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CHARACTERIZATION OF THE SEPARATION OF PACLITAXEL AND RELATED TAXANES BY REVERSED PHASE LIQUID CHROMATOGRAPHY ON HYDROCARBONACEOUS AND FLUORINATED STATIONARY PHASES

by

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Abstract

Characterization of the Separation of Paclitaxel and Related Taxanes by Reversed Phase Liquid Chromatography on Hydrocarbonaceous and Fluorinated Stationary Phases

by

Ralf Dolfinger

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Fluorinated columns have been reported to exhibit superior selectivity in the separation of taxanes. This study investigates the differences between fluorinated and hydrocarbonaceous stationary phases in the separation of taxanes and in reversed phase liquid chromatography in general. In this context the phenomenon of changes of elution order with variation of mobile phase composition and temperature is considered as well. Propyl-perfluorophenyl, ethyl-perfluorohexyl and ethyl-perfluoro(1-dimethyl-heptyl) phases are characterized in their selectivity to various compounds and compared to a regular hydrocarbonaceous octyl phase.

Using mobile phase and temperature optimization baseline

separation of 15 taxanes is achieved on all phases in 9.5 to 23 minutes in aqueous acetonitrile. The fluorinated columns do not show any definite advantage in the separation of taxanes or other model compounds including aromatic and non-aromatic structures with various functional groups.

Xylosyl-taxol compounds exhibit increasing retention with increasing temperature at mobile phase compositions around 50 % acetonitrile leading to exchanges of elution order with other taxanes. The effect slowly vanishes with increasing or decreasing concentration of acetonitrile. This aberrant temperature behavior is shown to be the caused by hydroxyl groups bound to non-aromatic backbone structures and is proportionally dependent on molecular size and degree of hydroxylation of the solute.

Taxanes, especially xylosyl taxol compounds, are found to show initially decreasing retention upon addition of water to the mobile phase followed by the usually observed increasing retention. At higher water concentrations the retention of xylosyl taxol compounds increases much more sharply compared to the other taxanes leading to multiple exchanges of elution order. The initial decrease of retention is attributed

to hydroxyl group interactions and is independent of the aromatic or non-aromatic nature of the backbone structure. The increase of retention with further increase in the water concentration is shown to depend on the molecular size of the carbon backbone structure.

A model based on the preferential solvation of the solute is proposed to explain the mechanism of the effects stated above. The retention of the solute does not depend on the chemical structure of the solute but on the structure of a solute-solvent complex. The composition of this complex changes with temperature and mobile phase composition affecting retention of the solute.

Dedicated to

My beloved mother Regina Dolfinger

for her 60th birthday

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I defended this thesis on September 11th between 8:50am and 10:25pm.

This is in memory of the thousands of people

who died during that time in the attacks on the

World Trade Center in New York.

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Purpose of This Study

1.1 Purpose of This Study

High Performance Liquid Chromatography (HPLC) is a very popular analytical technique used to separate, identify and quantify a multitude of compounds. The reversed phase mode of HPLC has developed into the most popular and most applied analytical technique in chemistry, especially in the pharmaceutical industry where up to 90% of all analytical work relies on reversed phase HPLC. It is therefore rather surprising that the mechanism of retention and selectivity of this technique is still not clearly known. There is a vast number of publications offering theories, models and studies which give various, often contradictory explanations of the mechanisms of reversed phase LC. Others just report how particular separations can be achieved providing only assumptions about the mechanistic aspect of the separation or simply ignoring it altogether. Many of the fundamental studies are performed with excessively simple analytes such as totally non-polar homologous series of compounds which exclude important aspects of the separation such as the presence of functional groups that can result in a variety of intermolecular interactions such as dipole-dipole or hydrogen bonding. Because of these

problems the chemical mechanism of reversed phase LC is dominated rather by questions and contradictions than solid answers and explanations.

This study is trying to overcome the restrictions stated above. The focus here is not only to develop methods for the separation of taxane compounds but to thoroughly characterize the separation in order to explain its dynamics on a molecular level. This objective is approached through the interpretation of the collected data with new and existing theories to create a new and more complete understanding of the separation mechanism and dynamics of this particular separation and of reversed phase LC in general.

Chapter II

Background

2.1 Paclitaxel

2.1.1 History

In 1964 Wall and co-workers found that extracts from the bark of the pacific Yew tree showed very high cytotoxicity [1]. The responsible compound was isolated and named taxol. The structure of taxol was discovered by Wani et al. in 1971 [2] and is shown in Figure 9. Bristol-Myers Squibb Pharmaceuticals registered the name Taxol several years later as a tradename for its clinical formulation of the compound and the name paclitaxel was assigned as the generic name of the compound. Paclitaxel was first approved for the treatment of advanced breast and ovarian cancer but also showed very good activity towards lung, head and esophageal tumors [3,4,6]. The efficacy of paclitaxel was found to be based on its cytotoxic and antimitotic effect, which is caused by the promotion of microtubule formation and bonding of paclitaxel to the microtubule structures through bonding to the tubulin protein. This causes irreversible polymerization of the microtubules and prevents the cancer cells

from concluding their cycle. The effect is that cancer cell division and therefore tumor growth is inhibited. It has also been suggested that paclitaxel has a radiosensitizing effect which would make it a promising drug for combination with radiotherapy [5,6]. There are, however, major drawbacks to the use of paclitaxel as an anti-tumor agent. The two major problems are the natural toxicity and the low water solubility of the compound, which makes effective delivery of the drug difficult. The doses that can be administered to the patient are limited by the cardiac- and neuro-toxicity of the drug. Additionally, hypersensitivty has been reported to be a serious problem in some cases. To overcome some of these problems, the current clinical formulation uses 30mg of paclitaxel dissolved in 5mL of a mixture of 50/50 Cremophore EL (polycarboxylated castor oil) and ethanol [6]. Cremophore EL acts as a solubilizing surfactant and can lead to serious allergic reactions [6-8].

Due to the problems stated above there is much research in progress to develop derivatives of paclitaxel that exhibit higher water solubility, greater activity and possibly tumor recognition to increase efficiency while lowering the necessary dosage [6].

2.1.2 Structure-Activity Relationships

An important aspect in the research on a drug compound is the structure-activity relationship. This describes which structural features of the compound are responsible for the observed clinical effect. The goal of these studies is to identify responsible groups and through replacement of one or more functional groups find derivatives that might show greater activity, fewer side effects, or both. Knowing the important features of the drug structure can also lead to better understanding of its mechanism of action, which can provide further help in developing more effective treatments.

Since the discovery of paclitaxel, intensive research on the structure-activity relationship of the compound has been done. Paclitaxel is known to have two distinct effects, antimitotic and cytotoxic. The cytotoxic effect leads to cell death and was found to be very complex. It is believed today that the mechanism of cytotoxicity of paclitaxel varies with concentration of the drug and more research is required to identify the distinct mechanisms [9,17]. It is well confirmed that there are four important features that determine the antimitotic activity [10-13,17]:

1. the C-13 side chain

2. the ester group at C-2

3. the ester group at C-4

4. the oxetane ring

It is believed that the conformation of the C-13 side chain is important for the way in which paclitaxel binds to the microtubules. In aqueous solution this chain undergoes "hydrophobic collapse" as indicated by NMR studies, which supposedly results in the appropriate conformation for biological activity [10,14]. The ester groups at C-2 and C-4 are believed to act as hydrogen bond acceptors while the oxetane ring provides rigidity to the entire structure that is necessary to provide spatial stability to the functional groups that are important in the interaction with the tubulin protein [15]. It was also found that substituents on the C-2 benzoyl group can have considerable effects on activity. Bulky substituents in the para position were found to reduce activity while the same substituents enhanced activity if placed in the meta position [16]. It was actually found that an azido group at the meta position can enhance biological activity to the point where the C-18 side chain is no longer required [17]. The complete details of the structure-activity relationship of paclitaxel are not known yet and are still the subject of intensive research.

2.1.3 Production

Paclitaxel was first discovered in the extract of the stem bark of the pacific Yew tree *Taxus brevifolia* in 1964 [1]. It has been found that the drug is also present in other parts of the plant such as needles and twigs but in much lower concentrations. The need for large amounts of paclitaxel for clinical trials and for the prospective use as a commercial drug raised concerns early on since even in the bark of the Yew tree the concentration of paclitaxel is rather low. The pacific Yew is a very slow growing tree and its use for paclitaxel extraction from its non-renewable parts such as the bark was perceived as an environmental threat to the tree population. Therefore, alternative sources for the drug had to be found. The three major alternatives today are:

1. complete synthesis
2. semi-synthesis
3. extraction from cell cultures

The first option, complete synthesis, has been achieved in the lab [1] but is complicated due to the highly complex structure of paclitaxel. This leads to a long and tedious multi-step synthesis, which usually gives low yields and is not usable for commercial-scale production [18].

The method mostly used for the commercial production of paclitaxel today is semi-synthesis from related naturally occurring taxanes like baccatin III and 10-deacetylbaccatin III, which are shown in Figure 9. 10-Deacetylbaccatin III can be extracted in reasonable amounts from needles of the European Yew tree *Taxus baccata*[17,18]. The needles are a renewable resource and contain 10-deacetylbaccatin III at concentrations 10 times higher (1g/Kg) than the concentration of paclitaxel in the bark of *Taxus brevifolia* [17] which make this natural resource environmentally and economically much more viable. Various methods of the conversion of 10-deacetylbaccatin III to paclitaxel have been reported [19-21].

A lot of effort has also been put into the development of methods that allow the production of paclitaxel from cell cultures [22]. Some of these studies use cells directly from the natural plant while others focus on the bioengineering of the cells to increase drug production and yield [18]. The cells are used in vitro in conditions and media selected and adjusted to maximize the production of the drug compound. If the cells store the drug in internal cell organelles like vacuoles a separate step is necessary for extraction from the culture. If the drug is stored on external parts of the cells like the membranes or is released into the medium it is possible to continuously remove the drug from the culture in a

flow- through setup using adsorbents or compatible extraction solvents [22]. The goal of this work is to develop cell cultures and methods that can efficiently, continuously and inexpensively produce large amounts of product in a simple and convenient laboratory setting. At this stage, however, low yield and reproducibility are still major obstacles and have not yet developed the technique to a level suitable for commercial purposes [22].

2.1.4 Separation and Analysis

The vast amount of research on paclitaxel and related derivatives requires methods for the separation and analysis of the various compounds. The technique of choice for this purpose is HPLC or, more accurately, reversed phase liquid chromatography (RPLC). A variety of RPLC methods have been published [23-31] focusing on different aspects related to the research efforts stated above. Determination of taxane compounds in extracts from bark, needles and other plant parts have been performed [25,26,28-31] to aid in the identification of the most useful sources of paclitaxel or alternative derivatives for semi-synthesis like baccatin III or 10-deacetylbaccatin III. Separation and analysis of taxanes have also been performed on extracts from cell cultures [27] to evaluate the dependence of yield and efficiency on the culture medium and conditions.

Many of the assays were developed for the separation of simple mixtures of 8 or even as few as 2 compounds [24,25,27,28,29,30,31]. Few studies focus on the separation of complex taxane mixtures of 12 to 15 compounds [23,26] and the analysis times of these complex separations tend to be long.

A variety of stationary phases have been used for the separation of

taxanes including octadecyl [26,27,29,31], phenyl [24,25,26,28,30,31], cyano [28], and proprietary phases [26,30,31] specially developed for taxane analysis. In recent years fluorinated stationary phases such as per-fluoro-phenyl (PFP) have increasingly been used in the RPLC analysis of taxol [23,24,30]. Many of the proprietary "taxol columns" are believed to use fluorinated bonded phases as well [23]. It is believed that these fluorinated columns have a greater selectivity to paclitaxel and its derivatives [24]. However, no detailed studies have been performed to characterize this particular selectivity or elucidate its chemical mechanism. Shao and Locke reported the separation of 13 and 15 taxanes on two different PFP columns in 19 and 30 minutes, respectively [23].Verpoorte et al. reported the separation of 12 and 13 taxanes on phenyl and "specialty taxane" columns in 30 to 55 minutes[26]. Kopycki et al. separated 8 taxanes on PFP, diphenyl and Curosil G columns and found identical elution orders on all 3 columns [30]. Raymond et al. compared the separation of 8 taxanes on octadecyl, phenyl and a "specialty" column (likely PFP) and found differences in elution order [31], but offered no explanation for possible chemical reasons. Some studies report problems in achieving resolution of paclitaxel and cephalomanine [24,27,29] and claim, that the superior separation on PFP columns is caused by the better selectivity of this bonded phase to the

taxane compounds [24]. The real mechanism of the observed improvement of selectivity on the fluorinated stationary phase, however, has not been explored.

2.2 High Performance Liquid Chromatography (HPLC)

2.2.1 Principles of HPLC

High performance liquid chromatography (HPLC) is probably the most widely used analytical technique. It is an expansion of liquid column chromatography, which was invented in 1903 by Tswett [32] and utilizes an instrument setup as shown in Figure 1. A solvent or solvent mixture called the mobile phase is moved continuously through a column containing particles of an insoluble chemical agent called the stationary phase and then through a detector using a high pressure pump. A sample mixture can be introduced into the mobile phase flow stream through the injector. The principle of the separation process is shown in Figure 2. If there is a difference of chemical affinities between each of the different solutes a, b and c and the stationary phase, each solute is retained to a different degree and needs a different time to get from the injector to the detector where it generates a response different from that of the mobile phase. The detector response versus time is recorded as a chromatogram; a typical chromatogram is shown in Figure 3. Through comparison with standard mixtures the retention time can be utilized to identify

a compound peak while the area under the peak is directly proportional to the amount of compound injected. Through the use of standard calibration curves, the amount of injected compound can therefore be determined. Each solute peak is characterized by its by its retention factor k' which is calculated as follows,

$$k' = (t_R - t_0) / t_0 \qquad (1)$$

where t_0 is the retention time of an unretained compound and t_R is the retention time of the compound to be characterized. The relative retention or separation factor α between two peaks is calculated as follows,

$$\alpha = k'_2 / k'_1 \qquad (2)$$

where k'_2 and k'_1 are the retention factors of solute 2 and 1, respectively. The resolution R is a better description of the separation of two compounds and is calculated as follows,

$$R = 2 (t_{R2} - t_{R1}) / (w_1 + w_2)$$

$$= 1.18 (t_{R2} - t_{R1}) / (w_{1/2\,1} + w_{1/2\,2}) \qquad (3)$$

where t_R, w and $w_{1/2}$ are the retention time, base peak width and peak width at half height, respectively. The subscripts 1 and 2 refer to peak 1 and peak 2, respectively. A resolution of about 1.5 indicates complete separation of two compounds [33] which is commonly referred to as "baseline separation" meaning that the detector response reaches baseline level between the two peaks.

There are a number of different separation modes in HPLC, including Normal Phase (NP), Reversed Phase (RP), Ion Pair (IP) and Ion Exchange (IE). The most popular mode by far is the reversed phase mode, which accounts for up to 90% of all HPLC separations [34]. This study will use the RP mode of HPLC, which will be explained further below.

Figure 1. HPLC Instrumental Setup

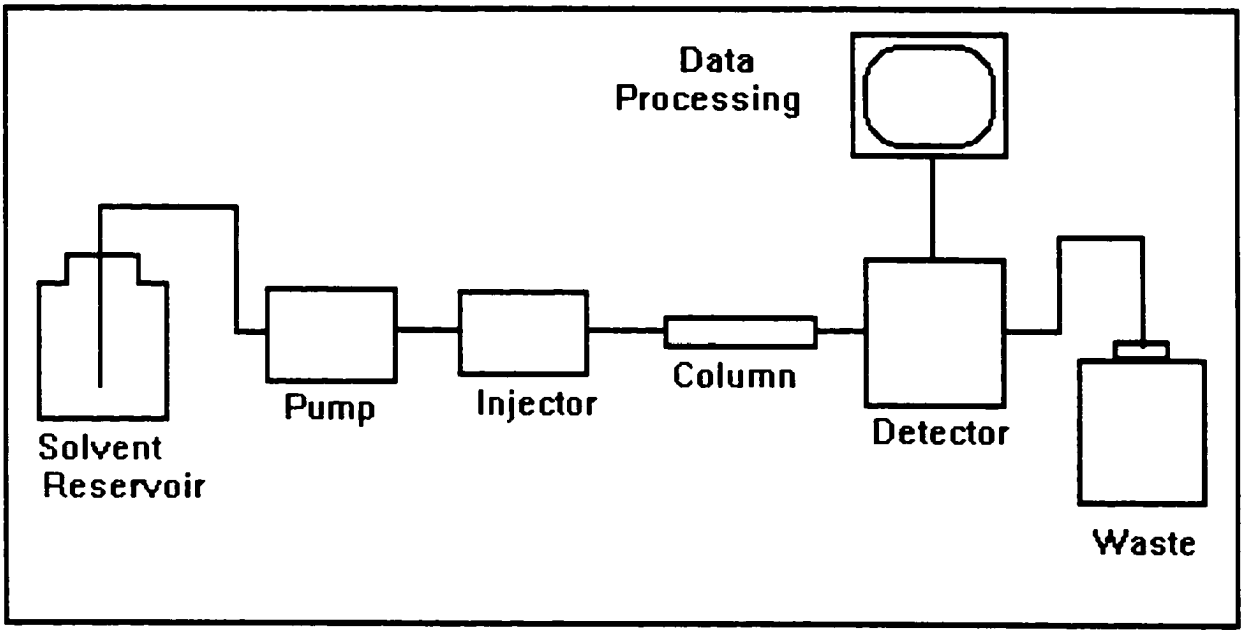


Figure 2. Principle of Separation in HPLC

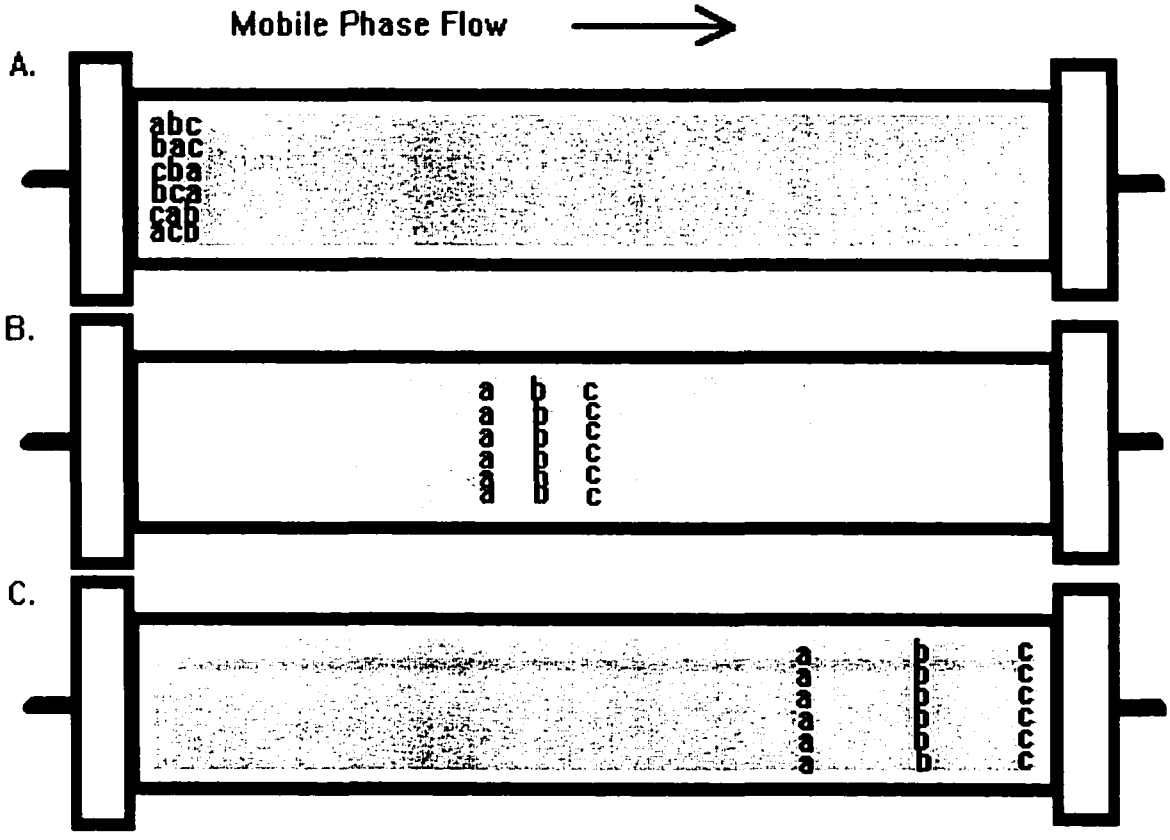
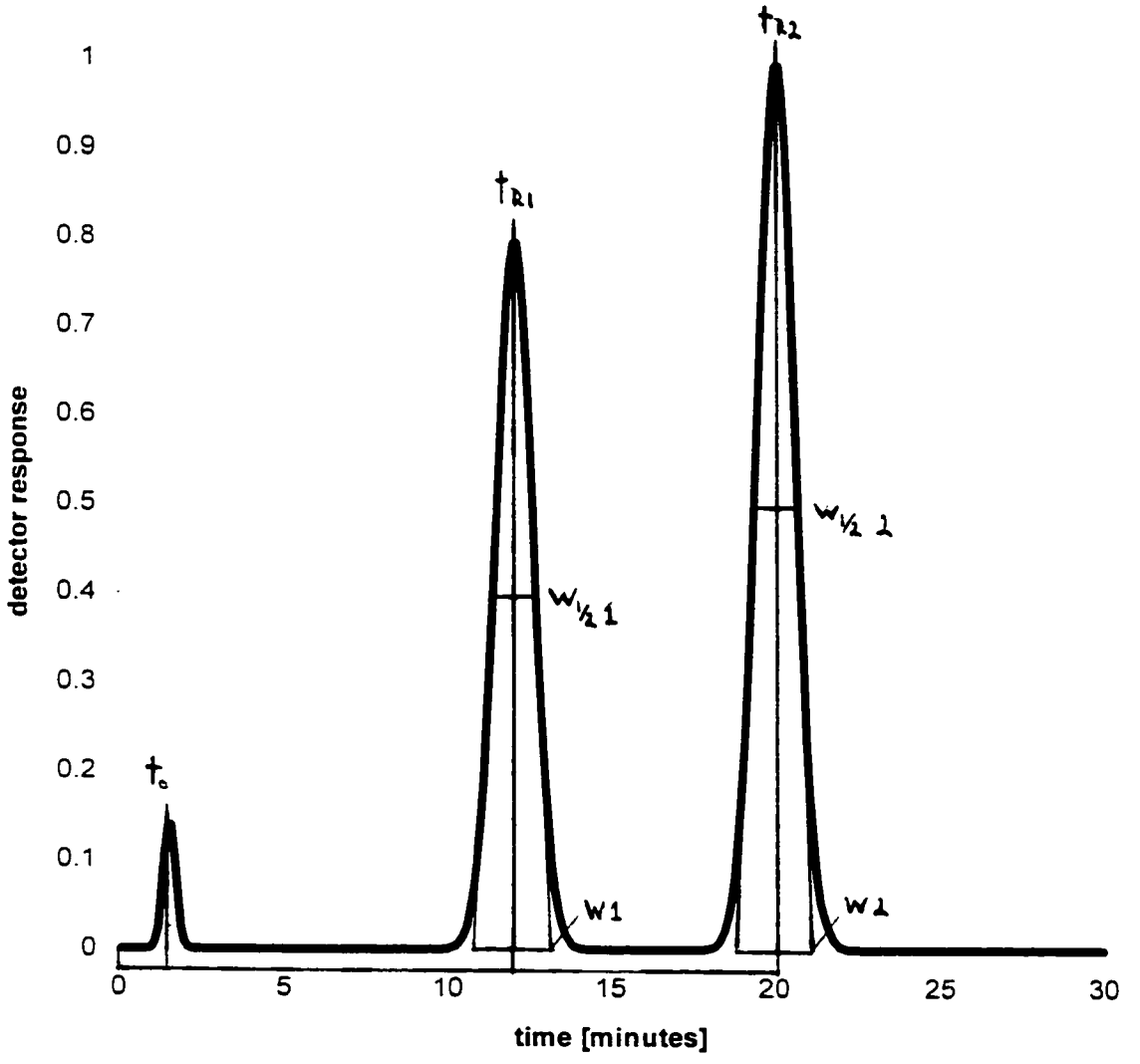


Figure 3. HPLC Chromatogram



2.2.2 History and Principles of Reversed Phase Liquid Chromatography

Reversed phase liquid chromatography (RPLC) is a particular mode of HPLC. While in normal phase liquid chromatography (NPLC) a relatively polar stationary phase is used in combination with a less polar mobile phase, RPLC employs a stationary phase, with a relatively non-polar surface and a more polar mobile phase. The mobile phase in RPLC usually consists of a mixture of two solvents; water and a organic solvent called a organic modifier. Generally, the organic modifiers of choice are either acetonitrile, methanol or, in some cases, tetrahydrofuran. Different organic modifiers are known to result in different selectivities for a given separation and the most suitable modifier is usually identified experimentally. To achieve intermediate selectivities the organic modifier can also be chosen as a combination of two or even all three of the above mentioned solvents. According to Snyder the three stated modifiers and their mixtures provide the full range of selectivities achievable with any other possible solvent [35]. In NPLC polar solutes are retained most strongly causing solutes to elute with increasing polarity. In RPLC the elution order is reversed; polar solutes elute first and the most non-polar solutes elute last.

