1 X~phe	2990	2640	2314
Trial 2 X~phe	3350	2770	2428
Trial 1 X~trp	4180	4060	2870
Trial 2 X~trp	4057	3680	2330

Table B.2 : Plate counts of Novapak C-18 column for X~phe and X~trp for efficiency determination.

APPENDIX C

Error distribution, summary of raw data

Date	X~phe mass in mg	X~phe mass out mg	% error	X~trp mass in mg	X~trp mass out mg	% error
9/16/96	1.776	1.953	10.0	1.704	1.8795	10.3
7/23/96	0.051	0.055	7.8	0.64	0.746	16.6
7/25/96	0.48	0.501	4.4	0.37	0.396	7.0
8/1/96	0.5	0.505	1.0	0.38	0.459	20.8
8/1/96	0.58	0.578	-0.3	0.41	0.452	10.2
8/5/96	0.09	0.081	-10.0	0.42	0.565	34.5
8/6/96	0.34	0.364	7.1	0.43	0.562	30.7
9/5/96	0.36	0.367	1.9	0.47	0.605	28.7
9/10/96	0.715	0.774	8.3	0.73	0.782	7.1
9/11/96	0.54	0.596	10.4	0.545	0.611	12.1
9/12/96	1.79	1.94	8.4	1.84	2.05	11.4
10/3/96	2.19	2.36	7.8	2.16	2.47	14.4
10/8/96	1.728	1.832	6.0	1.67	1.77	6.0
10/10/96	1.8	1.797	-0.2	1.8	1.86	3.3
10/29/96	2.19	2	-8.7	2.16	2.29	6.0
2/27/96	0.459	0.554	20.7	0.605	0.811	34.0
2/29/96	0.899	0.91	1.2	1.19	1.04	-12.6
3/6/96	0.94	0.934	-0.6	0.86	1	16.3
3/7/96	0.47	0.45	-4.3	0.43	0.47	9.3
3/14/96	0.57	0.479	-16.0	0.515	0.41	-20.4
3/20/96	0.565	0.517	-8.5	0.49	0.464	-5.3
4/3/96	0.465	0.482	3.7	0.485	0.454	-6.4
4/4/96	0.555	0.505	-9.0	0.495	0.464	-6.3
4/5/96	0.575	0.54	-6.1	0.495	0.463	-6.5
4/8/96	0.525	0.483	-8.0	0.5	0.447	-10.6
4/15/96	0.515	0.485	-5.8	0.535	0.46	-14.0
4/17/96	0.635	0.571	-10.1	0.67	0.553	-17.5
4/18/96	0.192	0.192	0.0	0.545	0.545	0.0
4/25/96	0.245	0.221	-9.8	0.51	0.49	-3.9
4/30/96	0.585	0.542	-7.4	0.22	0.189	-14.1
5/2/96	0.585	0.527	-9.9	0.21	0.186	-11.4
5/7/96	0.265	0.235	-11.3	0.53	0.472	-10.9
11/7/96	0.41	0.423	3.2	0.41	0.43	4.9

Error distribution, summary of raw data (continued)

Date	X~phe mass in mg	X~phe mass out mg	% error	X~trp mass in mg	X~trp mass out mg	% error
11/11/96	0.41	0.427	4.1	0.39	0.4	2.6
11/13/96	0.54	0.536	-0.7	0.53	0.561	5.8
11/15/96	0.525	0.535	1.9	0.515	0.546	6.0
11/19/96	1.176	1.216	3.4	1.08	1.08	0.0
11/21/96	0.39	0.374	-4.1	0.76	0.774	1.8
11/25/96	1.2	1.24	3.3	1.2	1.263	5.3
12/3/96	0.495	0.478	-3.4	0.545	0.51	-6.4
12/4/96	0.455	0.494	8.6	0.43	0.469	9.1
12/9/96	0.435	0.519	19.3	0.18	0.212	17.8
3/12/96				1.06	0.994	-6.2
4/9/96	0.31	0.229	-26.1			
4/9/96				0.715	0.583	-18.5
4/12/96				0.176	0.164	-6.8
4/12/96				0.265	0.25	-5.7
6/6/96	0.43	0.431	0.2			
6/11/96				0.305	0.323	5.9
6/13/96				0.77	0.796	3.4
6/17/96				1.74	1.85	6.3
6/18/96				0.8	0.819	2.4
6/21/96				0.118	0.142	20.3
6/24/96	0.49	0.518	5.7			
7/1/96				6.72	6.8	1.2
9/18/96				2.21	2.45	10.9
9/19/96	1.09	1.23	12.8			
1/15/97				7.27	6.94	-4.5
1/16/97				1.73	1.86	7.5

average X~phe error = 0.0184
standard deviation X~phe = 8.9997

average X~phe error = 3.6719
standard deviation X~trp =12.799