The first attempts at RPLC were made by Boldingh [36] who separated long-chain fatty acids on rubber powder in aqueous methanol and acetone in 1948. The term "Reversed Phase", however, was created by Howard and Martin in 1950 [37-39] when they used Kieselguhr coated with liquid paraffin and n-octane to separate fatty acids. It was initially thought that RPLC would be only suited for very non-polar analytes but it was soon discovered that the technique was applicable to a wide variety of separations. Modern bonded phases were developed in the mid sixties by reacting octadecylchlorosilane with silica gel [38]. This provided a non-polar, stable, long chain alkyl phase on a rigid, porous support with high surface area. Further improvements of the particle shape were achieved by Kirkland [40] and Majors [41] through development of totally porous, spherical, pellicular and microparticulate bonded phases resulting in greatly improved resolution and efficiency. Today RPLC columns mostly contain spherical 5 μm particles with pore sizes of 80-300Å, but smaller particles (3 μm) are used in short columns (3 cm) for fast LC. A variety of bonded phases have been developed for use in RPLC. Linear and branched chain alkyl phases with chain lengths from C1 to C22, phenyl, diphenyl, cyano and amino phases are commonly applied in the reversed phase mode. More recently perfluorinated, and partially fluorinated carbon chains have been

introduced as a new class of bonded phase [81-86]. They will be discussed in more detail below.

As mentioned earlier the bonded phase is grafted onto the silica gel through reaction of a chlorosilane derivative with the surface silanol groups as shown in Figure 4. The surface concentration of the silanol groups on silical gel is about 8 $\mu mol/m^2$ [39,42]. The usual bonded phase coverage that can be achieved is 2.5 to 3.0 $\mu mol/m^2$ [39,42] which can be increased to about 4.0 to 4.4 $\mu mol/m^2$ using optimized bonding reactions [39,43]. This leaves a large number of surface silanol groups unreacted. The remaining silanol groups affect the retention of solutes during chromatography and lead to tailing and poor efficiency, especially for basic and acidic compounds [39]. To avoid this problem a technique named "endcapping" is used in which the residual silanol groups are reacted with trimethylchlorosilane [44-46]. This process, however, is not totally effective and residual surface silanols influence the performance and properties of RPLC bonded phases.

The surface density of the bonded phase is a very important factor. Higher bonding densities provide more effective shielding of the residual surface silanols and protect the underlying silica matrix from aggressive solvents such as buffers at high or low pH. Additionally, it has been found that

higher bonding densities result in increased selectivity for certain compounds. For Example, the separation of polycyclic aromatic hydrocarbons has been shown to depend strongly on bonding density, which is believed to be caused by an increase in shape selectivity with more tightly spaced and more "ordered" bonded phases [47]. High bonding densities are therefore desirable, but they are limited due to steric constraints of the bonding reaction. The bonded moieties on the silica surface sterically prevent the large chlorosilanes access to the unreacted silanol groups. A fully reacted silica surface can therefore not be achieved.

The structural state of the bonded phase has been the subject of much research and discussion [39,45]. Early on it was believed that the alkyl chain bonded phases were extended in a brush like fashion as shown in Figure 5a [48]. This model has been dismissed as unrealistic since the chains have rotational freedom and therefore can coil into a more disordered, liquid like state as shown in Figure 5b. However, because the chains are restricted in their movement on one end through the attachment to the silica surface, the alkyl phase can not be expected to have properties equal to a liquid phase. It is known that the structure of the bonded phase depends on the mobile phase composition [49-51]. The

general perception is that at high to medium concentrations of organic modifier, the bonded phase is in a moderately extended state but tends to collapse at very low concentrations (<10% organic modifier) [52]. The degree of collapse depends on the nature of the bonded phase and leads to a decrease of retention and resolution of all solutes. A third opinion is that the bonded phase is in a collapsed state at any mobile phase composition as show in Figure 5c. Martire and Boehm [44] claim that methanol and acetonitrile are too polar and would lead to collapse of n-alkyl bonded phases. According to their study only THF has the ability to induce a partly extended configuration for the hydrophobic bonded phase. This view is supported by Kazakevich [53] who showed in yet unpublished work that the pore volume of the wet stationary phase is equal to the pore volume of the dry stationary phase. Since the bonded phase is necessarily collapsed in the dry state the equal pore volume in the wet stage suggests a equally collapsed configuration. The view of the permanently collapsed bonded phase, however, is still subject to debate and many researchers object to it. Further research will be needed to resolve this controversy.

The influence of temperature in RPLC is only partly understood. Until a few years ago elevated temperatures were only used to lower the viscosity of the mobile phase to achieve higher flow rates and reduced analysis times [54].

Higher temperatures were also recommended to increase solubility of some solutes [54]. It was generally believed that an increase in column temperature will reduce solute retention due to increased thermal motion of the solutes and correspondingly reduced interactions with the stationary phase [35]. Only in recent years it has been found that temperature is a parameter that can be used effectively to optimize selectivity and resolution in RPLC separations [55-61] but there has been no detailed study of the mechanism that facilitates this observed change in selectivity. In some cases a phase transition of the bonded phase has been named as the cause [39,59,62] but this interpretation is purely speculative.

Many of the studies published on stationary phase structure and effects are contradictory and strongly debated. A good example are studies on the efficiency of bonded phases with different alkyl chain length. Karger and Giese [63] reported that shorter chains lead to better efficiencies, whereas Grushka and Kikta [64] found the opposite trend. Karch et al [65] on the other hand found no effect at all.

Only very fundamental points about RPLC seem to be consistent amongst different studies. The most important ones are stated below:

1. retention of solutes increases with increasing concentration of water in the mobile phase [35,54]
2. retention of solutes increases with increasing chain length of the n-alkyl bonded phases[66]
3. selectivity of the RPLC system depends on:
   a. nature and composition of the mobile phase [35]
   b. chemistry of the bonded phase [39]
   c. bonding density and state of chain order on the silica surface [39]
   d. quality of the silica matrix [39,42]
   e. accessibility and surface concentration of residual silanol groups [39]
   f. temperature [55-61]

According to points 3d and 3e it is obvious that not only the bonded phase, but also the nature of the underlying silica matrix [67] and the manufacturing process are responsible for the characteristics of the column. Seemingly equivalent columns from different manufacturers or even different lots from the same manufacturer can therefore exhibit considerable variations in chromatographic behavior [39]. Consequently it might be hard to judge if the

result of a particular study describes the effect of the bonded phase (as is usually claimed) or the silica matrix.

Figure 4. Bonding Reaction of Chlorosilane with Silica Surface

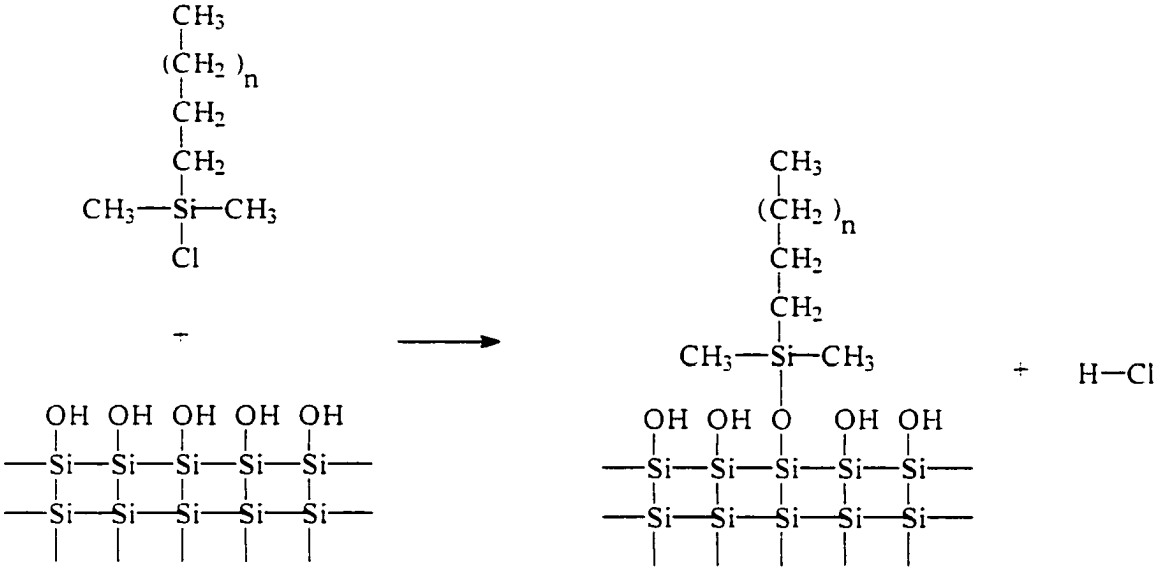
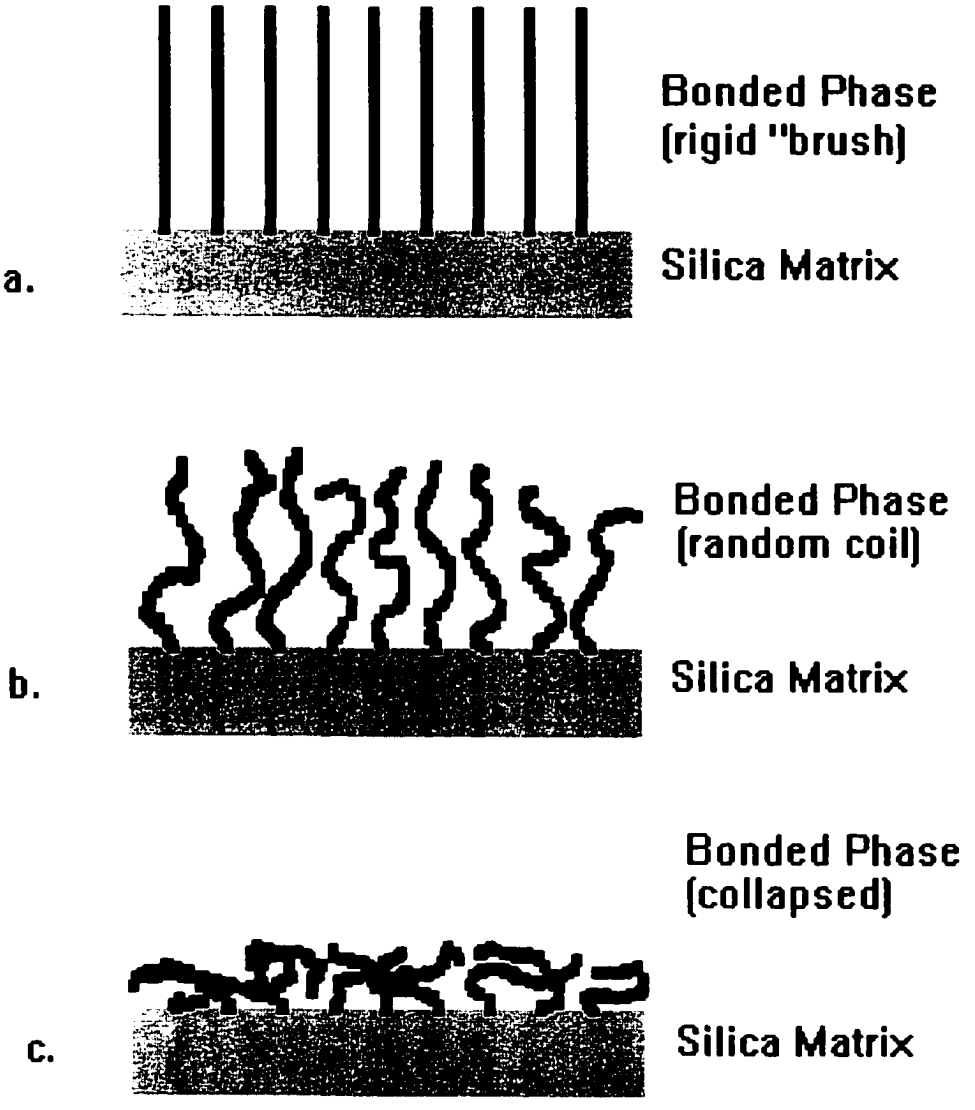


Figure 5. Bonded Phase Configuration



2.2.3 Mechanism of Reversed Phase Liquid Chromatography

On the surface the mechanism of RPLC seems quite simple. The stationary phase is non-polar and therefore interacts more strongly with non-polar solutes leading to increased retention as compared to more polar solutes. This leads to polar solutes eluting before non-polar ones[54]. The mechanism of the retention process, however, is still unclear and many theories and models have been proposed to explain the elution behavior of solutes in RPLC. Only the three major theories will be discussed here; the Solvophobic Theory, the Adsorption Theory and the Partitioning Theory.

The Solvophobic Theory is based on work by Sinanoglu and Abdulnur [68] and was used in further work by Horvath, Melander and Molnar [38]. According to this theory the major force of retention is solvophobic expulsion. The hydrophobic parts of the solute molecule have a relatively high surface area while dispersed in the mobile phase. The polar forces of the mobile phase, like the water hydrogen bonds, cause a very high surface tension, which "pushes" the solute out of the mobile phase. The solute effectively reduces its surface area by "clustering" onto the non-polar bonded phase. It is primarily the push of the mobile phase, not the attraction between solute and bonded phase that drives the

retention process [38]. According to this theory the chemistry of the bonded phase is not important as long as it is hydrophobic.

The partitioning theory is derived from the way retention is known to occur in liquid-liquid chromatography. In this case the solute distributes between the two liquid phases according to the relative solubility as determined by the partition coefficient K. An important feature of this process is that the solute totally penetrates the liquid stationary phase. According to the partitioning theory, retention RPLC occurs in the same way with the bonded phase acting like an immobilized liquid into which the solute is dissolved. The driving force for retention is the relative chemical affinity of the solute for the mobile and stationary phase[39].

In the adsorption model the driving force of the retention process is also the chemical affinity of the solute to the stationary phase but the solute is not believed to penetrate into the bulk bonded phase. The only area of contact is the surface of the bonded phase [39].

There are many studies providing evidence for all of the three theories. Horvath et al. found support for the solvophobic theory through the comparison of solvophobic strength of the mobile phase with the capacity factor of several solutes. But many studies found that the nature of the stationary phase can

strongly influence retention and retention order of solutes, which is in direct contradiction to the solvophobic theory. Berendsen and Galan [66] reported that the retention of a variety of solutes increases with increasing chain length of the bonded phase, which indicates the influence of the stationary phase on the retention process. The solvophobic theory does not account for any influence of the stationary phase and is therefore at least incomplete. Berendsen and Galan also found that the retention of long chain solutes increases with longer bonded phase chains until they are about equal in length. After that point the retention reaches a plateau [66]. The same study indicated that the plateau was reached at shorter bonded phases when the solute contained polar functional groups. These finding strongly suggest that the solute partitions into the stationary phase and that retention depends on the degree of penetration. A further interpretation would be that for short bonded phases and polar solutes the retention process becomes more adsorption-like while for non-polar solutes and long chain bonded phases a more partitioning-like retention process is possible.

It is reasonable to assume that adsorption occurs on the surface of cyano and amino bonded phases through dipole-dipole interactions and hydrogen bonding; π-π interactions might lead to adsorption of certain solutes on phenyl and diphenyl phases.

There is a clear indication that the nature of the retention mechanism can not be generally defined. The most reasonable interpretation of the studies cited above is that all three mechanisms probably are in competition with one another and affect retention to varying degrees. Depending on the chemical structure of each particular solute and stationary phase one mechanism might dominate the retention process. This, however, should not be used as evidence that the other mechanisms do not occur. The complexity and variety of data collected in RPLC strongly suggest that more than one effect plays a role in the retention of solutes.

It should be mentioned that there are several factors and phenomena that are being ignored or remain unexplained by most if not all theories of the retention mechanism. The most important ones are listed below:

1. change of elution order of solutes with change in mobile phase composition
2. different elution order with different organic modifiers
3. effect of solvation of the stationary phase on solute retention
4. effect of solvation of the solute on retention

It is well known that in many separations solutes exhibit different elution

orders at different mobile phase compositions. Solute mixtures that show this behavior are described by Snyder et al. [57] as "irregular samples" but they are actually quite common.

The use of different organic modifiers can have the same effect [35]. The solvation of the stationary phase is a phenomenon investigated in many studies [67,69-73] and will be discussed in detail below. It is agreed that the solvation of the stationary phase affects solute retention but no mechanism for this effect has been suggested yet. The effect of solute solvation on retention in RPLC has been virtually ignored. There are only a handful of studies[74,75,76] most of them theoretical models developed by Jaroniec et al. [76-78], but the treatment is only mathematical and does not provide a conceptual picture of the effect of solvation in RPLC.

All of the topics mentioned include the mobile phase as a major factor in the retention of solutes in RPLC, however, none of the mainstream theories above includes the mobile phase as part of the separation mechanism and they may therefore be flawed or at least incomplete.

2.2.4 Characterization of the Stationary Phase

The structure and chemistry of the stationary phase can not be defined without considering its interaction with the mobile phase. Like any molecule in solution, the bonded phase and the accessible silica matrix are solvated with the mobile phase components. The non-polar bonded phase is believed to be solvated preferentially with the organic modifier, which leads to a swelling or "breathing" of the stationary phase surface [44]. It is believed that the adsorbed mobile phase components form a mostly organic rich layer on the liquid solid interface. The relative amounts of adsorbed organic modifier and water vary at different mobile phase compositions and temperatures and can be determined experimentally as sorption isotherms or sorption excess isotherms (also called surface excess isotherms) using various methods [67,69-73,79-80]. The sorption isotherm describes the amount of mobile phase component adsorbed at different mobile phase compositions whereas the sorption excess isotherm describes the excess of organic modifier (or water) adsorbed at different mobile phase compositions. The most popular methods to characterize mobile phase sorption are listed below:

1. gas chromatographic analysis of solvent stripped from the column [67,70]
2. frontal analysis [108]
3. injection of labeled mobile phase components [69,70,72,73]

In method 1, the HPLC column is equilibrated with a certain mobile phase composition and then the bulk mobile phase and the adsorbed components are stripped from the column using a suitable solvent like dioxane. The dioxane "wash" is then analyzed by gas chromatography to determine the amounts of organic modifier and water, which can be used to calculate the adsorbed amounts. McCormick and Karger [70] state that the use of this method is restricted to mobile phase compositions with organic modifier concentrations below about 50 % due to the decreasing accuracy at higher concentrations, but Burke and Zwier [67] used the method for the entire mobile phase range. A disadvantage of this method is that it is very work intensive, has low accuracy and requires large amounts of solvents.

Method 2 is based on the fact that the adsorbed amounts change with changing mobile phase composition. The concentration of the organic modifier in the mobile phase is changed and the column eluent is monitored using a

refractive index detector. The detector will "see" two shifts of its baseline plateau, one corresponding to the new water concentration and one to the new organic modifier concentration. If the stationary phase is solvated with the same volume of water as organic modifier, both fronts will emerge at the same time. The difference of elution volume between the two fronts/shifts is equivalent to the difference of volume of the adsorbed mobile phase components. Since the shifts of the baseline plateau are not sharp, however, it is problematic to determine precise elution volumes which affects the accuracy of the method.

In method 3. deuterated species of the mobile phase components are injected onto the column and the effluent is monitored with a refractive index detector. Since the two deuterated solvents undergo diffusional exchange with their adsorbed counterparts on the stationary phase the difference in their retention volumes can be used to calculate the difference in the adsorbed amounts. A problem in this method is that the deuterated species undergo isotopic exchange with the mobile phase components while moving through the column, which can result in changes of retention times and calculated excess [79]. This effect, however, is not believed to be strong enough to prevent the generation of useful data as indicated by the frequent use of the method.

For practical reasons method 3 seems to be the method of choice since it

only requires the purchase of the deuterated solvents. The experimental part is identical to running a regular HPLC sample and can be executed with any common HPLC system setup.

2.3 Fluorinated Stationary Phases in Reversed Phase Liquid Chromatography

2.3.1 History

The use of fluorinated stationary phases for RPLC was first reported by Galan et al. in 1980 [81,82]. They prepared a stationary phase equivalent to a C10 phase with the eight terminal carbons of the bonded phase being perfluorinated, and compared it to regular C3 and C10 phases. All columns showed comparable properties in the separation of fluorinated and non-fluorinated compounds, respectively. The perfluorinated phase, however, was found to show increased selectivity for separations of mixtures of fluorinated compounds and their corresponding non-fluorinated analogs. This was caused by the observed increased retention of fluorine-containing solutes by the perfluorinated phase. Billiet and Galan [82] reported overall lower retentivity of the fluorinated stationary phase and special selectivities for some non-fluorine-containing compounds. The results of the study are questionable, however, due to the use of different mobile phase compositions on the fluorinated and non-fluorinated columns [82]. Haas and Köhler [83]

synthesized and tested a perfluorinated aliphatic and a perfluorinated phenyl bonded phase. They reported results comparable to previous studies for the aliphatic fluorinated phase but reported that the perfluorinated phenyl phase had a higher retentivity than a non-fluorinated analog in the separation of aromatic hydrocarbons. Krafft et al. [84] tested the effect of chain length of fluorinated compounds on perfluorinated phases and found direct correlation between increased retention and the number of fluorine atoms in the solute.

The lower retentivity of fluorinated alkyl phases can be useful in achieving separations of lipids in more advantageous mobile phase compositions as described by De Miguel et al. [85].

The lower retentivity of fluorinated alkyl phases has been attributed to the low polarity and greater rigidity of fluorinated chains due to the greater covalent radius of the CF unit [83,85] and a greater rotational energy barrier [86]. The chemical mechanism responsible for the observed differences in selectivity to some compounds as compared to regular bonded phases is not clearly understood and explanations at this time are mostly speculative.

2.3.2 Fluorinated Bonded Phases in Taxane Separations

In recent years fluorinated phases, especially perfluorinated phenyl phases have been used increasingly in the separation of taxanes [23,24,26,30,31]. Fluorinated bonded phases are believed to exhibit better selectivity to taxane compounds but the chemical mechanism of the taxane interactions with the fluorinated surface has not been investigated at this point. A detailed discussion of the application of fluorinated bonded phases in the separation of taxanes is given above in section 2.1.4.

Chapter III

Experimental

3.1 Instrumentation

All experiments were performed using a Hewlett-Packard HP-1090, Series II Liquid Chromatograph equipped with three solvent channels, an autosampler and a UV photodiode array detector. Data acquisition and processing were performed on a Hewlett-Packard Vectra 486/66U computer using Hewlett-Packard Chemstation software (Rev. A.03.03).

Column temperature was controlled using a laboratory-constructed water jacket fed by a Lauda RM20 water thermostat with heating and cooling options. The temperature within the jacket was monitored with a Fisher temperature probe connected to a Fisher Accumet 925 pH meter. The instrumental setup of the column thermostating equipment is shown in Figure 6. The temperature was controlled within ±0.1 °C.

For the surface excess isotherm measurements, the mobile phase flow rate was measured using a burette with an accuracy of ±0.1mL immersed in a graduated cylinder, which was fed by and immersed in a Lauda RM20 thermostated water bath with heating and cooling option so that the water

overflowing from the cylinder could return into the water bath. Time measurements were performed using a digital stopwatch over periods of 30 seconds.

For the surface excess isotherm measurements a Waters 410 refractive index detector was used. It was connected to the Hewlett-Packard HP-1090 Chromatograph and Chemstation software using a Hewlett-Packard 35900E A/D interface. The refractive index cell temperature was adjusted to 40.0 °C. The complete chromatographic instrument set-up is shown in Figure 7.

All substances were weighed using a Fisher XA-200DS analytical balance with an accuracy of ±0.1mg.

Calculations and graphics were executed on Microsoft Excel for Windows 5.0.

Figure 6. Column Thermostating Set-Up

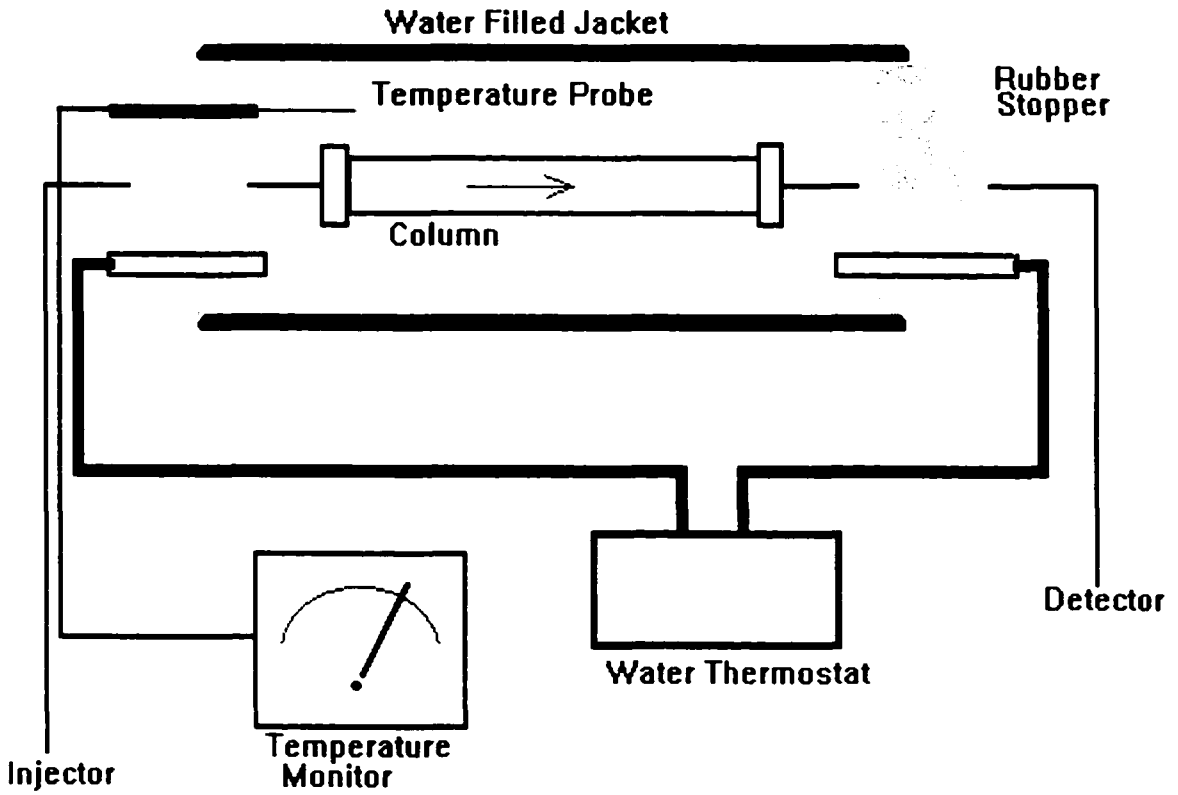
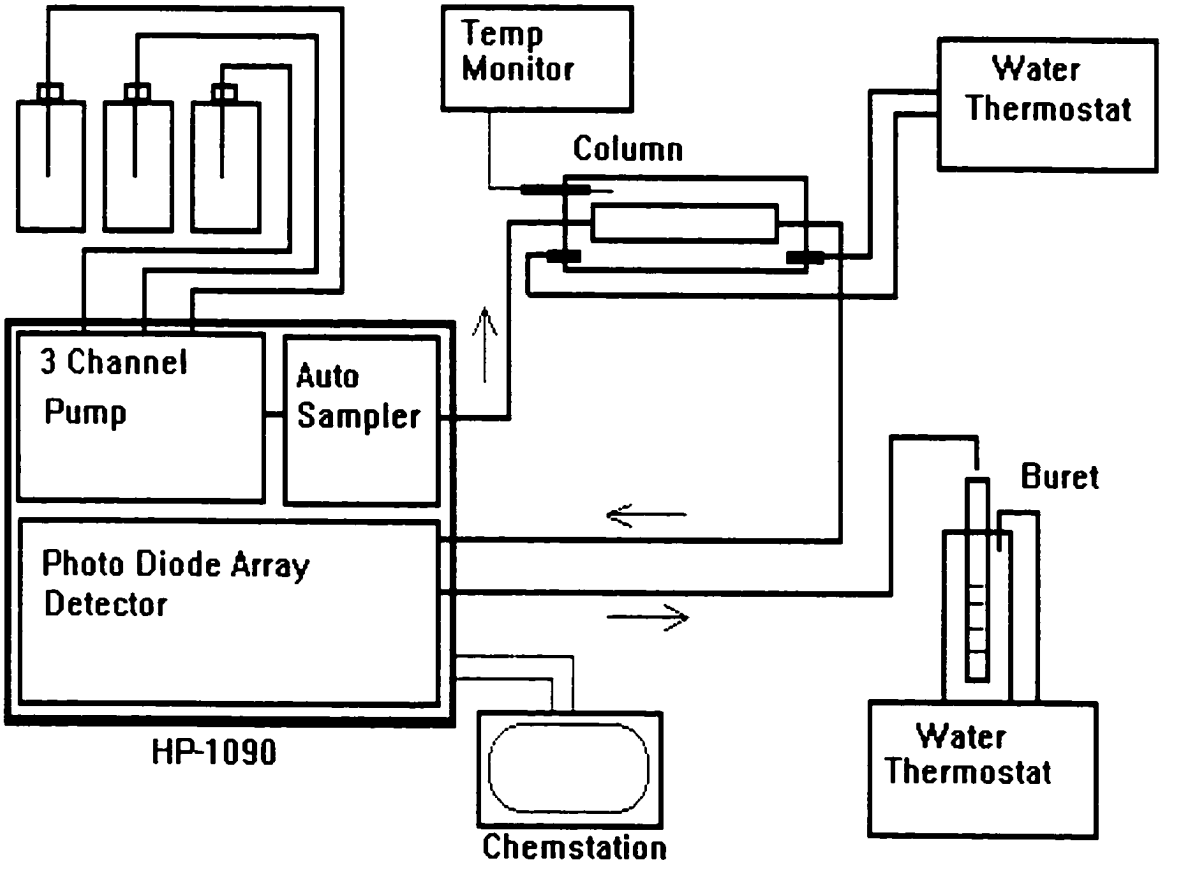


Figure 7. Chromatography Instrument Set-Up



3.2 Stationary Phases

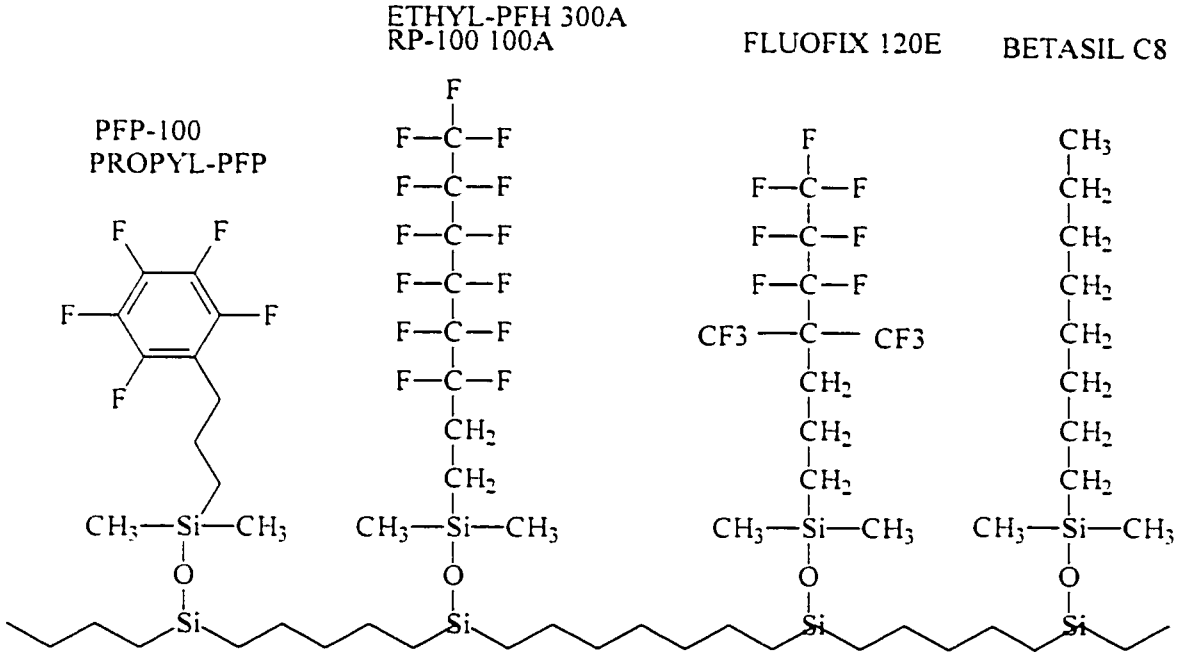
Six columns were donated by Keystone Scientific Inc.:

1. Betasil C8 (linear octyl), 150 x 4.6mm, particle size 5μm, pore size 100A, Lot # P9G09
2. Ethyl-PFH (linear perfluoro hexyl), 150 x 4.6mm, particle size 5μm, pore size 300A, Lot # R8G02
3. RP-100 (linear perfluoro hexyl), 150 x 4.6mm, particle size 5μm, pore size 100A, Lot # R9E14
4. Fluofix 120E (branched propyl perfluoro hexyl), 150 x 4.6mm, particle size 5μm, pore size 100A, Lot # KKA222
5. Propyl-PFP (perfluoro phenyl), 150 x 4.6mm, particle size 5μm, pore size 100A, Lot # R9A06
6. PFP-100 (perfluoro phenyl), 150 x 4.6mm, particle size 5μm, pore size 100A, Lot # R9E08

Columns 2 and 3 are identical bonded phases on silicas with different pore

sizes. Columns 5 and 6 are identical stationary phases of different production batches. The structures of all stationary bonded phases are shown in Figure 8.

Figure 8. Structure of Bonded Phases



3.3 Chemicals

3.3.1 Solvents

HPLC grade acetonitrile and glacial acetic acid (Baker Analyzed) were purchased from Fisher Scientific, Pittsburgh, PA. Water was glass distilled and then passed through a Milli-Q system from Millipore Corp., Bedford, Massachusetts.

For the determination of surface excess isotherms deuterated water, D_2O, and deuterated acetonitrile, CD_3CN, were purchased from Aldrich Chemical Company Inc.

Mobile phases for all experiments except the surface excess isotherms were mixed using the gradient mixing option of the HP-1090 Liquid Chromatograph.

Mobile phases for the surface excess isotherm experiments were prepared through weighing of the pure solvents with an accuracy of ±0.01g and using the premixed mobile phase through a single channel on the chromatograph.

Buffered mobile phases were prepared using potassium hydrogen phosphate. The pH was adjusted with sodium hydroxide and hydrochloric acid

using a calibrated Corning 430 pH meter.

All solvents and premixed mobile phases were degassed through helium purging for 90 seconds prior to use. This procedure ensures sufficient degassing without changing the composition of the premixed mobile phases through selective evaporation.

3.3.2 Taxane Standards

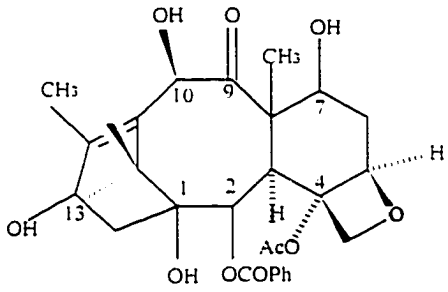
Fifteen individual solid taxane standards were donated by Hauser Chemical Research, Boulder, Colorado:

taxane #1	10-deacetyl baccatin III
taxane #2	10-deacetyl-7-xylosyl taxol B
taxane #3	baccatin III
taxane #4	10-deacetyl-7-xylosyl taxol
taxane #5	13-acetyl-dihydro baccatin III
taxane #6	10-deacetyl-7-xylosyl taxol C
taxane #7	7-xylosyl taxol
taxane #8	taxinine M
taxane #9	10-deacetyl taxol
taxane #10	cephalomannine
taxane #11	10-deacetyl-7-epitaxol
taxane #12	paclitaxel (taxol, taxol A)
taxane #13	benzyl-analog
taxane #14	taxol C

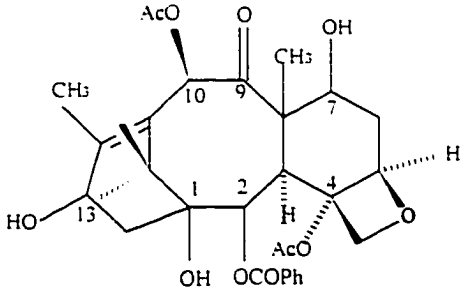
taxane #15 7-epitaxol

The structures of the taxanes are shown on Figure 9. All taxane standards were dissolved at a concentration of about 40μg/mL in acetonitrile/water/acetic acid (70/30/0.1 v/v/v) to ensure stability of the solutions [23].

Figure 9. Structure of Taxane Compounds

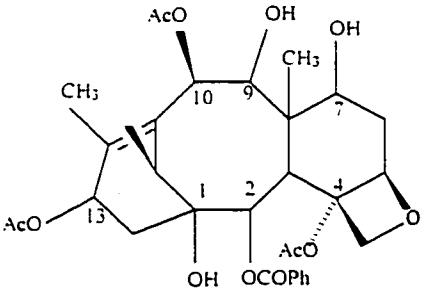


1: 10-deacetyl baccatin III

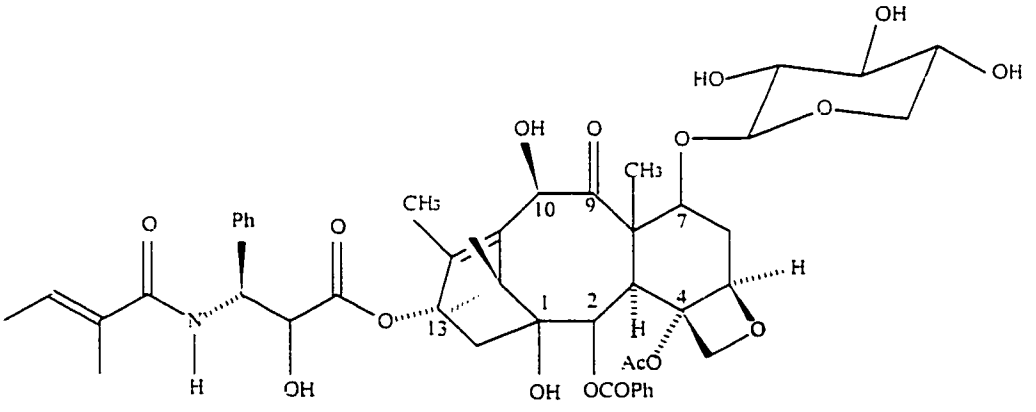


2: baccatin III

Figure 9. Structure of Taxane Compounds (continued)

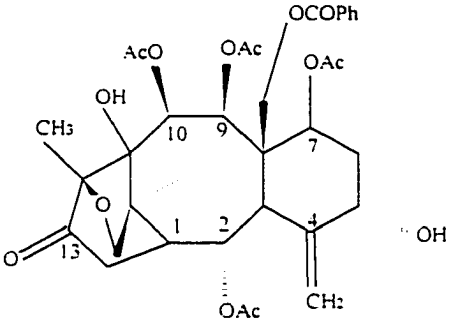


3: 13-acetyl-9-dihydrobaccatin III

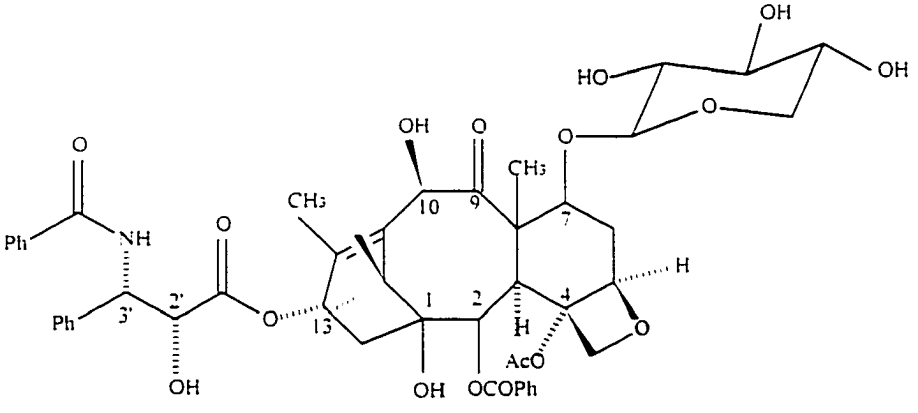


4: 10-deacetyl-7-xylosyl taxol B

Figure 9. Structure of Taxane Compounds (continued)

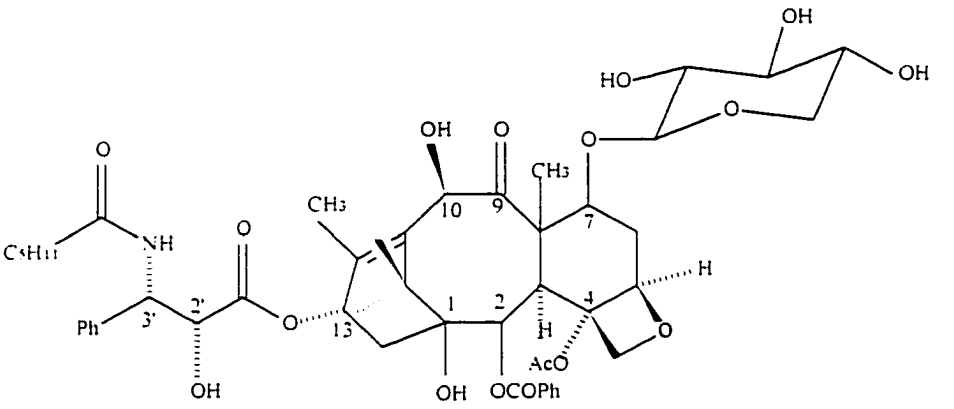


5: taxinine M

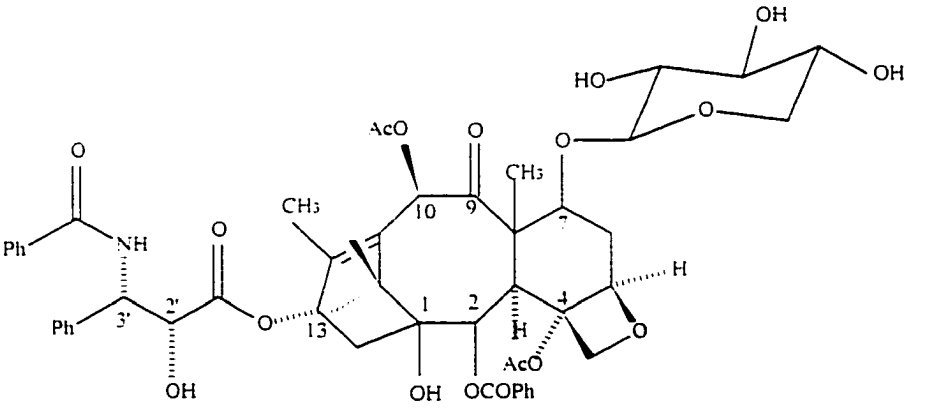


6: 10-deacetyl-7-xylosyl taxol

Figure 9. Structure of Taxane Compounds (continued)

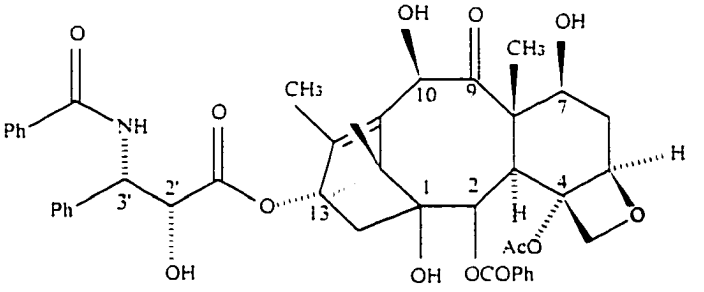


7: 10-deacetyl-7-xylosyl taxol C

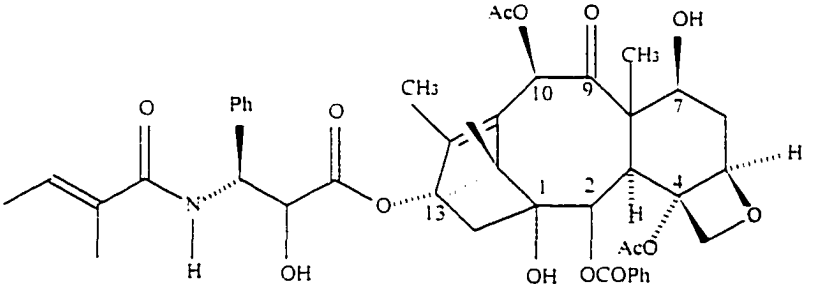


8: 7-xylosyl taxol

Figure 9. Structure of Taxane Compounds (continued)

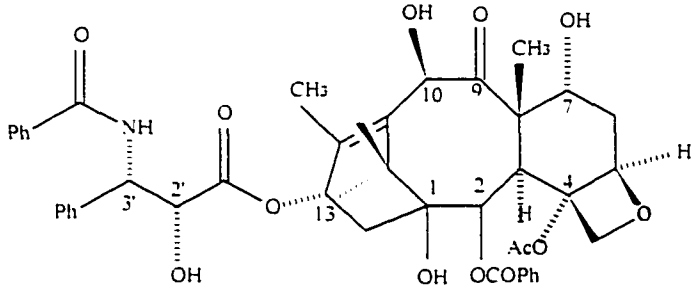


9: 10-deacetyl taxol

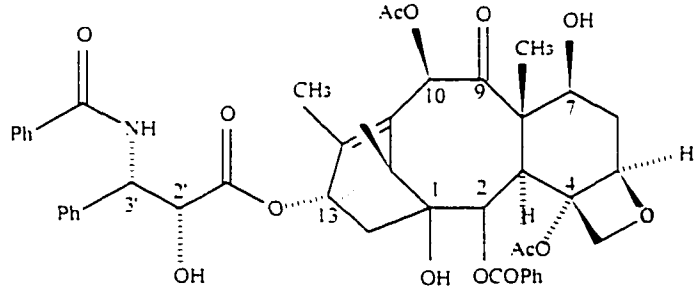


10: cephalomanine (taxol B)

Figure 9. Structure of Taxane Compounds (continued)

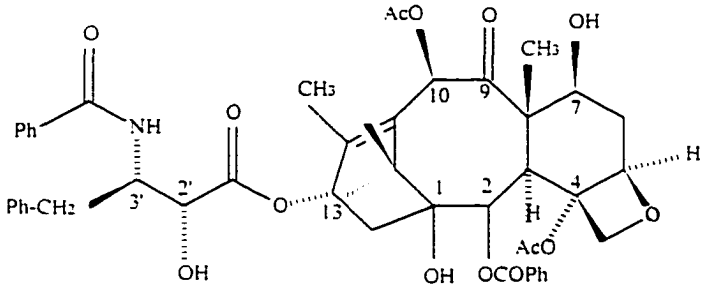


11: 10-deacetyl-7-epitaxol

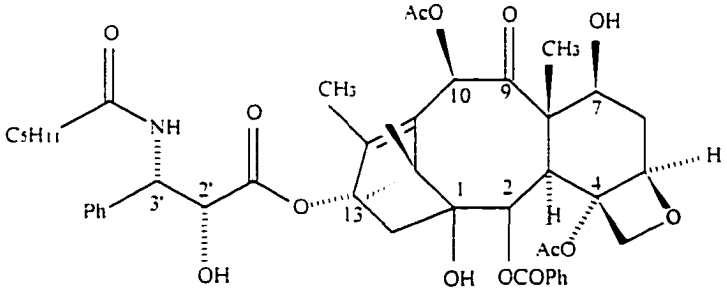


12: paclitaxel (taxol A)

Figure 9. Structure of Taxane Compounds (continued)

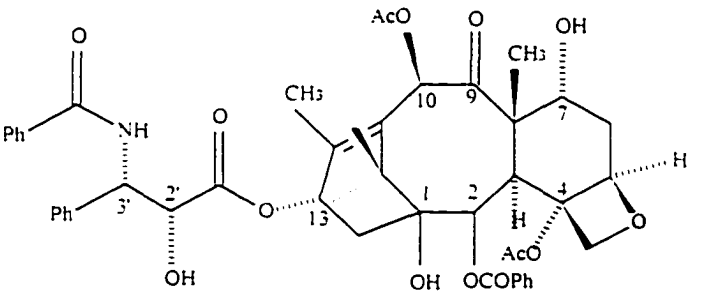


13: benzyl analog
(N-debenzoyl-N-phenylacetyl taxol)



14: taxol C

Figure 9. Structure of Taxane Compounds (continued)



15: 7-epitaxol

3.3.3 Naphthalene Derivatives

Six naphthalene derivatives were selected to evaluate functional group selectivity of the stationary phases. All compounds were purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin:

1-naphthylacetic acid, 95%

1-naphthoic acid, 96%

1,8-naphthalic anhydride

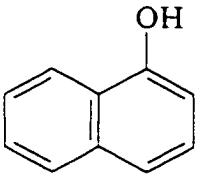
1-naphthaldehyde, 95%

1-naphthol, 99+%

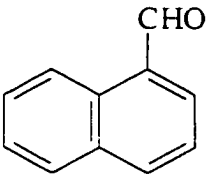
1-naphthylacetonitrile, 97%

The structures of the compounds are shown in Figure 10. All compounds were dissolved in acetonitrile/water 50/50 at a concentration of about 0.5mg/mL.

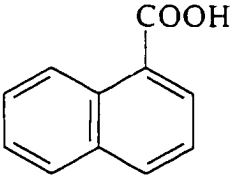
Figure 10. Structure of Naphthalene Compounds



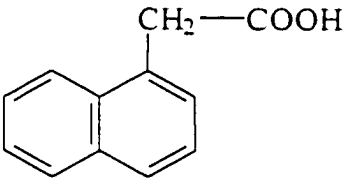
1-naphthol



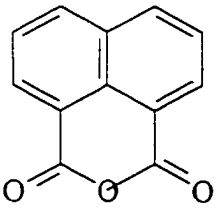
1-naphthaldehyde



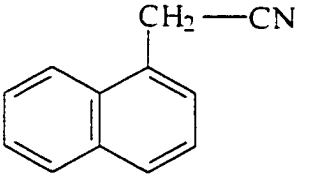
1-naphthoic acid



1-naphthylacetic acid



1,8-naphthalic anhydride



1-naphthyl acetonitrile

3.3.4 Cyclic Compounds for Selectivity Study

Several cyclic compounds with different functional groups and numbers of double bonds were selected to evaluate selectivity to π-systems and aromaticity of the different stationary phases. The compounds were purchased from various suppliers, which are indicated below. Ethyl acetate was used as non-cyclic standard for comparison:

1. cyclohexyl bromide, Aldrich Chemical Company, Inc., Milwaukee, Wisconsin
2. nitro-cyclohexane, Aldrich Chemical Company, Inc., Milwaukee, Wisconsin
3. cyclohexyl acetic acid, Aldrich Chemical Company, Inc., Milwaukee, Wisconsin
4. 1,3-cyclohexadiene, Aldrich Chemical Company, Inc., Milwaukee, Wisconsin
5. 1,4-cyclohexadiene, Aldrich Chemical Company, Inc., Milwaukee, Wisconsin
6. benzene, Fisher Scientific, Pittsburgh, PA.

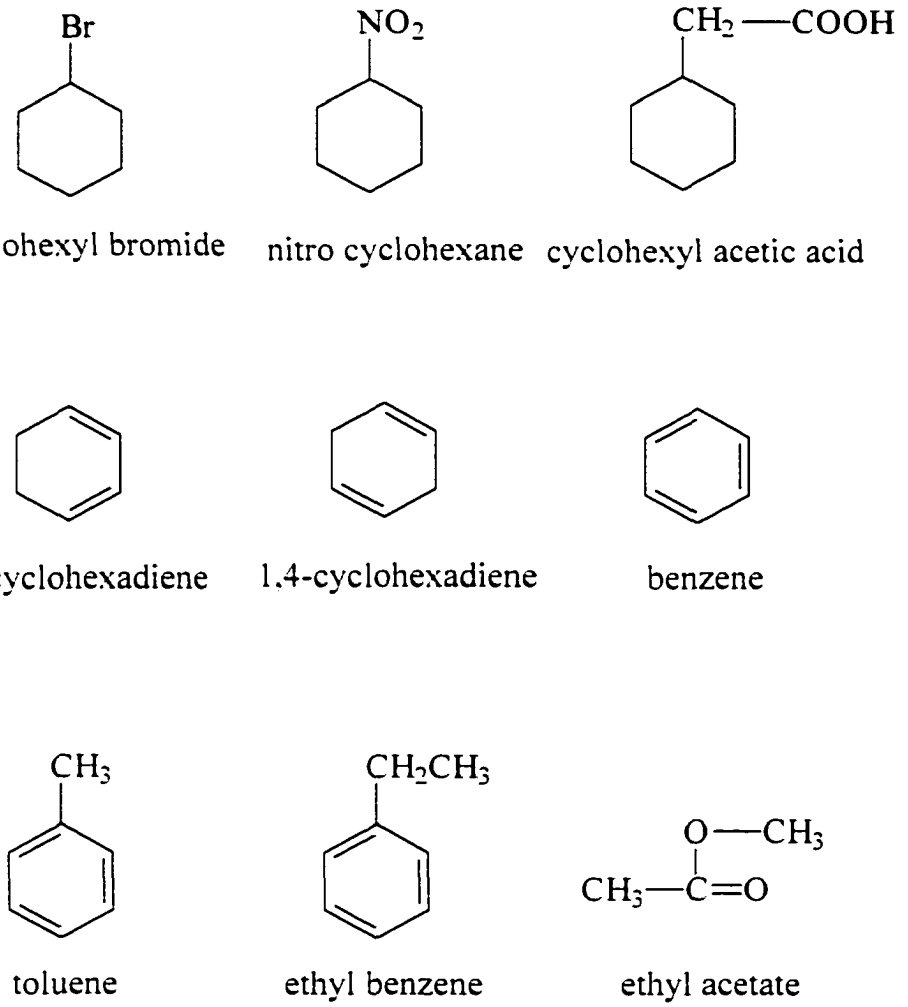
7. toluene, Fisher Scientific, Pittsburgh, PA.

8. ethyl benzene, Fisher Scientific, Pittsburgh, PA.

9. ethyl acetate, Fisher Scientific, Pittsburgh, PA.

The structures of the compounds are shown in Figure 11.

Figure 11. Structure of Cyclic Compounds for Selectivity Study



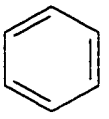
3.3.5 Hydroxylated Benzene Compounds

Five compounds were purchased from various suppliers to evaluate effect of solute solvation:

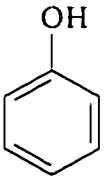
1. benzene, from Fisher Scientific, Pittsburgh, PA
2. phenol (hydroxy benzene), from Fisher Scientific, Pittsburgh, PA
3. 1,3-dihydroxy benzene, from Lancaster Synthesis, Inc., Windham, NH
4. 1,4-dihydroxy benzene, from Lancaster Synthesis, Inc., Windham, NH
5. 1,3,5-trihydroxy benzene, from Lancaster Synthesis, Inc., Windham, NH

The structures of the compounds are shown in Figure 12. All compounds were dissolved in acetonitrile/water 50/50 at a concentration of about 0.5mg/mL.

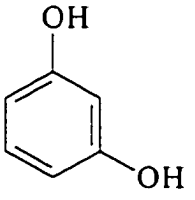
Figure 12. Structure of Hydroxylated Benzene Compounds



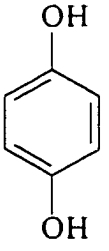
benzene



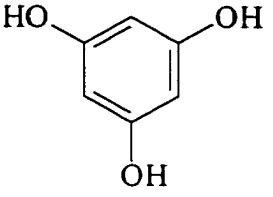
phenol



1,3-dihydroxy benzene



1,4-dihydroxy benzene



1,3,5-trihydroxy benzen

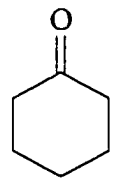
3.3.6 Compounds for Solute Solvation Study

Nine compounds were purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin and from Fisher Scientific, Pittsburgh, PA. to evaluate the effect of solute solvation:

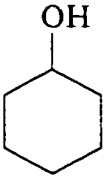
1. cyclohexanone, Aldrich
2. cylcohexanol, Aldrich
3. hydroxycyclohexanone, Aldrich
4. γ-butyrolactone, Aldrich
5. α-hydroxy-γ-butyrolactone, Aldrich
6. 1,2-butanediol, Fisher
7. 1,2-hexanediol, Fisher
8. 1,2,3-hexane triol, Fisher
9. 1,8-octanediol, Fisher

The structures of the compounds are shown in Figure 13. The compounds were dissolved in acetonitrile/water 50/50 at a concentration of about 0.5mg/mL.

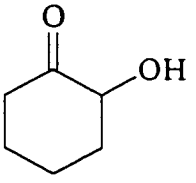
Figure 13. Structure of Compounds for Solute Solvation Study



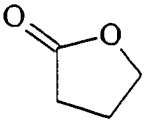
cyclohexanone



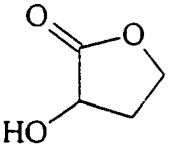
cyclohexanol



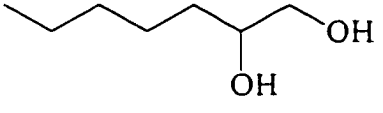
2-hydroxycyclohexanone



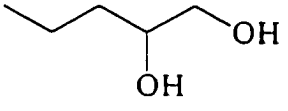
gamma-butyrolactone



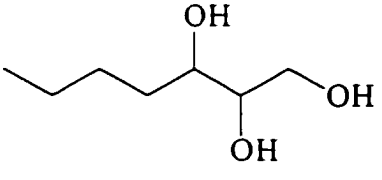
alpha-hydroxy-gamma-butyrolactone



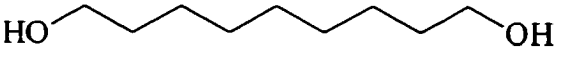
1,2-hexanediol



1,2-butanediol



1,2,3-hexanetriol



1,8-octanediol

3.3.7 Determination of the Retention Time of Unretained Compound

The retention time of an unretained compound was determined using uracil purchased from Eastman Kodak, Rochester, NY.

Chapter IV

Results and Discussion

4.1 Separation of Taxanes on Hydrocarbonaceous and Fluorinated Stationary Phases

Optimized conditions for the separation of a mixture of fifteen taxanes were determined on the six stationary phases described above using aqueous acetonitrile (ACN) as the mobile phase. The methodology applied was as follows: The column temperature was set to 21°C and the column was equilibrated with a mobile phase of ACN/water 95/5. Consecutive runs of the taxane mixture were performed lowering the ACN concentration in the mobile phase by 5% each time until the peak capacity allowed for complete separation of all fifteen compounds (the peak capacity is the number of completely resolved peaks that can be spatially fitted between the first and the last eluting peak). Once sufficient peak capacity had been achieved, the mobile phase composition and temperature were changed independently in small increments to evaluate their effect on the selectivity of poorly resolved peak pairs. This information was then used to evaluate conditions for optimum separation. If possible mobile phase gradient steps were added to shorten the separation time.

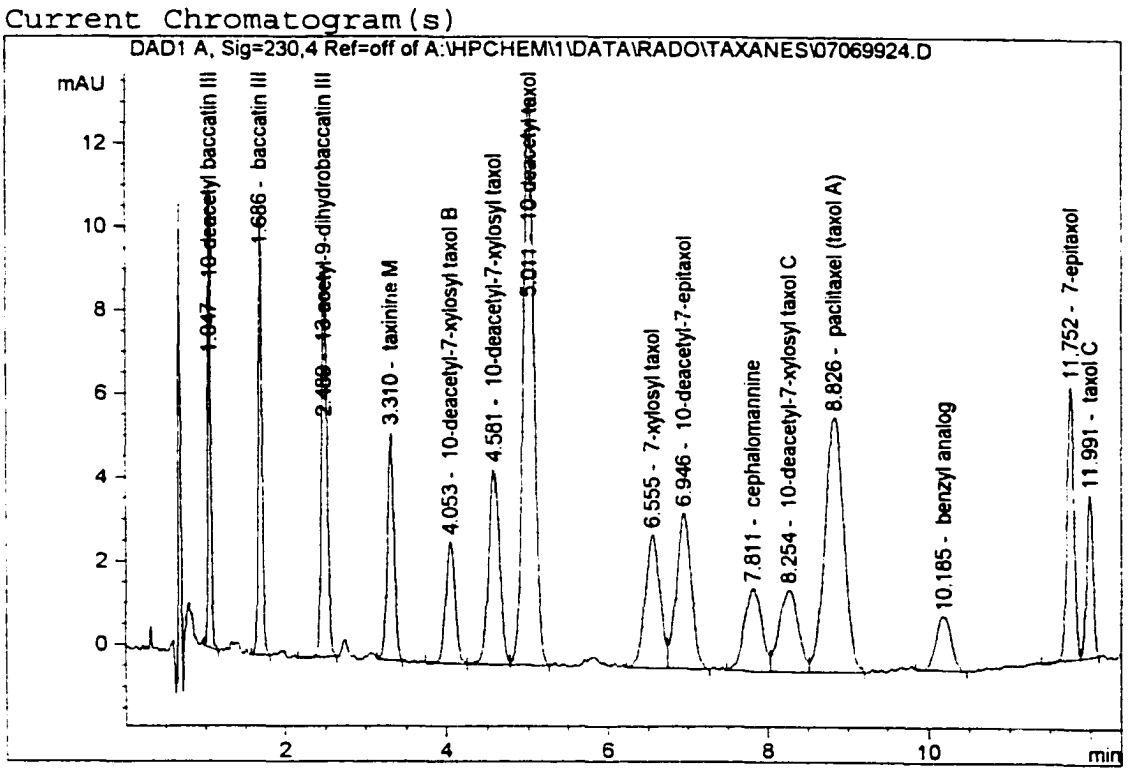
The resulting separation methods are listed below.

4.1.1 Taxane Separation Methods

4.1.1.1 Separation of Taxanes on Ethyl-PFH

Separation of all fifteen taxane compounds was achieved on Ethyl-PFH at a column temperature of 40.0 °C and a mobile phase flow rate of 3.0 mL/minute using ACN/water 27/73 in isocratic mode for 9.0 minutes followed by a gradient to ACN/water 70/30 in 10.0 minutes until the elution of taxol C. The method will be referred to as method A. The required analysis time is about 12 minutes. The separation is shown in Figure 14.

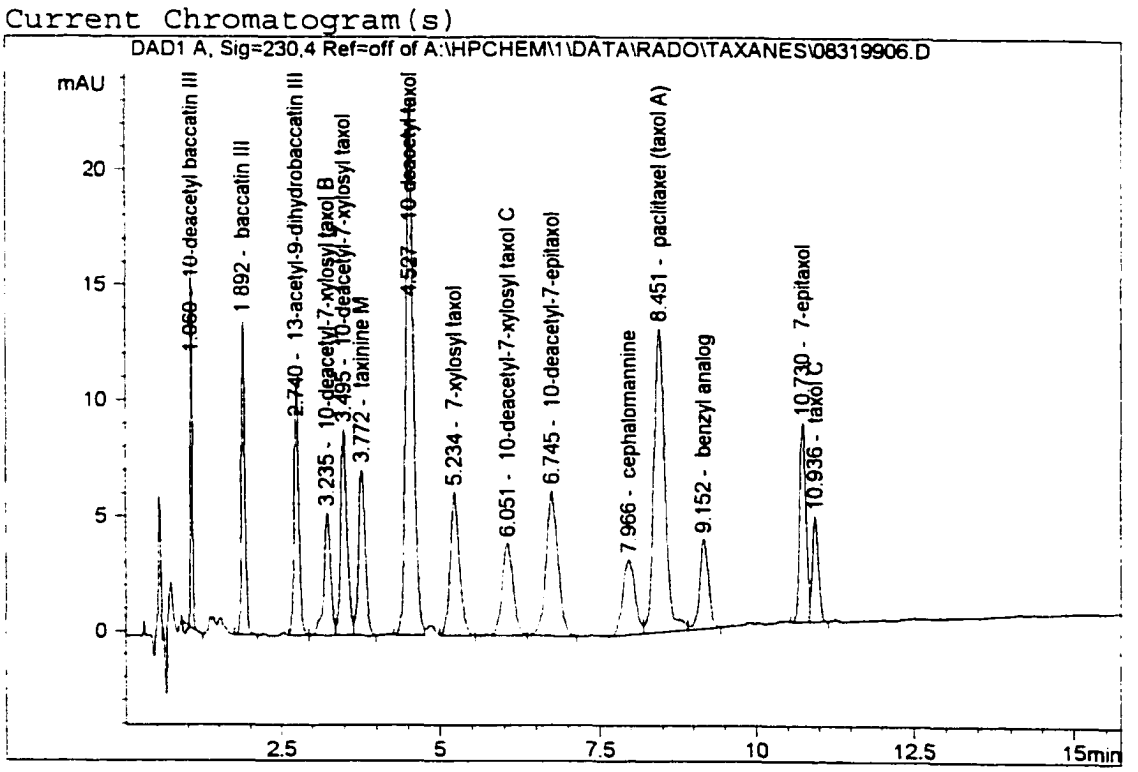
Figure 14. Separation of 15 Taxanes on Ethyl-PFH; Method A



4.1.1.2 Separation of Taxanes on RP-100

Separation of all fifteen taxane compounds was achieved on RP-100 at a column temperature of 50.0 °C and a flow rate of 3.0 mL/minute using a mobile phase of ACN/water 32/68 in isocratic mode for 7.0 minutes followed by a gradient to ACN/water 50/50 in 6.5 minutes until the elution of taxol C. The method will be referred to as method B. The analysis time is about 11 minutes. The separation is shown in Figure 15.

Figure 15. Separation of 15 Taxanes on RP-100; Method B



4.1.1.3 Separation of Taxanes on Fluofix 120E

On Fluofix 120E the separation was accomplished by two different methods referred to here as method C and method D.

Method C is performed at a column temperature of 40.0 °C and a flow rate of 3.0 mL/minute using a mobile phase of ACN/water 33/67 in isocratic mode for 5.5 minutes followed by a gradient to ACN/water 70/30 in 10.0 minutes until the elution of 7-epitaxol. All compounds are baseline separated in about 9.5 minutes. The separation is shown in Figure 16.

Method D is performed at a column temperature of 60.0 °C and a flow rate of 2.0 mL/minute using a mobile phase of ACN/water 33/67 in isocratic mode for 9.0 minutes followed by a gradient to ACN/water 70/30 in 10 minutes until the elution of taxol C. All compounds are baseline separated in about 13 minutes. The separation is shown in Figure 17.

Figure 16. Separation of 15 Taxanes on Fluofix 120E; Method C

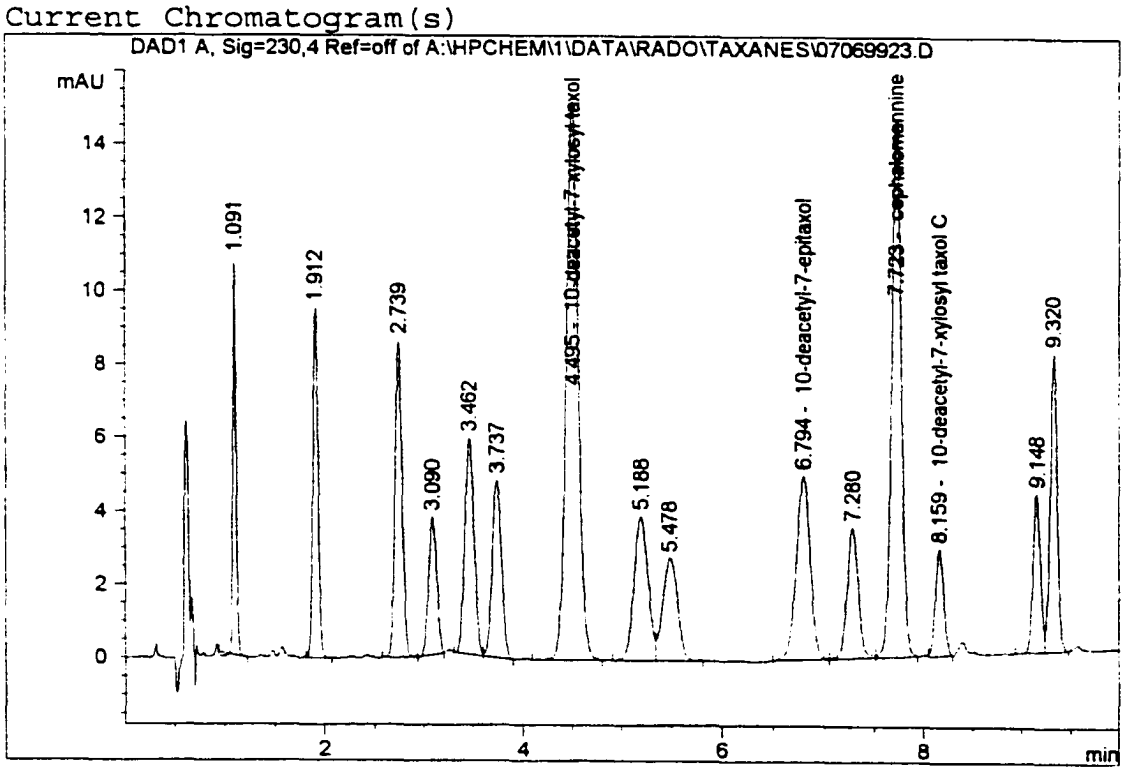
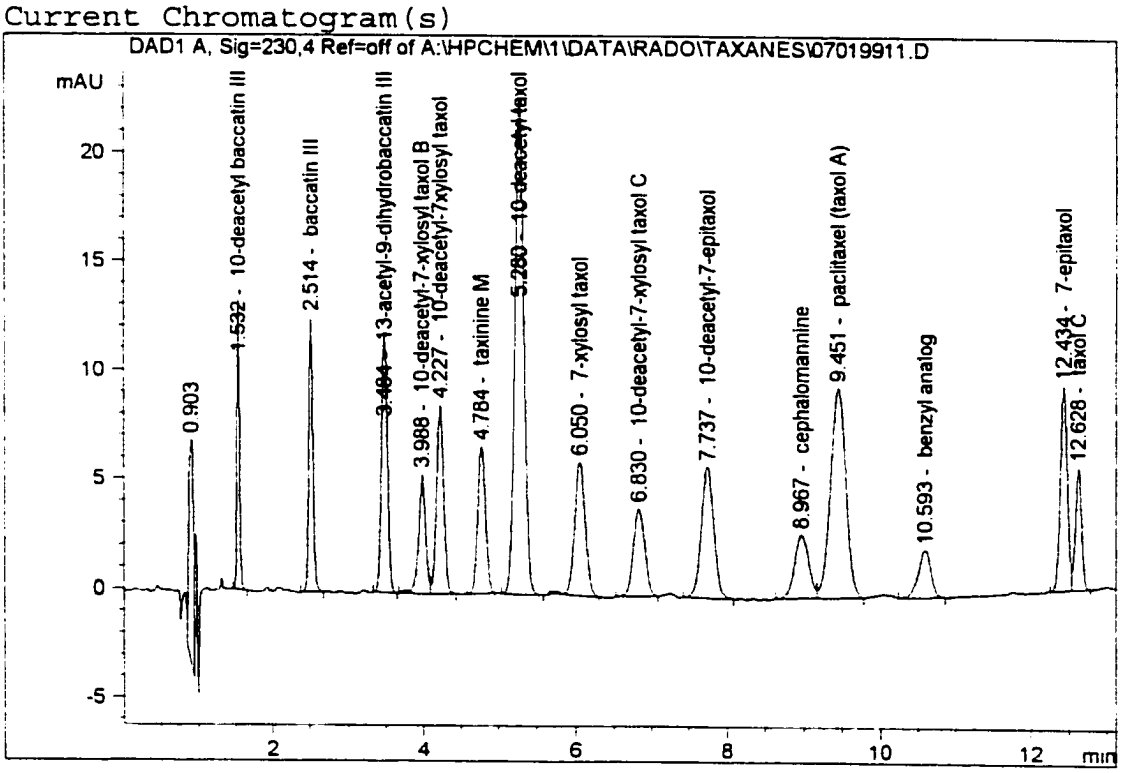


Figure 17. Separation of 15 Taxanes on Fluofix 120E; Method D



4.1.1.4 Separation of Taxanes on Propyl-PFP and PFP-100

Propyl-PFP and PFP-100 are two identical columns from two different batches. Separation of all fifteen compounds was achieved at a column temperature of 22.0 °C and a flow rate of 2.0 mL/minute using a mobile phase of ACN/water 35/65 in isocratic mode for 12.5 minutes followed by a gradient to ACN/water 58/42 in 13.0 minutes until the elution of 7-epitaxol. The analysis time is about 22 minutes. The method will be referred to as method E. The separation on Propyl-PFP and PFP-100 are shown in Figure 18 and Figure 19, respectively.

Figure 18. Separation of 15 Taxanes on Propyl-PFP; Method E

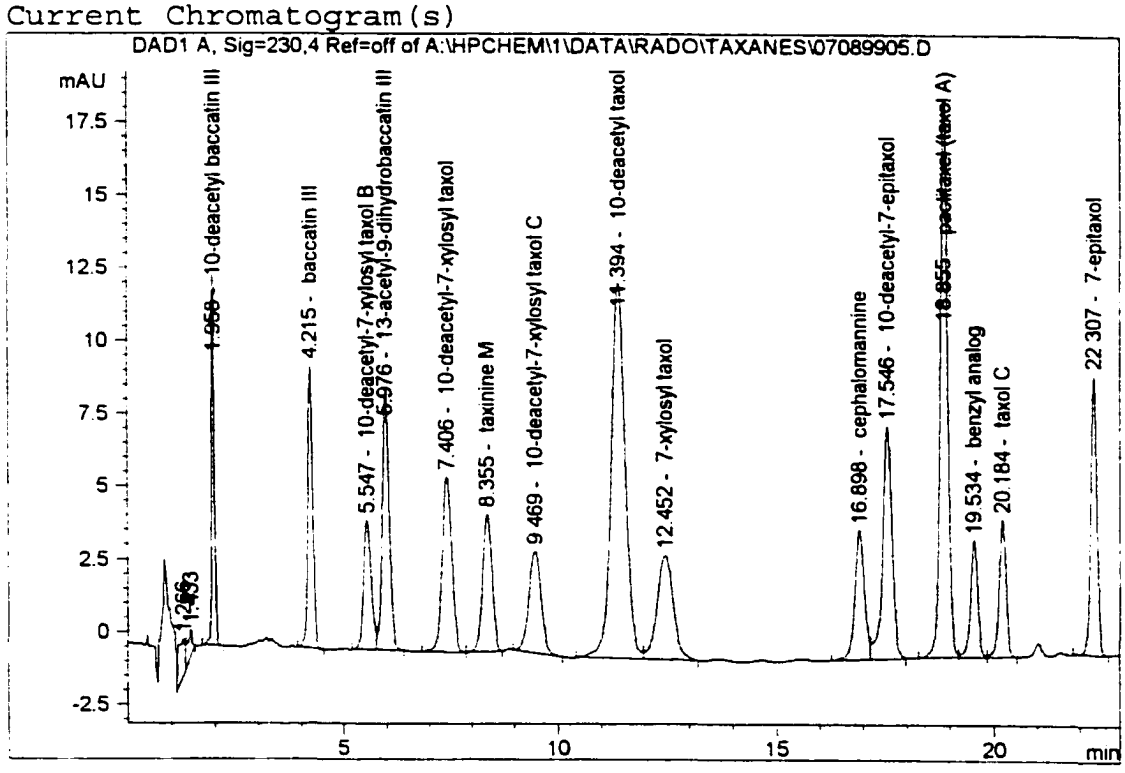
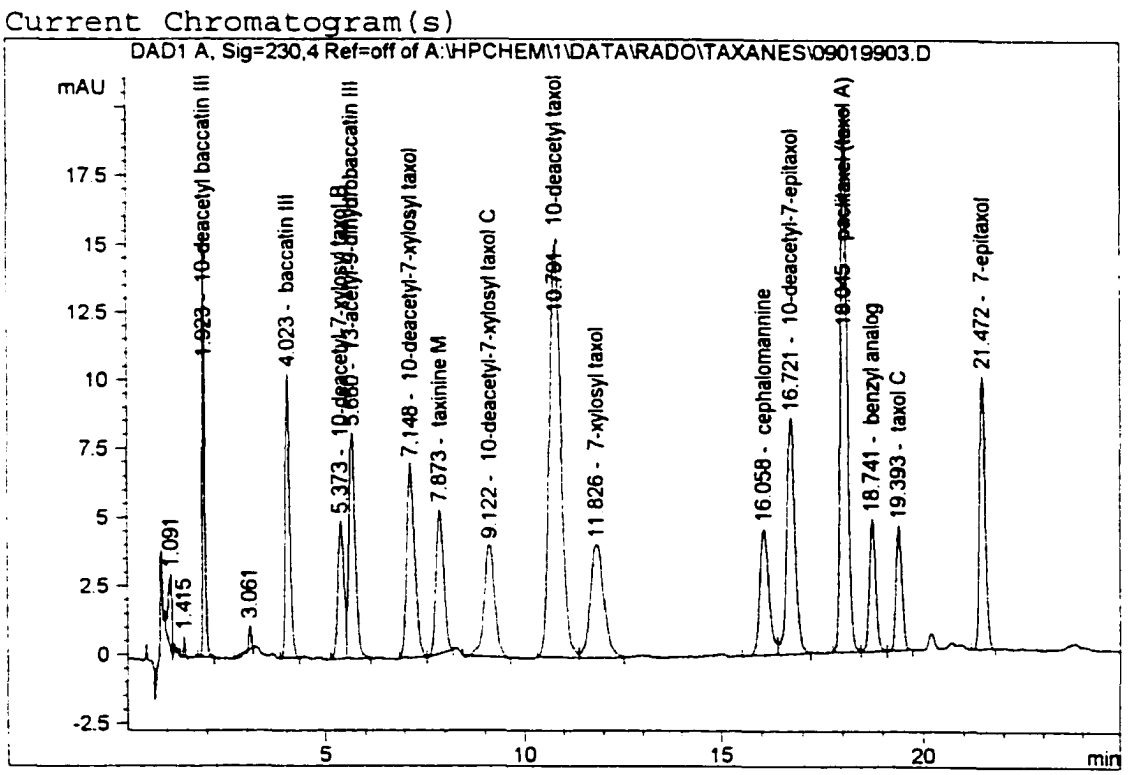


Figure 19. Separation of 15 Taxanes on PFP-100; Method E



4.1.1.5 Separation of Taxanes on Betasil C8

Three methods were developed for the separation of taxanes on Betasil C8 referred to as methods F, G and H, respectively.

All fifteen compounds were separated with method F at a column temperature of 21.0 °C and a flow rate of 2.5 mL/minute with a mobile phase of ACN/water 35/65 in isocratic mode for 19.0 minutes and then with a gradient to ACN/water 85/15 in 8.0 minutes. The separation is shown in Figure 20.

Fourteen taxane compounds were separated in about 13.5 minutes with method G at a column temperature of 21.0 °C and a flow rate of 1.0 mL/minute using a mobile phase of ACN/water 47.5/52.5 for 6.5 minutes in isocratic mode followed by a gradient to ACN/water 80/30 in 10 minutes until the elution of 7-epitaxol. The separation is shown in Figure 21. With this method baccatin III and 10-deacetyl-7-xylosyl taxol coelute.

Fourteen taxane compounds were separated in about 15.5 minutes with method H at a column temperature of 21.0 °C and a flow rate of 1.0 mL/minute using a mobile phase of ACN/water 45/55 in isocratic mode for 8.0 minutes followed by a gradient to ACN/water 80/30 in 10.0 minutes until the elution of

7-epitaxol. The separation is shown in Figure 22. With this method baccatin III and 10-deacetyl-7-xylosyl taxol are completely separated but 7-xylosyl taxol and taxinine M coelute.

Figure 20. Separation of 15 Taxanes on Betasil C8; Method F

Current Chromatogram(s)

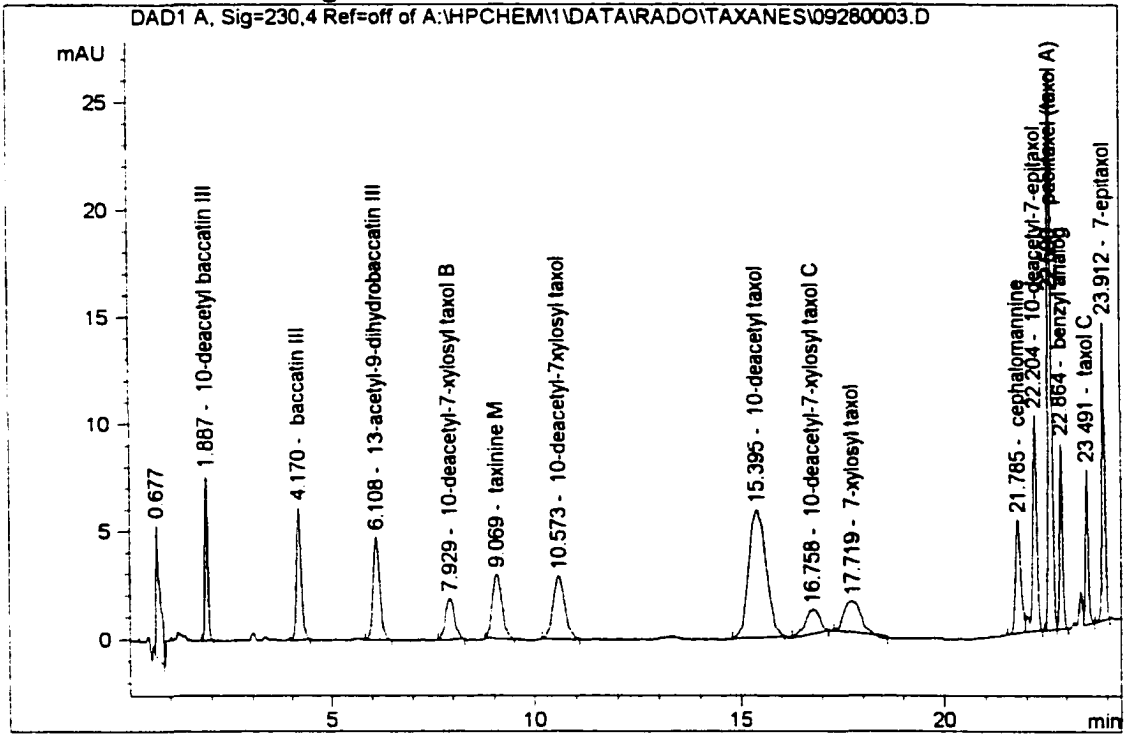


Figure 21. Separation of 14 Taxanes on Betasil C8; Method G

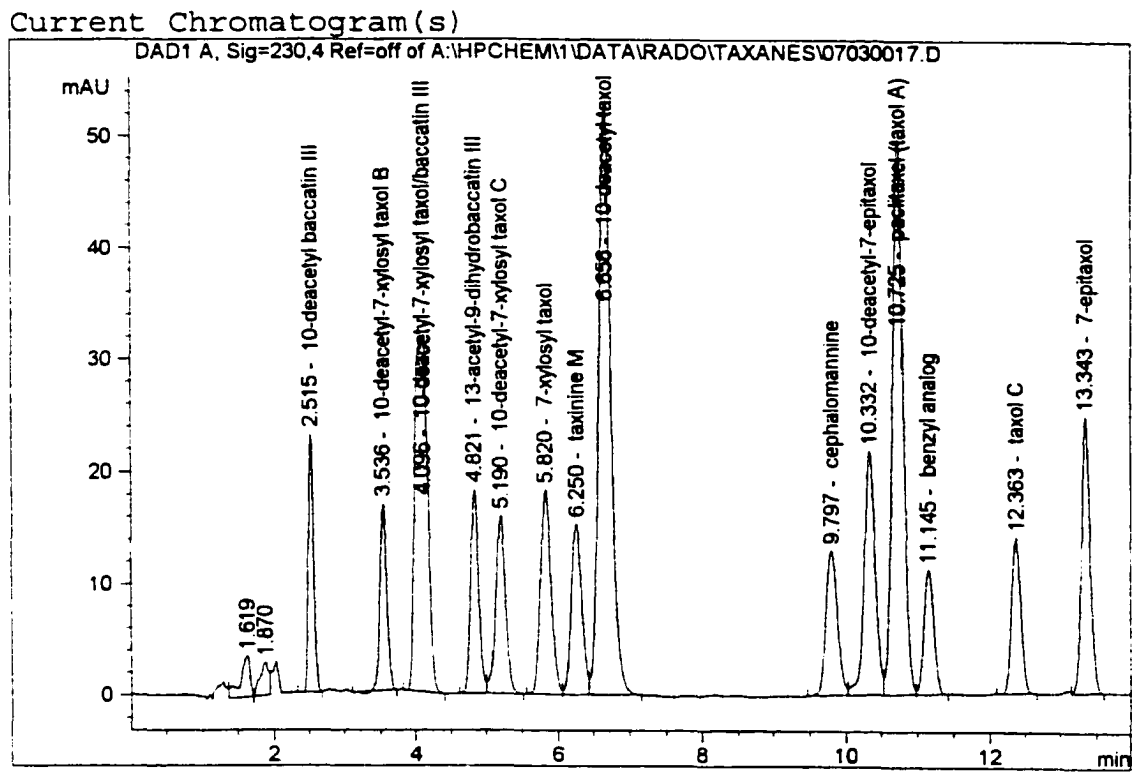
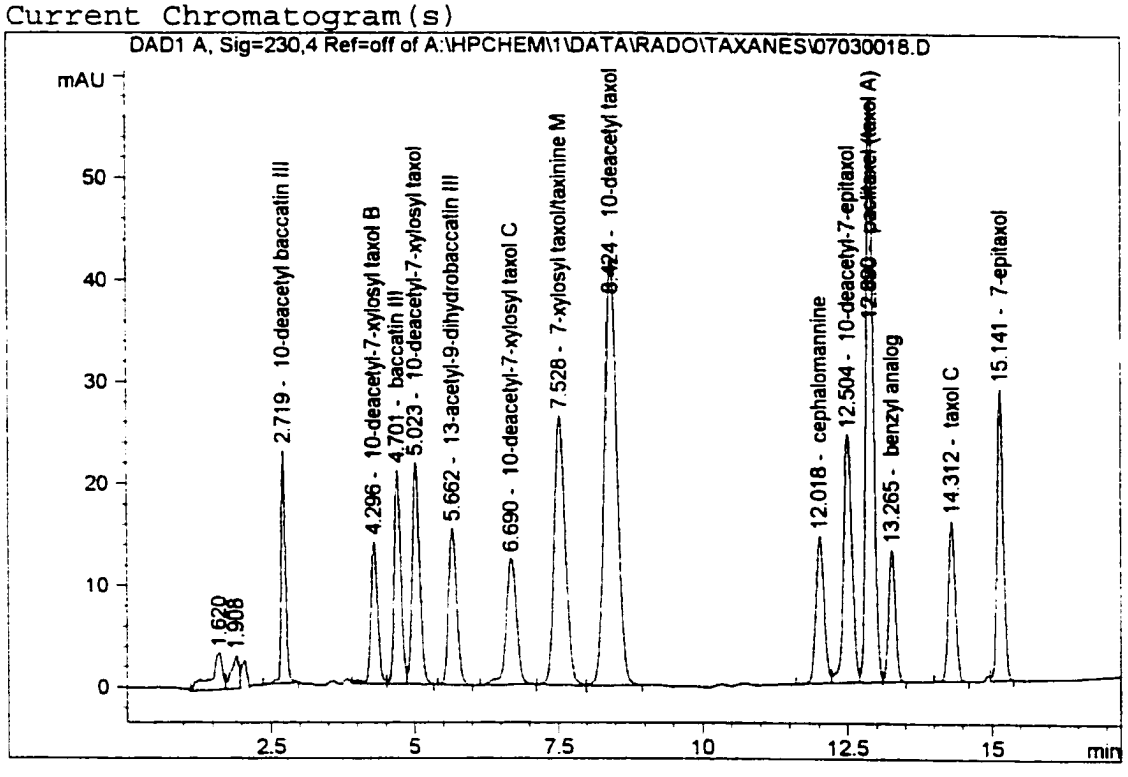


Figure 22. Separation of 14 Taxanes on Betasil C8; Method H



4.1.2 Comparison and Discussion of Taxane Separation Methods

4.1.2.1 General Features of the Taxane Separations

There are several common features in all the taxane separations described above. The fifteen taxanes can be categorized into several groups. One group is formed by baccatin III and its derivatives (taxane #1-#3). These compounds have the basic structure of taxanes without the C-13 side chain. The next group consists of 7-xylosyl taxol and its derivatives (taxane #4 and taxane #6-8). This group of compounds distinguish themselves from other taxanes through a sugar group attached at the C-7 position. The next group consists of paclitaxel and 10-deacetyl taxol (taxol A, taxane #9 and taxane#12, respectively). The last group is formed by 7-epitaxol and its 10-deacetyl derivative (taxol #11 and #15). The epitaxols differ from paclitaxel in the isomeric bonding of the hydroxyl group on the C-7 position. There are four taxanes left that can not be included in any group and stand by themselves; taxinine M (taxane #5), cephalomanine (taxol B, taxane #10), the benzyl analog (taxane #13) and taxol C (taxane #14). The elution pattern observed between the groups and the "independent" taxanes is very similar with all the methods described above. The first group to elute is the

baccatin III compounds followed by the xylosyl taxol compounds. Taxinine M elutes between these two groups. The paclitaxel group elutes after the xylosyl taxol group partially overlapping with it. Cephalomanine elutes between paclitaxel and 10-deacetyl taxol. The last group to elute is the 7-epitaxols partially overlapping the paclitaxel group. Taxol C and the benzyl analog usually elute within this group as well.

In all the separation methods described above there are consistent trends in the elution order within each group. Deacetyl compounds always elute before their non-deacetylated analogs. This trend can be seen within the baccatin III group, the xylosyl taxol group, the paclitaxel group and the epitaxol group. The early elution of the deacetyl compounds can be explained by the presence of an additional hydroxyl group which replaces an acetyl group in the 10-deacetyl compounds. The more polar hydroxyl group results in a more polar molecule and a reduced retention in the reversed phase mode.

Another common trend in the separations is that taxol B, A and C derivatives of the otherwise analogous structure elute in the stated order. This trend can be seen within the xylosyl taxol group (7-xylosyl taxol is a taxol A) and in the elution order of cephalomanine (taxol B), paclitaxel (taxol A) and taxol C. The three derivatives show different structures at the end of the C-13

side chain. The A, B and C derivative side chain ends with a phenyl group, a butenyl group and a pentyl group, respectively. The butenyl group is more polar than the phenyl group resulting in the lower retention of the B derivative. In the same manner the pentyl group is less polar than the phenyl group leading to the greater retention of the C derivatives.

The major difference between the separations shown in Figures 14-22 above seems to be the degree of overlap between the groups defined above and the placement of the independent taxols within the overlaps. This leads to the observed differences in elution orders among the different methods.

An interpretation of the elution order with respect to chemical interactions between the taxane compounds and the different bonded phases will not be made. The methods used in the separations on the different columns used different temperatures and mobile phase compositions which leads to differences in the solvation state of the stationary phases and the solutes as explained above. The effect of these processes on the retention of solutes in RPLC is not known and an interpretation of elution order with different methods is therefore not possible. A more detailed discussion of these phenomena will be provided in section 4.4.

4.1.2.2 Comparison of the Taxane Separations

Past publications on the separation of taxanes mostly reported rather time consuming methods [23-31]. Even simple separations of a little as 2 taxanes could take up to 60 minutes [29]. Few studies attempted complex separations of more than 10 taxanes [23,26]. The fastest separation was reported by Shao et al. with the separation of 13 and 15 taxanes in 19 and 35 minutes, respectively [23]. None of the previously reported methods used temperature as a parameter to optimize the separation. However, during development of the methods described above it was found that temperature has a major influence on the selectivity of most of the columns and has to be controlled carefully to ensure efficiency and ruggedness of the methods. The effect of temperature on the separation of taxanes will be discussed in detail in section 4.4.2.

The slowest method developed in this study used the penta fluoro phenyl bonded phases, Propyl-PFP and PFP-100, with an analysis time of about 22 minutes for 15 taxane compounds which still makes them faster than any previously published method. The two columns are actually identical stationary phases of different production batches but were assigned different names by the manufacturer, Keystone Scientific. For this reason the same method was used

for both columns. As can be seen in Figures 18 and 19 the separations are very similar with only slight differences in retention times and resolution. PFP-100 shows slightly lower resolution of 10-deacetyl xylosyl taxol B and 13-acetyl dihydro baccatin III compared to Propyl-PFP.

The separations on Fluofix 120E required only 13 and 9.5 minutes, respectively. The two methods differ only in column temperature (40.0 °C vs. 60.0 °C). The increased temperature results in the reversal of the elution order of 7-epitaxol and taxol C and shifts of resolution between other compounds such as 7-xylosyl taxol and 10-deacetyl-7-xylosyl taxol C.

Ethyl-PFH and RP-100 are columns with the same bonded phase on silica matrices with different pore diameters. Ethyl-PFH has a pore diameter of 300A while RP-100 has 100 A pores. As can be seen in Figure 14 and 15 the two columns show some considerable differences in elution order; taxinine M and 10-deacetyl-7-xylosyl taxol C elute in very different places with respect to the other compounds. The reason for this might be a result of the different separation methods used on the two columns. RP-100 has a higher retentivity, probably caused by the larger surface area resulting from the smaller pore size. To keep the retention times short with the high retentivity, an increase in the concentration of ACN in the mobile phase is necessary. As mentioned above

this can lead to changes in the solvation of the stationary phase and the solute, which can lead to differences in selectivity. This phenomenon will be investigated in further detail in section 4.4.

The most surprising separations are the ones developed on Betasil C8. Betasil C8 is a regular hydrocarbonaceous octyl bonded phase, which according to previous studies was believed to lack sufficient selectivity for the separation of complex taxane mixtures. Figure 20 shows the separation of 15 taxanes in 24 minutes using method F. The method is not very good due to the long isocratic run resulting in broad peaks for some of the compounds. Method G has the advantage of considerably shorter separation time and sharper peaks, but can only separate 14 taxanes. Baccatin III and 10-deacetyl-7-xylosyl taxol coelute. To separate these two compounds method H can be used. In this method the previously coeluting compounds are perfectly separated but taxinine M and 7-xylosyl taxol coelute. Due to the low concentration of ACN in the mobile phase, the relatively short analysis time and the low flow rate of only 1.0 mL/minute methods G and H are very economical, consuming considerably less solvent than any other method introduced here or elsewhere. The two methods prove that a hydrocarbonaceous column has the selectivity to separate all taxane compounds with analysis times that compete with any of the fluorinated

stationary phases or any previously published method.

The C8 phase showed a considerably higher retentivity than the fluorinated stationary phases, which is in agreement with the literature [81-86]. In an overall comparison, Betasil C8 showed the highest retentivity followed by the PFP phases. The PFH phases and Fluofix 120E showed the lowest retentivity. This phenomenon leads to the use of a higher concentration of ACN in the mobile phase on Betasil C8, which is the likely reason for the markedly different elution order as compared to the fluorinated phases. The dependence of elution order of taxane compounds on mobile phase composition and temperature will be discussed in detail in section 4.4.

4.2 Characterization and Comparison of Fluorinated and Hydrocarbonaceous Stationary Phases using Surface Excess Isotherms

As mentioned above the stationary phase in chromatography is covered with an adsorbed layer of mobile phase. The composition of this adsorbed layer is different from the composition of the bulk mobile phase and changes with temperature and concentration of organic modifier in the mobile phase. A schematic description of the stationary phase is shown in Figure 23. The adsorbed phase is believed to consist mostly but not exclusively of the organic modifier. Dipole-dipole interactions between the modifier molecules and water facilitate accommodation of water in the adsorbed layer. Residual silanol groups on the silica surface provide further adsorption sites for water [69-73] .It is not clearly known yet how much the adsorbed layer penetrates into or extends beyond the bonded phase. It is believed, however, that it affects the retention of solutes and influences the chromatographic behavior of the stationary phase [72]. During retention the solute either has to permeate into or replace parts of the adsorbed layer to get into contact with the bonded phase [80] which is an effect that is virtually ignored in explanations of the retention mechanism in

RPLC.

A detailed discussion of the different methods to characterize mobile phase adsorption onto the stationary phase is given in section 2.2.4. In this study surface excess isotherms were determined using injection of labeled compounds. A thorough description of the method is given by Kovats et al. [69]. The method is based on the comparison of the retention times of the deuterated components of the mobile phase, which can be monitored using a refractive index detector. The surface excess of the organic modifier is then calculated using the following equation,

$$\Psi_{ACN/vNA} = (V_{R.ACN^*} - V_{R.\,H2O^*})\,\phi_{ACN^\circ}\,\phi_{H2O^\circ} / S \qquad (4)$$

where $\Psi_{ACN/vNA}$ is the surface excess of ACN in $\mu L/m^2$ according to the "nothing is absorbed" convention, V_R is the retention volume, ACN* and H_2O* are the deuterated species of acetonitrile and water, respectively, ϕ_{ACN° and ϕ_{H2O° are the volume fractions of ACN and water in the mobile phase, respectively and S is the surface area of the stationary phase. Data for the surface areas of the tested stationary phases was provided by Richard Henry of Keystone Scientific and Neos Company, Japan and are listed in Table 1.

Table 1. Surface Areas of Stationary Phases

	Surface Area [m^2/g]	Data Source
Betasil C8	325	Keystone Scientific
RP-100	322	Keystone Scientific
Ethyl-PFH	115	Keystone Scientific
Fluofix 120E	300	Neos Company, Japan
Propyl-PFP	333	Keystone Scientific
PFP-100	311	Keystone Scientific

The "nothing is absorbed" convention implies that at 100% of any solvent there will be no excess of any mobile phase component. Mathematically, this is accomplished through the multiplication by the volume fractions of water and the organic modifier in equation (4). Some studies chose to report the surface excess in mass or mol units per mass of stationary phase, but there is a possibility to introduce errors or ambiguities with conversion to these units. The adsorption of the organic modifier is believed to change its molar volume [69],

which makes it impossible to accurately calculate the adsorbed amount. An approximation is usually made using the molar volume of the non-adsorbed species. Adsorption is a surface phenomenon and the amount of organic modifier adsorbed therefore strongly depends on the total surface area of the stationary phase material in the column. Differences in particle size, pore size and bonded phase length cause considerable variations in surface area per mass of stationary phase material. Surface excess data can therefore not be compared from column to column if it is expressed per mass of stationary phase. Since this study aims to identify differences between the investigated mobile phases, the surface excess was calculated in $\mu L/m^2$.

The retention volume was calculated as follows,

$$V_R = t_R F^* \qquad (5)$$

where t_R is the retention time of the labeled compound and F* is the corrected mobile phase flow rate. The corrected mobile phase flow rate was calculated as follows,

$$F^* = F \exp [\alpha_\mu (T_c - T_f) - \kappa_\mu P_{av}] \qquad (6)$$

where α_μ is the coefficient of the thermal expansion of the mixture, T_c and T_f are the temperature of the column and the flow measuring system, respectively, κ_μ is the isothermal compressibility and P_{av} is the average column pressure. The average column pressure is sufficiently well approximated as

$$P_{av} = P_i / 2 \qquad (7)$$

where P_i is the pressure measured at the column inlet. The coefficient of thermal expansion and the isothermal compressibility at any given mobile phase composition are calculated as follows,

$$\kappa_\mu = \phi_A \kappa_A + \phi_B \kappa_B \qquad (8)$$

$$\alpha_\mu = \phi_A \alpha_A + \phi_B \alpha_B \qquad (9)$$

where κ_A is the coefficient of isothermal compressibility of component A, α_A is the coefficient of thermal expansion and ϕ_A is the volume fraction of solvent

A in the mobile phase. The coefficients are listed in Table 2. and were supplied by reference [69].

Table 2. Isothermal Compressibility and Coefficient of Thermal Expansion

	H_2O	ACN
$\kappa \times 10^{4}\ atm^{-1}$	0.45	1.60
$\alpha \times 10^{4}\ K^{-1}$	3.03	13.7

The identification of the labeled compound peaks is complicated by the appearance of a concentration perturbation, which is a peak that results from differences in concentration between the mobile phase and the injected sample solution. This can be seen in Figure 24 with the injection of pure water. Only through multiple injections of pure labeled and unlabeled solvents and mixtures of the same at concentrations equal to the mobile phase peak identification is possible. The retention times of the deuterated compounds at each mobile phase composition were determined from seven injections to ensure unambiguous identification of the peaks. An example is shown in Figure 24.

In this study the surface excess isotherm will not be used for any interpretation about the surface structure of the solvated stationary phase but rather serve as a tool to compare the solvation behavior of the different bonded phases.

Figure 23. Adsorption of Mobile Phase onto the Stationary Phase

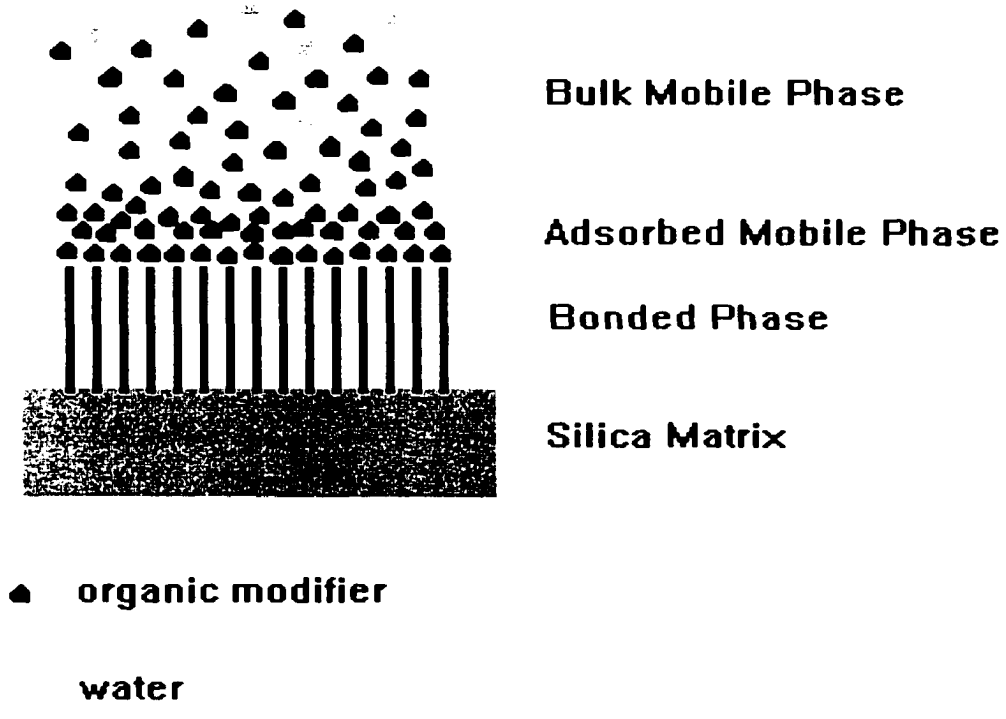
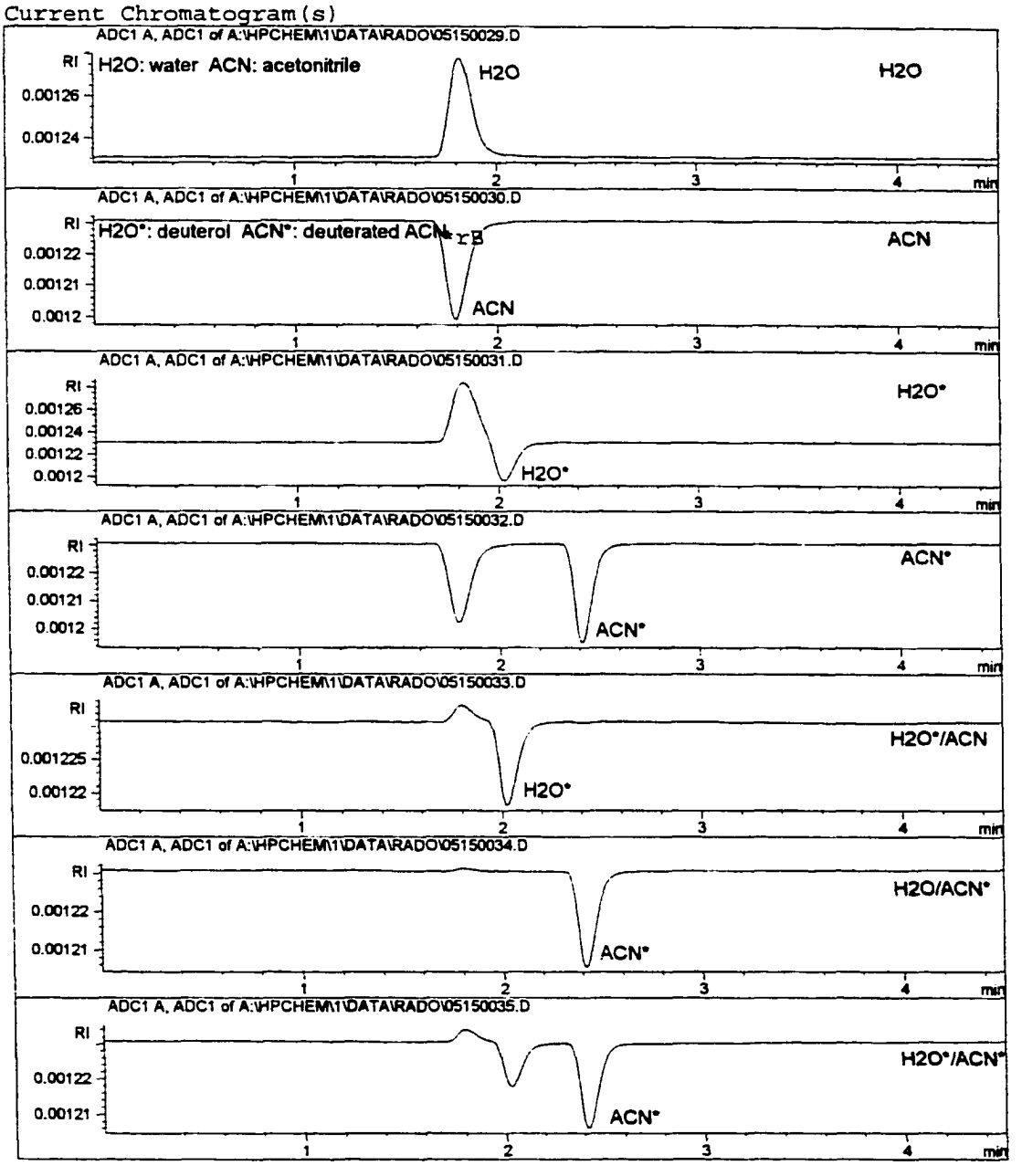


Figure 24. Injections for Determination of Surface Excess Isotherm



4.2.1 Surface Excess Isotherms

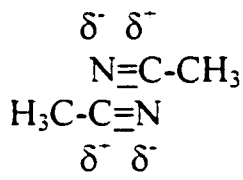
There are no published studies at this time on the solvation of fluorinated bonded phases in comparison with hydrocarbonaceous surfaces.

In this study surface excess isotherms were determined at 25 °C and 45 °C for Betasil C8, Fluofix 120E, Propyl-PFP, PFP-100, Ethyl-PFH and RP-100 and are shown in Figures 25-30, respectively. The experimental data points were fitted with a 6th degree polynomial function.

The surface excess isotherms all show a maximum excess adsorption of ACN at about 40 % and 35 % ACN at 25 °C and 45 °C, respectively. The excess of ACN is lower at 45 °C, which indicates that the adsorption of ACN on the non-polar stationary phase is exothermic. These findings are in agreement with previous studies [69,72] of various hydrocarbonaceous phases.

The amount of adsorbed ACN increases rapidly from 0-30 % ACN, which is caused by the preferential solvation of the bonded phase with the organic modifier. It reaches a maximum at 35-40 % ACN and then decreases almost linearly until 85-90 % ACN. At about this point the surface excess of ACN becomes negative with a minimum at about 95 % ACN. It then increases again to terminate at 100 % ACN. The point at which the isotherm passes

through 0 is sometimes referred to as the "azeotropic" point [71]. It is believed that at high concentrations of ACN in the mobile phase the remaining water is pushed onto the stationary phase by hydrophobic expulsion [69,71,72]. This theory becomes more plausible if one looks at a study about the solvation structure in ACN/water mixtures conducted by Harris and Rowlen [87]. They found that ACN tends to form dimers and clusters at high concentrations in water. These clusters and dimers form through the alignment of the CN dipoles leading to partial cancellation of the dipole moments as shown below.



This results in more non-polar ACN species [87] that will exert a more hydrophobic effect on the water leading to its expulsion onto the stationary phase. The adsorption of water on the hydrophobic stationary phase under those conditions is facilitated by residual silanol groups [69,71].

While the above interpretation is generally accepted as the explanation for the negative excess of CAN, the data are in strong contrast with the direct measurement of the adsorbed amounts of the mobile phase components. Burke and Zwier [89] measured the amount of adsorbed ACN and water on an octadecyl phase through GC analysis and found that the volume of water adsorbed to the stationary phase is lower than the volume of adsorbed ACN at all mobile phase compositions and especially at high concentrations of ACN in the mobile phase. This totally contradicts the results found through injection of labeled compounds and frontal analysis. To find an explanation for this discrepancy the theoretical foundation of the method has to be reevaluated. The labeled compound method is based on the assumption that the retention of the deuterated species is affected exclusively by the amount of its non-deuterated analog adsorbed to the stationary phase. A basic fact of RPLC is, however, that the retention of any solute strongly depends on the mobile phase composition due to the changing solubility in the mobile phase. The more soluble the solute the shorter its retention time. This means that if the concentration of ACN in the mobile phase is increased, the bonded phase will adsorb more ACN leading to stronger retention of the deuterated species. At the same time, though, the high ACN concentration will "wash" the adsorbed deuterated species faster out of the

adsorbed layer and consequently reduces its retention. Another way of looking at the problem is to consider the case of a small volume of water adsorbed to the stationary phase at high concentrations of ACN (as indicated by the data of Burke et al. [89]). A deuterated water molecule then has very few adsorbed water molecules on the stationary phase that it can change places with leading to short retention. But once it is adsorbed it has to wait for a longer time for another water molecule to come by to replace it from the stationary phase and get it back into the mobile phase leading to long retention. This effect would be much stronger for water since it is bound to the surface by strong hydrogen bonds which means it needs another water molecule that can engage in hydrogen bonds to dislodge it. ACN is bound to the stationary phase by weak hydrophobic-hydrophobic interactions. So it can probably be easily removed even by water through dipole-dipole interactions.

This mechanism suggests that there may be two competing effects in the labeled compound method. The effect of mobile phase strength on retention interferes with the effect of stationary phase solvation and is the likely cause for the differences of the surface excess results when compared to the GC results. Surface excess data determined by labeled compound injection should therefore be interpreted with caution.

Since this study does not intend to make quantitative interpretations of the surface excess isotherm, but focuses on identifying differences between the selected stationary phases, the results from the labeled compound injection method are usable.

It is still reasonable to attribute the apparent negative excess of ACN to the residual surface silanols. Hydroxyl groups on the stationary phase should increase the retention of water or its deuterated analog, especially at high ACN concentrations at which the polarity of the mobile phase is very low due to lack of water and dimerization and clustering of ACN as explained above. If that is the case, then stationary phases that were prepared from the same base silica with the same bonding and endcapping procedure should show comparable surface excess isotherms in the extent of negative sorption excess at high %ACN. The results of this study support this hypothesis. According to Richard Henry, founder of Keystone Scientific, [88] all columns except Fluofix 120E were produced in house by Keystone Scientific with the same base silica. Fluofix 120E is manufactured by Neos Company in Japan with a different silica matrix. The Keystone columns all show a very small negative excess of about $0.01\mu L/m^2$ while Fluofix 120E shows a pronounced negative peak with a negative excess of about $0.05\mu L/m^2$. This indicates that Fluofix 120E has a

larger surface concentration of residual silanol groups than the Keystone phases.

The maximum surface excess values range from 0.60 $\mu L/m^2$ on Betasil C8 to 0.67 $\mu L/m^2$ on Ethyl-PFH for the Keystone phases at 25 °C. Comparing these values it has to be considered that the determination of the surface area S in equation (4) is only precise to $\pm 10 m^2$ which leads to a possible error of the surface excess values of up to 0.02 $\mu L/m^2$. There are also variations in packing density of the column that result in variations of the calculated bonded phase surface in the column. Considering additional possible errors from the chromatographic experiment, the differences in the maximum surface excess are surprisingly small and probably insignificant. Fluofix 120E has a lower maximum surface excess with 0.53 $\mu L/m^2$. This lower value is most likely an effect of the larger surface concentration of silanol groups, which will allow for an overall increased adsorption of water and a consequently lower excess of ACN.

The isotherms of the Keystone phases are quite similar considering profile and magnitude, which is surprising considering the differences in the chemical structure of the bonded phases. The surface excess on Betasil C8 is only slightly lower than the ones on the fluorinated phases and there is hardly a difference between the aromatic PFP phases and the aliphatic Ethyl-PFH and

RP-100 phases. The differences between these results are so small that they do not warrant interpretation. The fluorination does not seem to markedly affect the solvation of the stationary phase.

In its chemical structure the Fluofix phase is very similar to the RP-100 phase, the only difference being the branching of the fluorinated chain. Nevertheless, the surface excess isotherm of the RP-100 is much more similar to the chemically very different aromatic PFP-100 phase made by the same manufacturer with the same silica matrix. This suggests that the nature of the silica matrix and possibly the manufacturing process have a much greater effect on the solvation behavior of the stationary phase than the chemistry of the bonded phase.

The results of the surface excess isotherms indicate that there are no differences in the solvation behavior of fluorinated phases as compared to hydrocarbonaceous phases that could account for the alleged better selectivity to taxane compounds.

Figure 25. Surface Excess Isotherm; Betasil C8

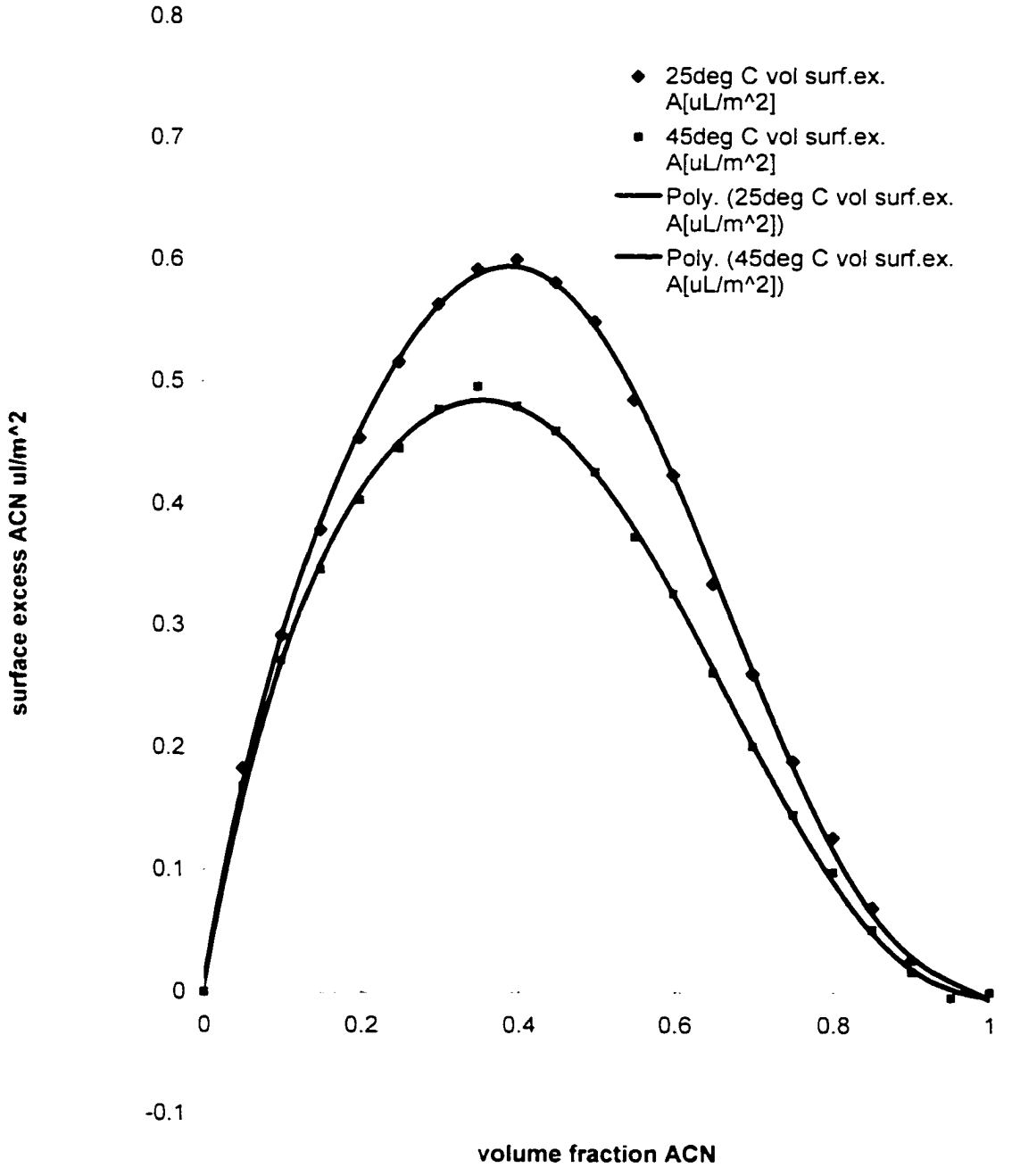


Figure 26. Surface Excess Isotherm; Fluofix 120E

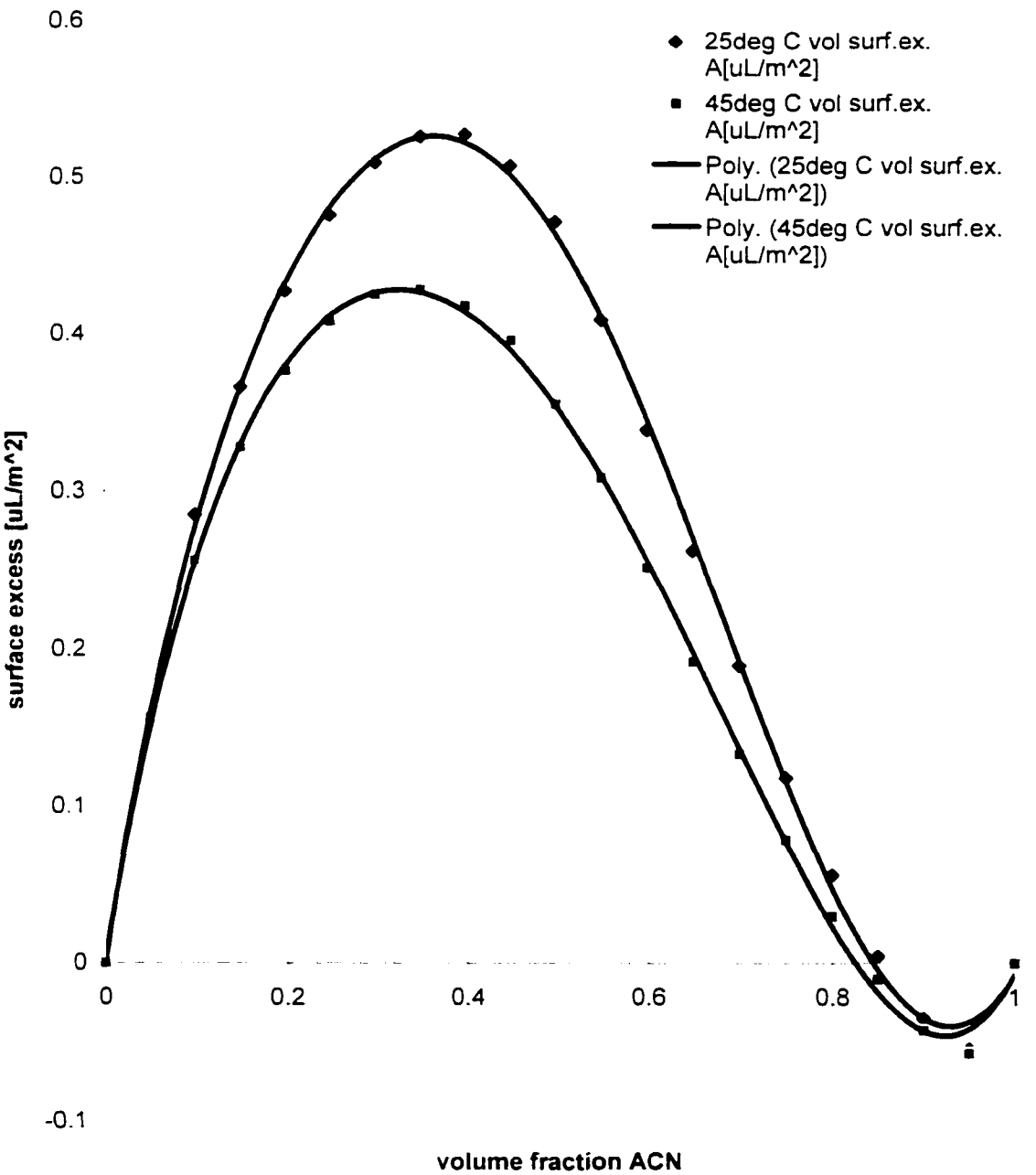


Figure 27. Surface Excess Isotherm; Propyl-PFP

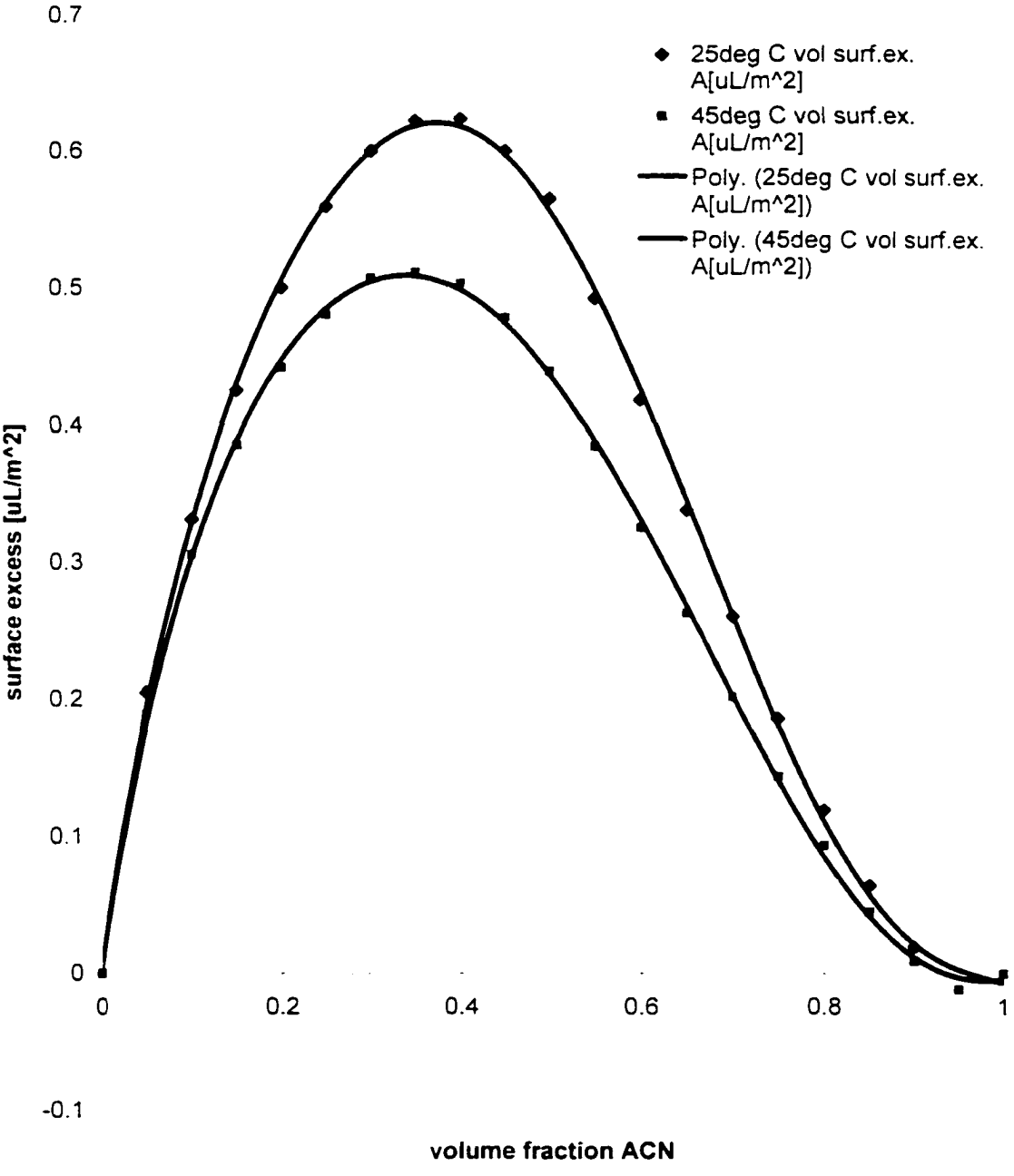


Figure 28. Surface Excess Isotherm; PFP-100

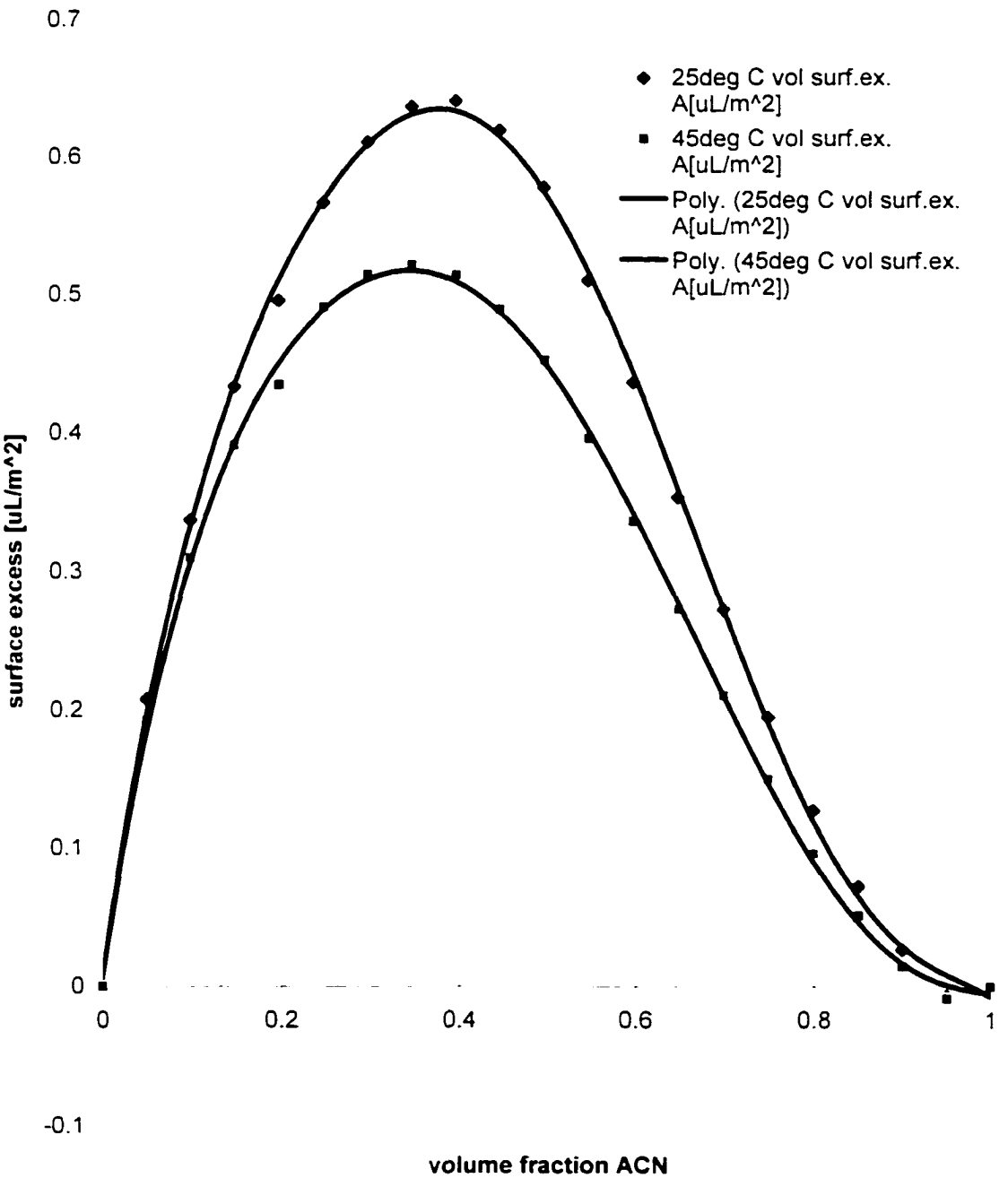


Figure 29. Surface Excess Isotherm; Ethyl-PFH

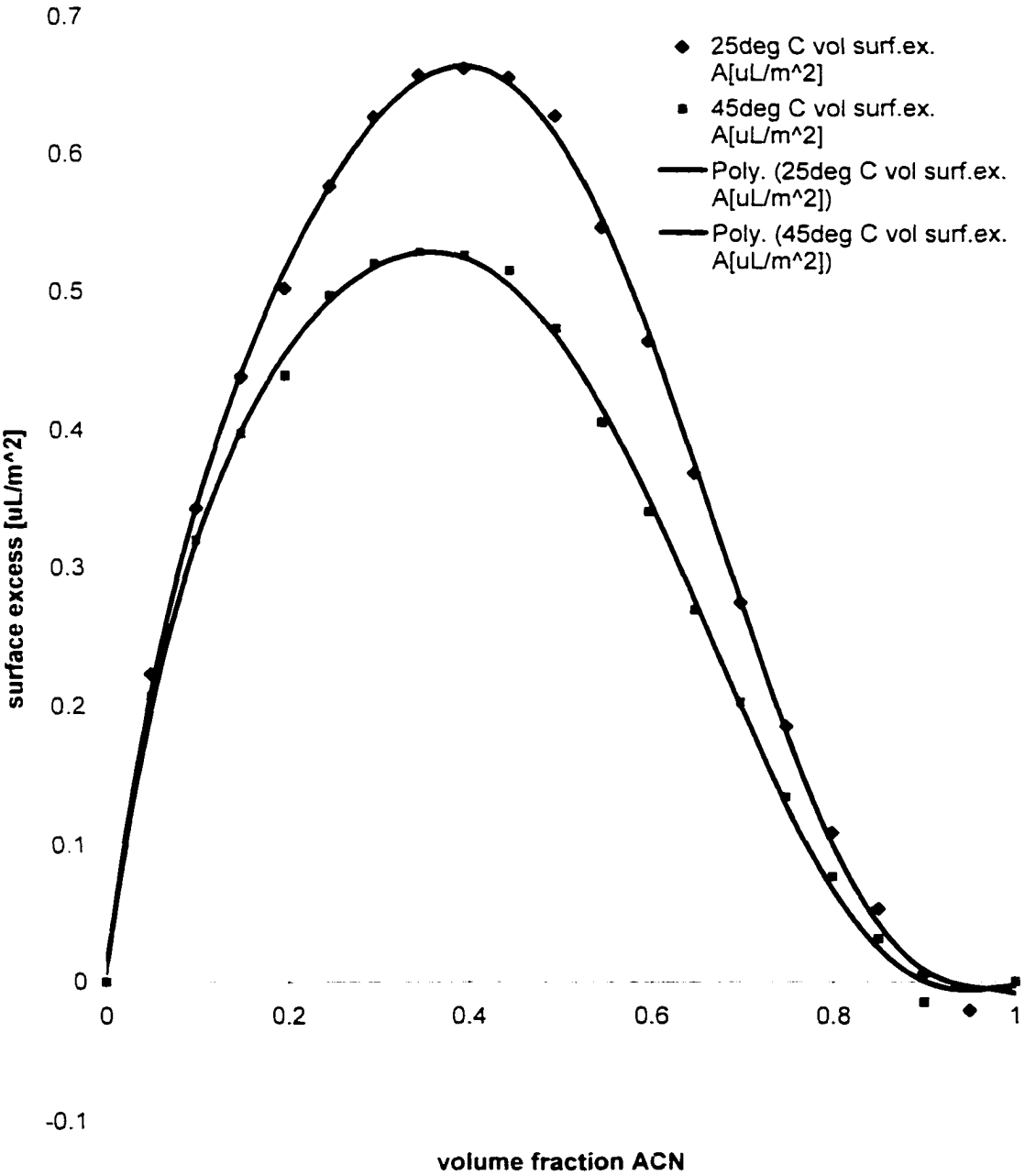
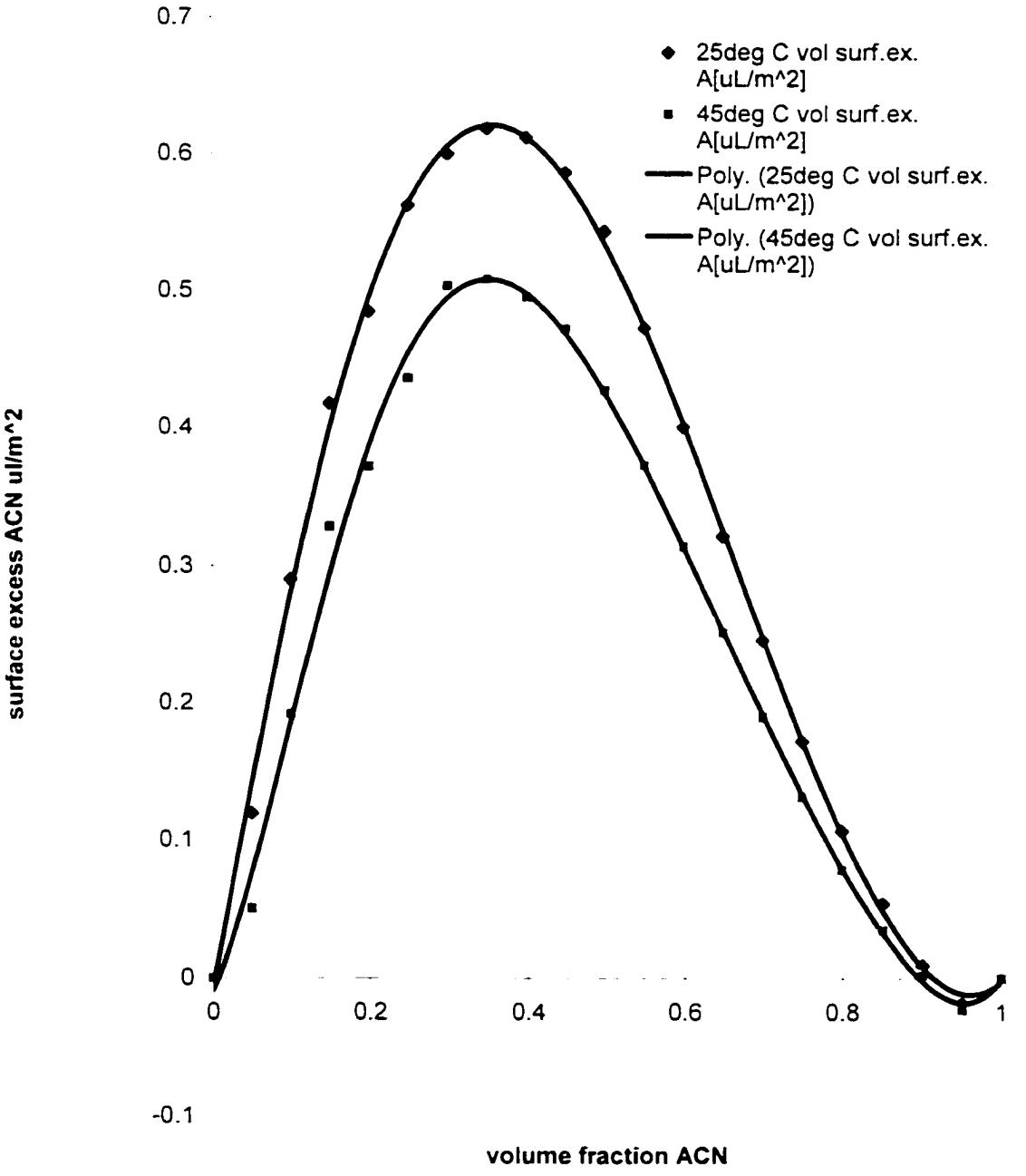


Figure 30. Surface Excess Isotherm; RP-100



4.2.2 Bonded Phase Collapse of RP-100

It has been discussed in section 2.2.2 that the bonded phase tends to collapse at low concentrations of organic modifier in the mobile phase. The threshold at which this phenomenon occurs is believed to be below 10% organic modifier. The collapse of the bonded phase is caused by the high water concentration in the mobile phase which forces the hydrophobic, non-polar bonded phase to reduce its surface area through self association in a "folding up" process. The reduced surface area in turn results in reduced interaction with the solutes and consequently reduced retention and resolution of solutes. In 1980 Galan et al. reported related problems with an aliphatic, partially perfluorinated C10 bonded phase [81]. The performance of the column decreased gradually when operated below 40% methanol in the mobile phase. At highly aqueous mobile phases the solutes would elute with drastically reduced retention times making separation impossible. Galan et al. attributed this problem to reduced pore wettability at high water content [81]. In 1980 the theory of bonded phase collapse was not yet widespread, which might explain the different interpretation of Galan's data.

In the determination of surface excess isotherms it is desirable to cover

mobile phase compositions from 0% to 100% organic modifier. Since it is implied that the surface excess at the pure solvents is zero, surface excess isotherms were determined from 95% to 5% ACN. RP-100 showed a problem at mobile phases with high water content especially at 45 °C as can be seen in Figure 31. The surface excess drops sharply and remains low. This problem was reproducible but kept occurring at different mobile phase compositions, sometimes at ACN concentrations as high as 20%. The Surface Excess Isotherm shown in Figure 30 was a "lucky" experiment that showed only minor effects of the stated problem. Upon close inspection, however, it can be seen that the surface excess drops to fast at low %ACN resulting in a slightly sigmoidal shape of the 45 °C isotherm. Multiple runs of the experiment indicated that the problem was most pronounced if the ACN concentration was lowered over a long period of time and that it might be triggered by mechanical shock such as turning the pump off and on. A probable explanation for this phenomenon is that the bonded phase collapses which prevents the effective solvation with ACN. This theory seems reasonable but bonded phase collapse has never been reported to occur at organic modifier concentrations as high as 20% or to be triggered by mechanical effects. To test this hypothesis, the RP-100 column was slowly brought to a mobile phase composition of ACN/water 95/5 and

equilibrated for about 30 minutes. 1-naphthol was injected repeatedly over a period of several hours with turning the pump off and back on several times in the process. The results of the experiment are shown in Figure 32.

As can be seen the retention time of naphthol slightly decreases from 36.4 to 35.6 minutes from the first to the second injection. However, once the pump has been turned off and back on the retention time drops to 21.7 minutes. Repeating the process results in a even further decrease to 6.6 minutes. With the pump kept running a further injection leads to only a slightly lower retention time of 6.2 minutes. A further pump turn off reduces the retention time again to 3.2 minutes. An injection about 90 minutes later shows two peaks which indicates that further changes occur on the stationary phase. The results of this experiment clearly indicate the collapse of the RP-100 bonded phase and shows that the onset and progression of the collapse is sensitive to mechanical shock. The cause for the two peaks could be either peak splitting or the separation of an impurity, but this question is secondary to this study and was not further pursued.

Figure 31. Surface Excess Isotherm with Bonded Phase Collapse; RP-100

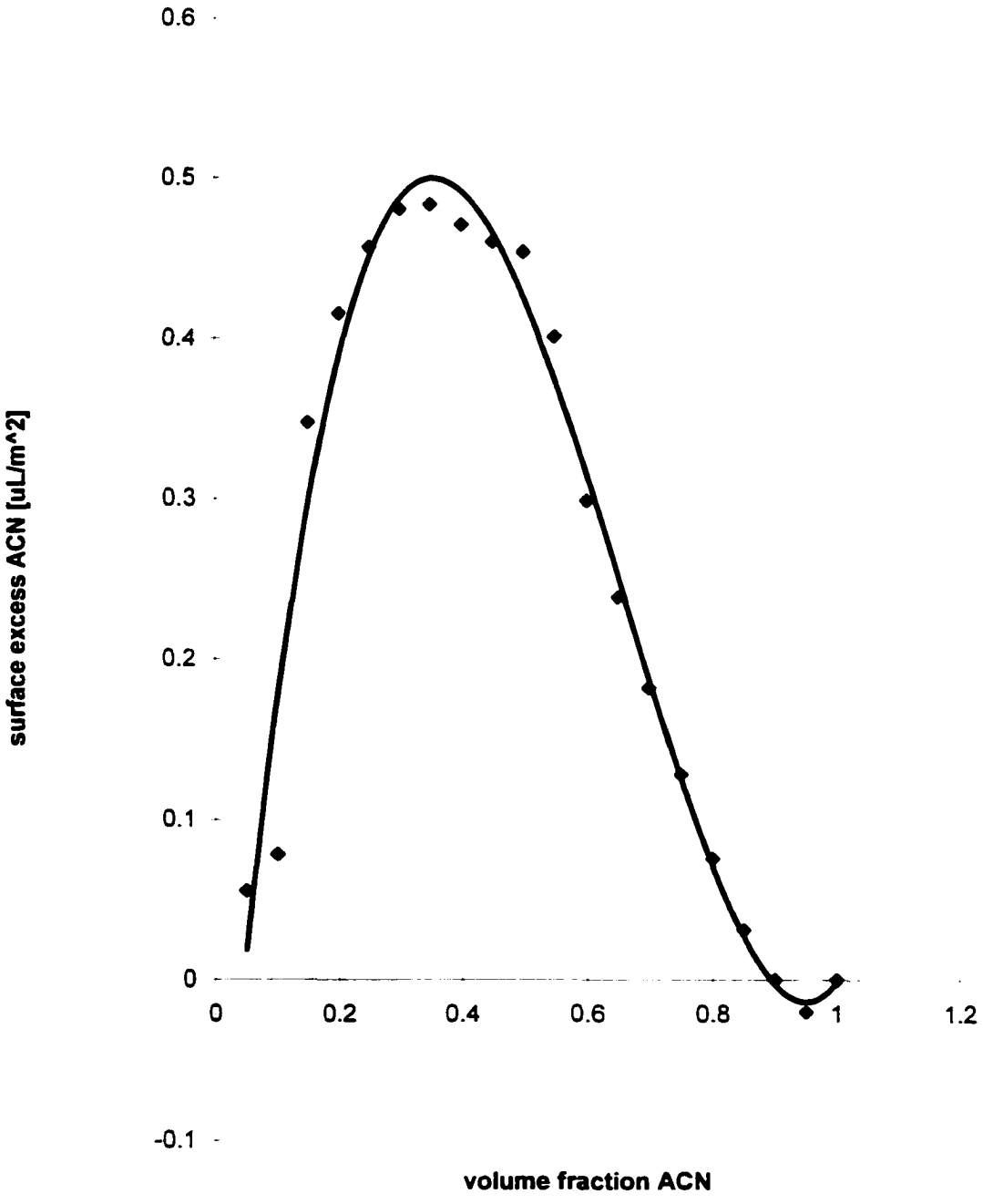
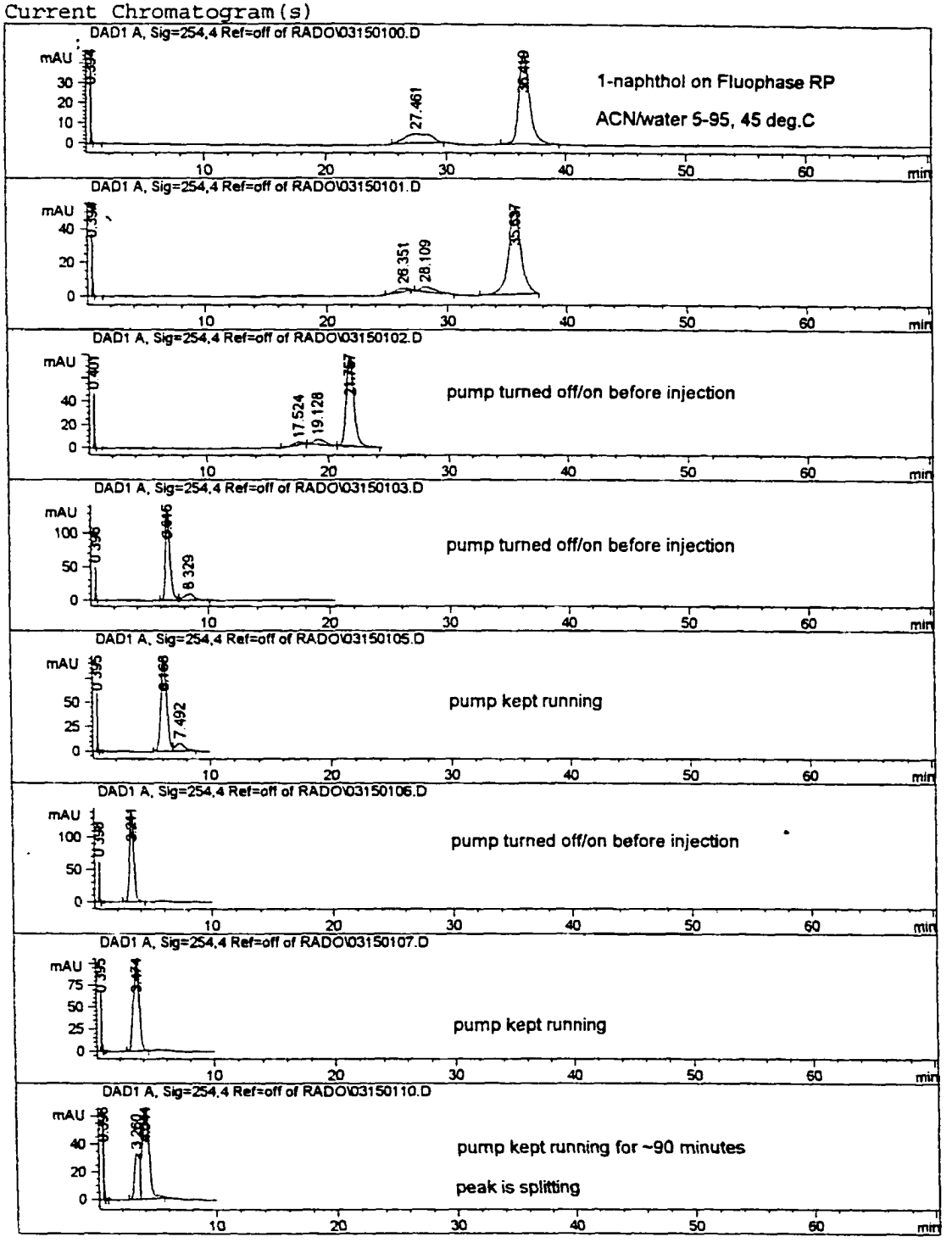


Figure 32. Injection of 1-naphthol; Effect of Bonded Phase Collapse

Current Chromatogram(s)



4.3 Comparison of Elution Order

The comparison of elution order of compound mixtures has been widely employed in the characterization of stationary phases in liquid chromatography [81-84]. Through comparison of peak resolution and relative retention, differences in the chromatographic behavior of different stationary phases can be identified. There can be problems with this approach and the interpretation of the results. Often the chromatographic conditions applied on two different columns are not identical because the investigator tries to develop separations with comparable retention times sometimes referred to as isoelutropic systems [82]. Changes in the chromatographic conditions such as temperature and mobile phase composition, however, can change the solvation state of the stationary phase and lead to changes in interactions between the solute and the mobile phase. This raises the question as to whether the observed differences in the separations on the two columns are a result of the bonded phase chemistry or the effects stated above [82]. To avoid this ambiguity, this study expands the idea of comparing elution order to comparing elution behavior. According to Snyder [35] plots of the logarithm of the capacity factor, k', versus the concentration of the organic modifier in the mobile phase yield virtually linear

plots. Therefore, if the retention times of a compound mixture at several mobile phase compositions are entered into such a plot the elution order of the compounds and changes of them can be shown continuously over a wide mobile phase range through interpolation. In his way the elution behavior of the compounds on different stationary phases can be described, and compared effectively.

4.3.1 Elution Order of Naphthalene Derivatives

The retention times of a series of naphthalene derivatives were determined at various mobile phase compositions, using ACN as the organic modifier. The naphthalene derivatives were introduced in chapter 3. Acetic acid was added to the mobile phase (2%) to prevent tailing of the acidic compounds. It was determined experimentally that naphthyl acetic acid and naphthoic acid are non-dissociated at pH 2.5. This was accomplished by checking the pH dependence of the retention time of the compounds as shown in Figure 33. At pH values close to the pKa of the compounds, the average polarity and with that the retention time depends on the degree of dissociation of the acid which in turn depends on the pH. At a low enough pH the acid is completely protonated and is relatively constant with further decreases in pH. The results in Figure 33 indicate that complete protonation of the acid is achieved at a pH of about 3.2. The elution experiments were performed at pH values of 2.5 and 4.7 to investigate the chromatographic behavior of both the protonated and dissociated acids, respectively. One column of each of the four different bonded phase structures was used for this study. The plots of ln k' versus %ACN for Ethyl PFH, Fluofix 120E, Propyl PFP and Betasil C8 are shown in Figures 34, 35, 36

and 37, respectively. The mobile phase composition ranges of the graphs for each column vary since they were chosen to cover the maximum, experimentally practical range depending on the retentivity of each particular stationary phase. It is important to consider the varying ranges when comparing the graphs since they lead to different overall appearances even for identical trends.

The three fluorinated phases show very similar elution behavior of the naphthalene derivatives. The two acids elute first and show virtually identical retention times at pH 2.5 on Ethyl-PFH and Fluofix 120E. Propyl-PFP shows some selectivity to the acids, especially at higher concentrations of ACN at which 1-naphthoic acid elutes before 1-naphthyl acetic acid. The two compounds converge and change elution order at low concentrations of ACN. The two acids are separated on all columns at pH 4.7 due to their differences in pKa and the consequent difference in dissociation. Again, on Propyl-PFP an exchange of elution order takes place at about 50% ACN.

The next two compounds to elute are 1-naphthol and 1,8-naphthalic anhydride. Both Propyl-PFP and Fluofix 120E separate these compounds well from the two acids. On Ethyl-PFH the acids converge with the two compounds at lower concentrations of ACN. On all three fluorinated columns, 1-naphthol

and 1,8-naphthalic anhydride elute very close to one another with 1-naphthol eluting first at high ACN concentrations, but the compounds change elution order at about 50% ACN on all fluorinated columns.

The last eluting compounds are 1-naphthaldehyde and 1-naphthyl acetonitrile. On all three fluorinated columns naphthyl acetonitrile elutes first at low concentrations of ACN, but the two compounds either converge (as on Ethyl-PFH) or exchange elution order (as on Fluofix 120E and Propyl-PFP) with increasing ACN concentration. This change of elution order occurs at different mobile phase compositions depending on bonded phase structure and pH.

The elution behavior on Betasil C8 is comparable to Ethyl-PFH, which is its fluorinated analog. Naphthyl acetic acid and naphthaldehyde show the same elution order and the convergence at around 50% ACN. The two acids are very slightly separated on Betasil C8 but coelute on the fluorinated phase. On both columns naphthalic anhydride elutes very close to the acids, especially at low concentrations of ACN, but on Betasil C8 napthol is better separated from these three compounds than on Ethyl-PFH. The differences observed between Betasil C8 and its fluorinated analog are very small. They are of the magnitude expectable for batch to batch or manufacturer deviation of the same bonded

phase. A defined difference in chemical selectivity to the naphthalene compounds could not be identified. The slight variations observed in the elution behavior might be caused by the difference in chain stiffness caused by the larger atomic radius of the fluorine compared to hydrogen, but this interpretation is purely speculative.

Figure 33. Retention of 1-Naphthoic Acid and 1-Naphthylacetic Acid versus pH

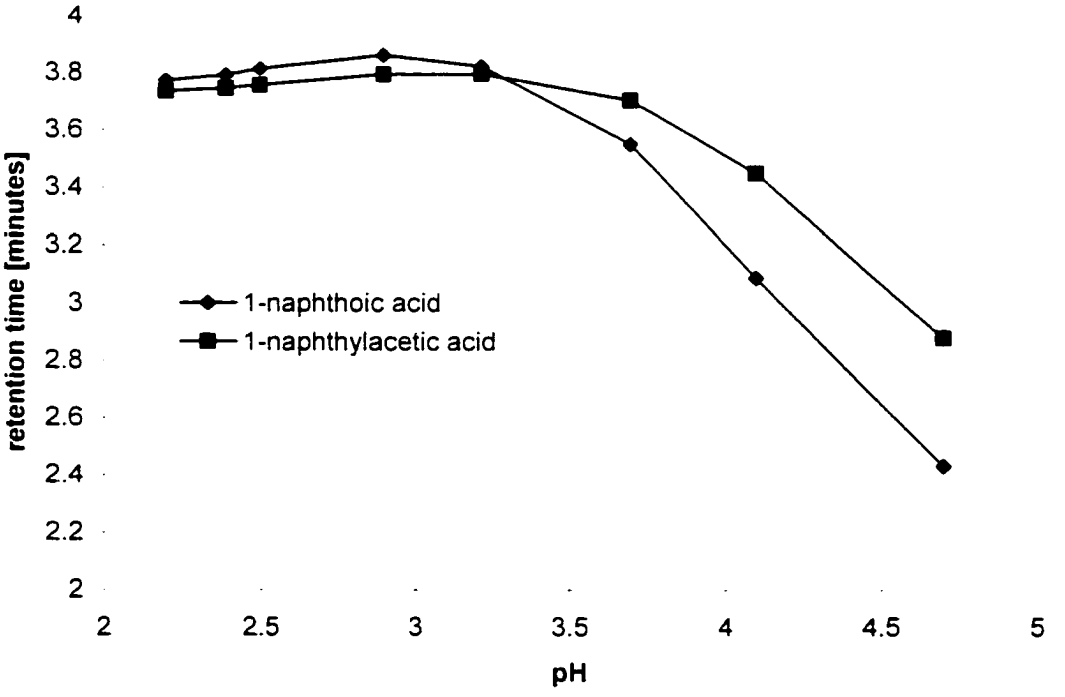


Figure 34. Elution Behavior of Naphthalene Compounds on Ethyl-PFH

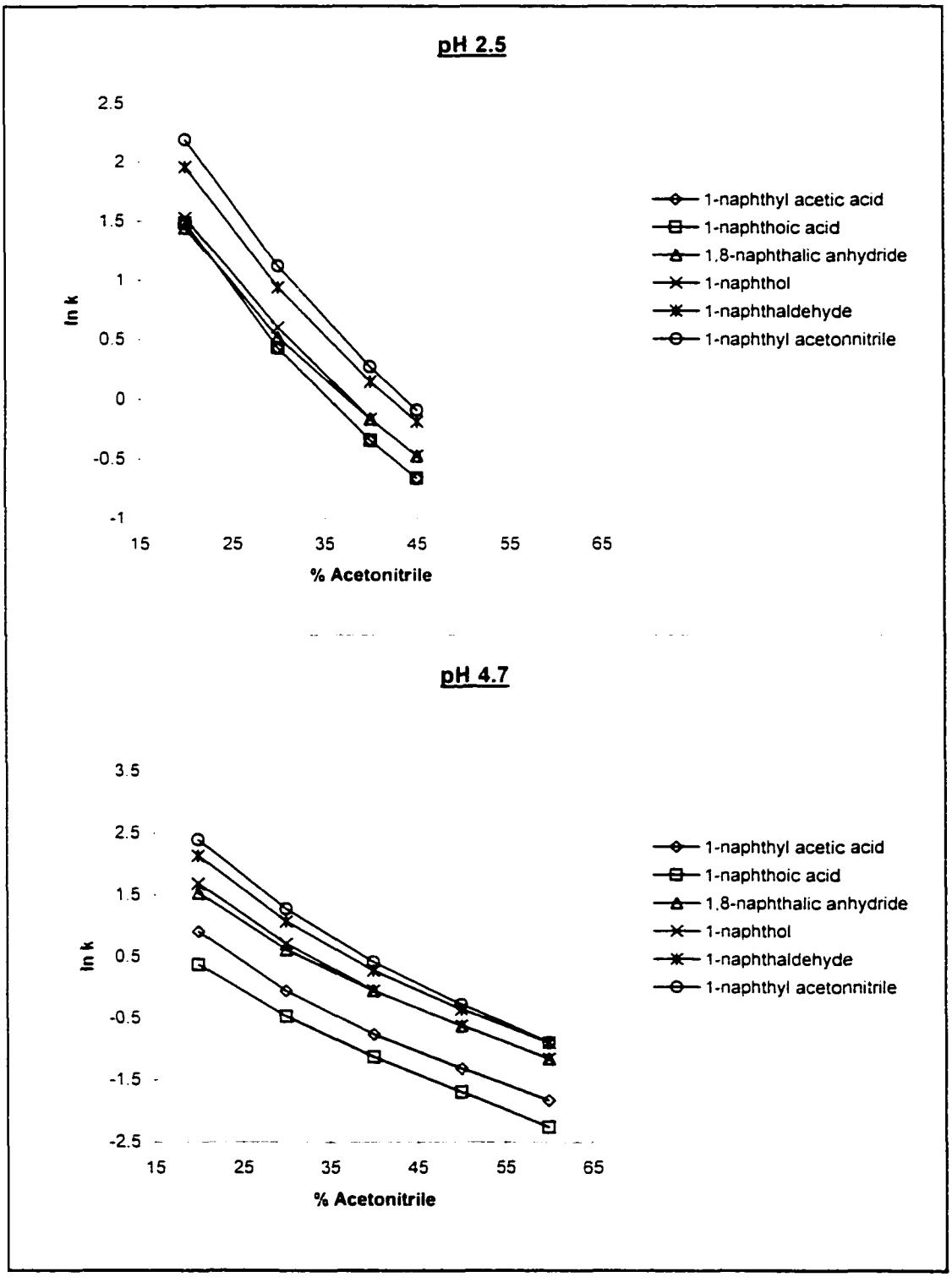


Figure 35. Elution Behavior of Naphthaiene Compounds on Fluofix 120E

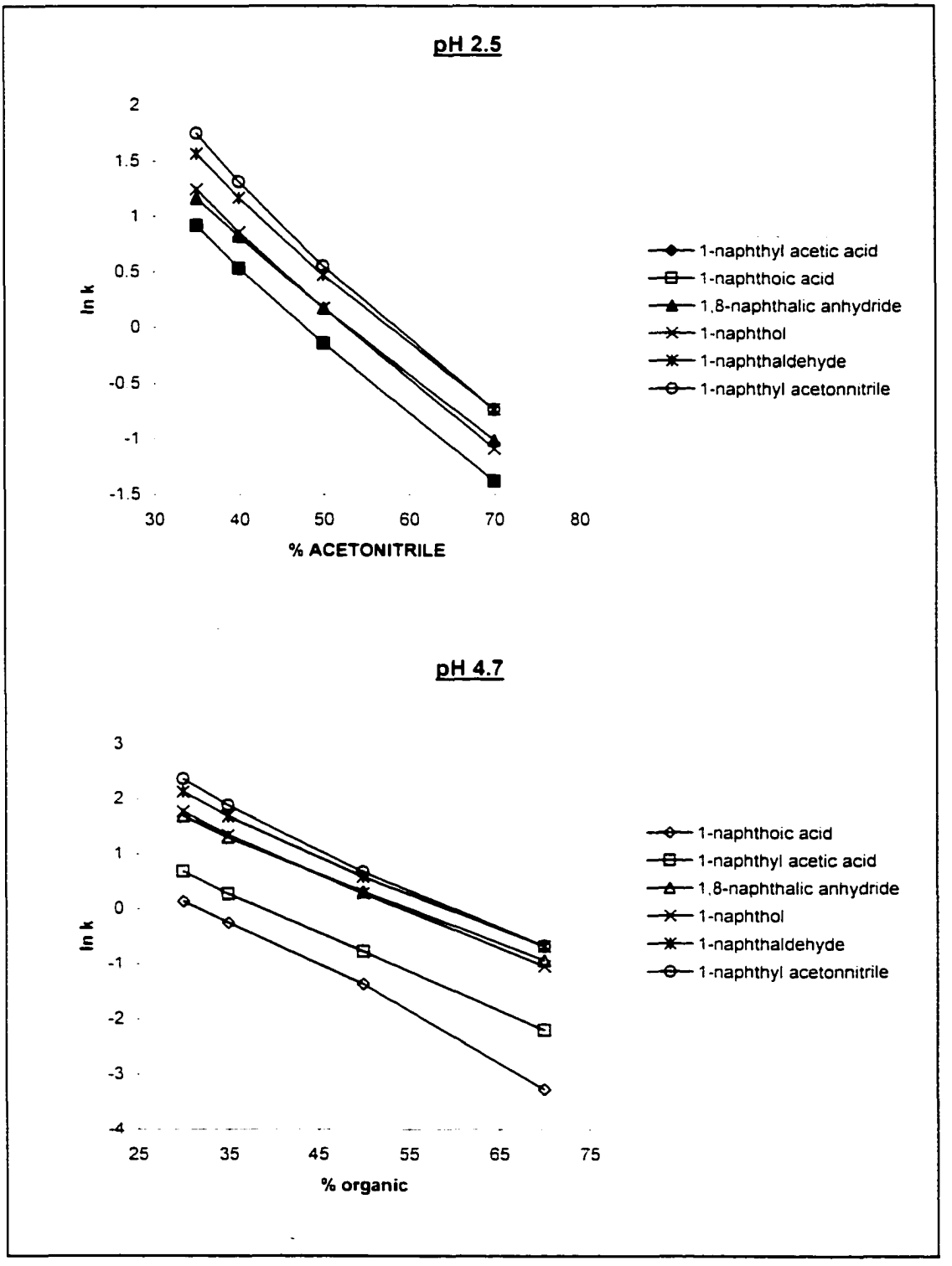


Figure 36. Elution Behavior of Naphthalene Compounds on Propyl-PFP

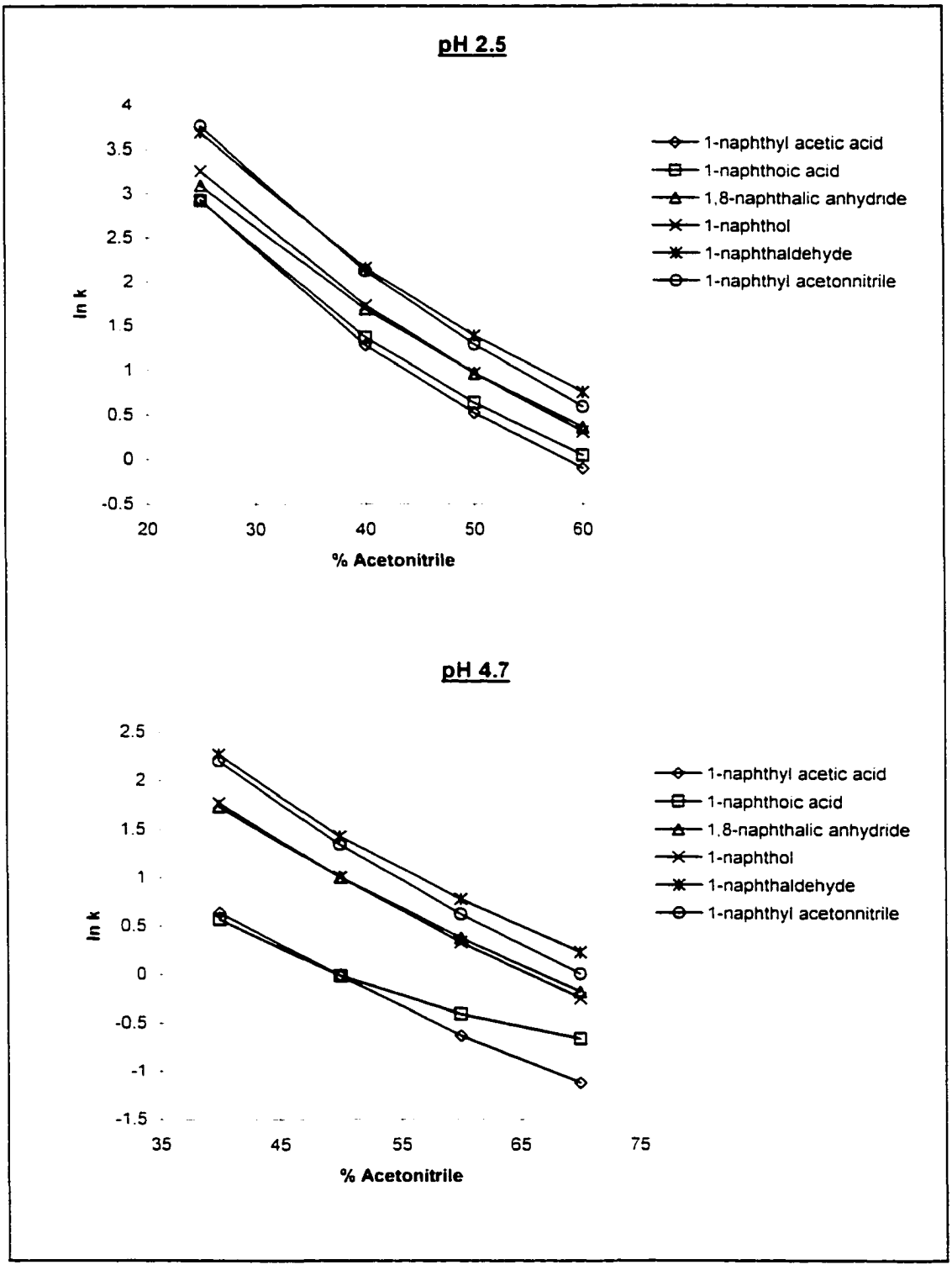
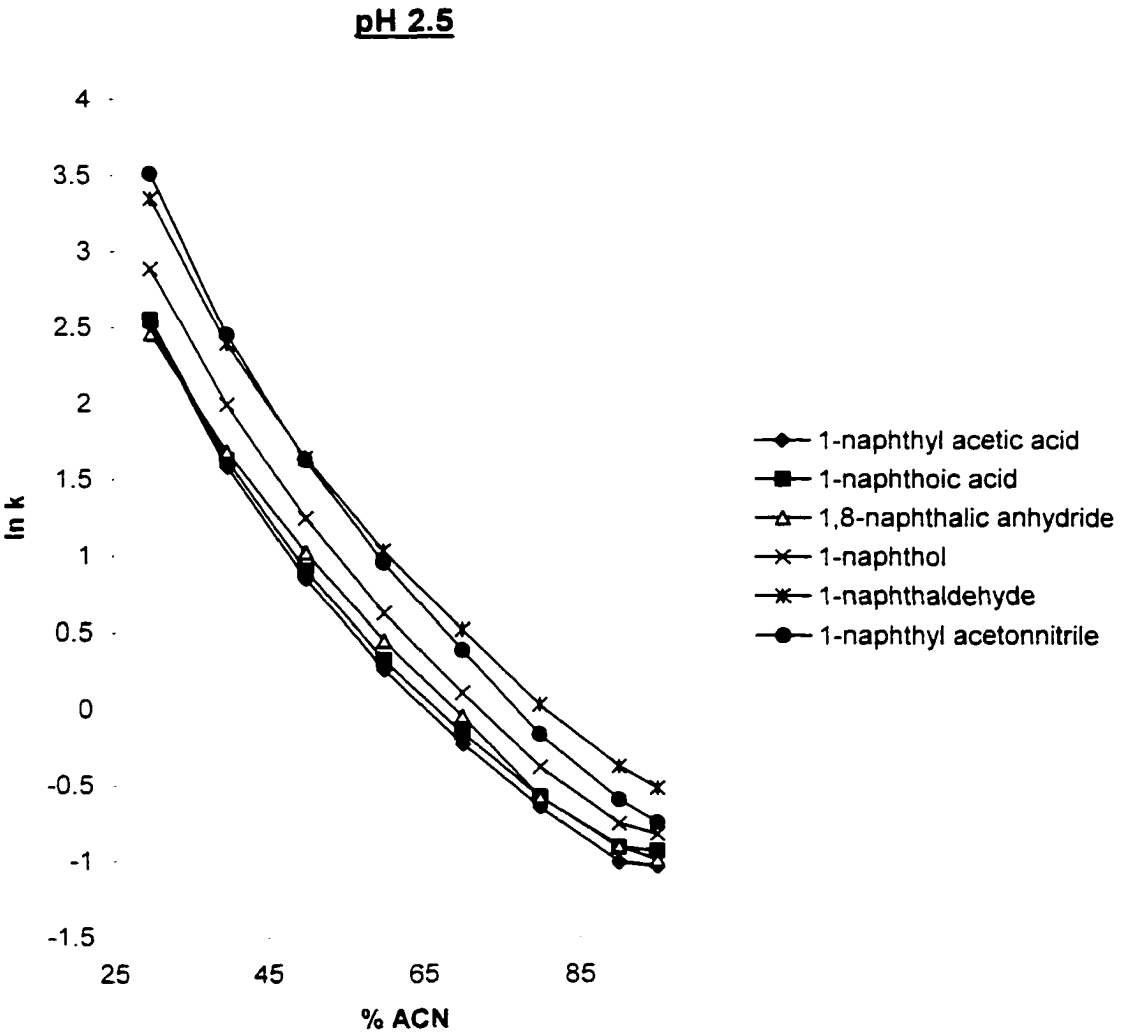


Figure 37. Elution Behavior of Naphthalene Compounds on Betasil C8



4.3.2 Elution Order of Cyclohexane Derivatives

Five cyclohexane derivatives were used to determine the influence of π-electrons and several functional groups on the selectivity of the hydrocarbonaceous and fluorinated phases. The compounds and their structures were given in chapter III. The elution order was investigated at various mobile phase compositions to prepare plots of ln k' versus %ACN. Since one of the compounds is acidic (cyclohexyl acetic acid) the experiment was performed once with a mobile phase of ACN/water and once with ACN/ 25mM phosphate buffer pH 2.50 to ensure complete protonation of the acid. Acetic acid (2%) was added to the aqueous phase to prevent tailing of the cyclohexyl acetic acid peak. The acidic compound was excluded from the ACN/water study since the retention of the dissociated acid under these conditions depends primarily on the pH of the mobile phase and not on the bonded phase chemistry.

The focus of this study was to identify differences in selectivity caused by the fluorination of the bonded phase. Variations in the elution order of model compounds between the octadecyl phase and most of the other fluorinated phases would be hard to interpret due to the considerable differences in chemical structure. The experiment was therefore only performed on Betasil C8

and its fluorinated analog RP-100. Plots of ln k' versus %ACN for Betasil C8 and RP-100 are shown in Figures 38 and 39, respectively.

It can be seen that on each column, the elution behavior of the non-acidic compounds is virtually the same. On both columns cyclohexyl bromide elutes last, well separated from the other compounds. The next two compounds to elute are 1,3-cyclohexadiene and 1,4-cyclohexadiene. They are not separated from one another on Betasil C8, but RP-100 shows a slight difference in selectivity towards the two compounds that is consistent through the entire mobile phase range (50%-90% ACN). De Miguel et al. speculated that fluorinated phases show better separations of aromatic polycyclic hydrocarbons due to interactions of the π-electrons with the C-F dipoles [85]. The results shown here, however, do not show an increased retention of the cyclohexadienes compared to the other compounds. The increased sensitivity towards the spatial arrangement of the double bonds in the diene compounds on the fluorinated phase might be a result of the better shape selectivity that is observed on more ordered bonded phases.

The most prominent difference on the two phases is the selectivity towards nitrocyclohexane. On the octyl phase the compound elutes between the cyclohexadienes and cyclohexyl acetic acid, well separated from both. On RP-

100, however, nitrocyclohexane elutes very close to the cyclohexadienes at high ACN concentrations and it quickly converges with them at decreasing ACN concentrations. This indicates that the fluorinated phase has an increased retention to the nitro compound. With the buffered mobile phase system, cyclohexyl acetic acid elutes last on both columns well separated from the other compounds. At close to 100% ACN, the cyclohexyl acetic acid shows non-linear behavior on Betasil C8 which leads to a change of elution order with nitro- cyclohexane. This phenomenon has been observed for many taxane compounds as well and will be discussed in detail in section 4.3.4.

Figure 38. Elution Behavior of Cyclohexyl Derivatives on Betasil C8

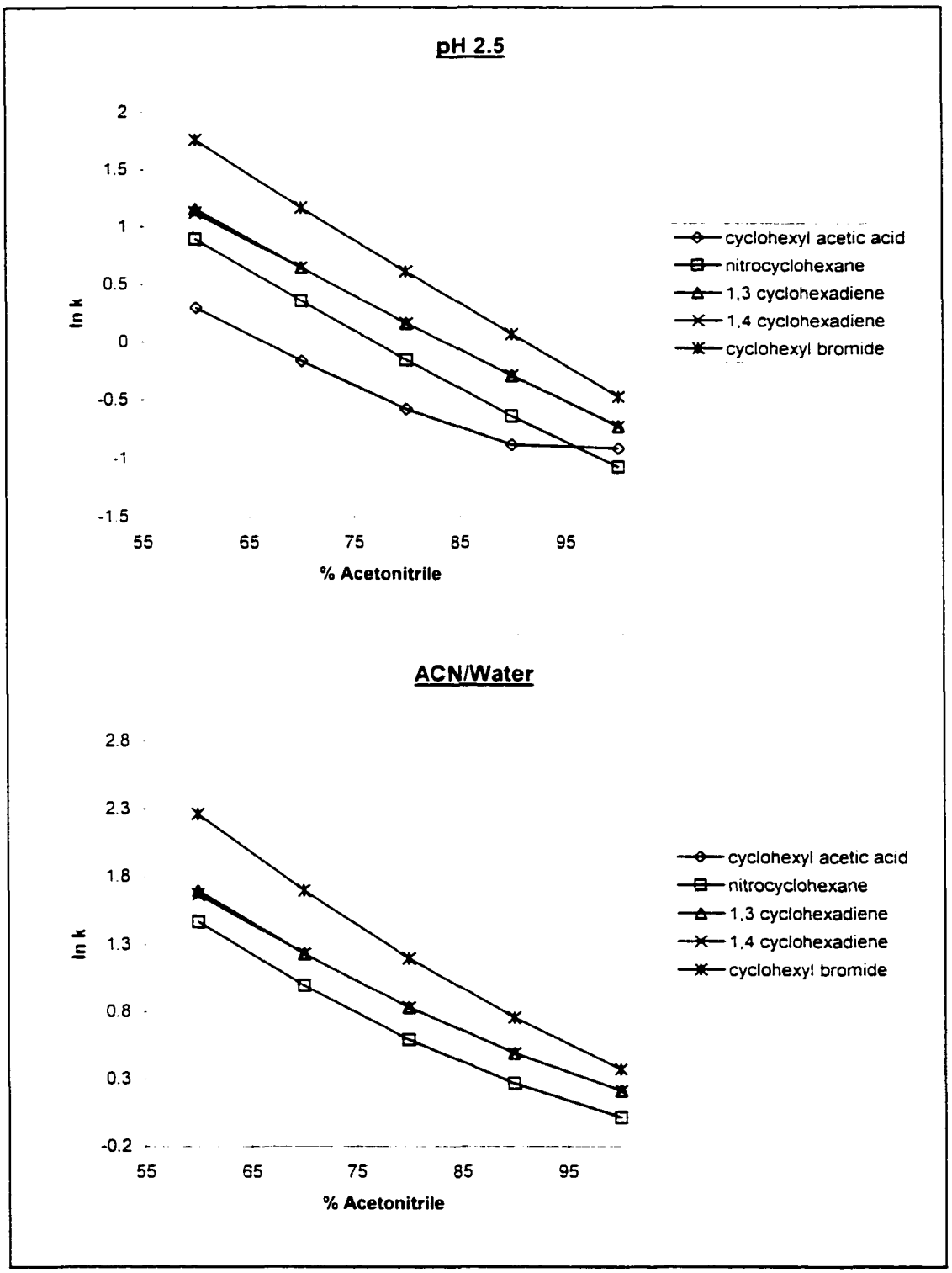
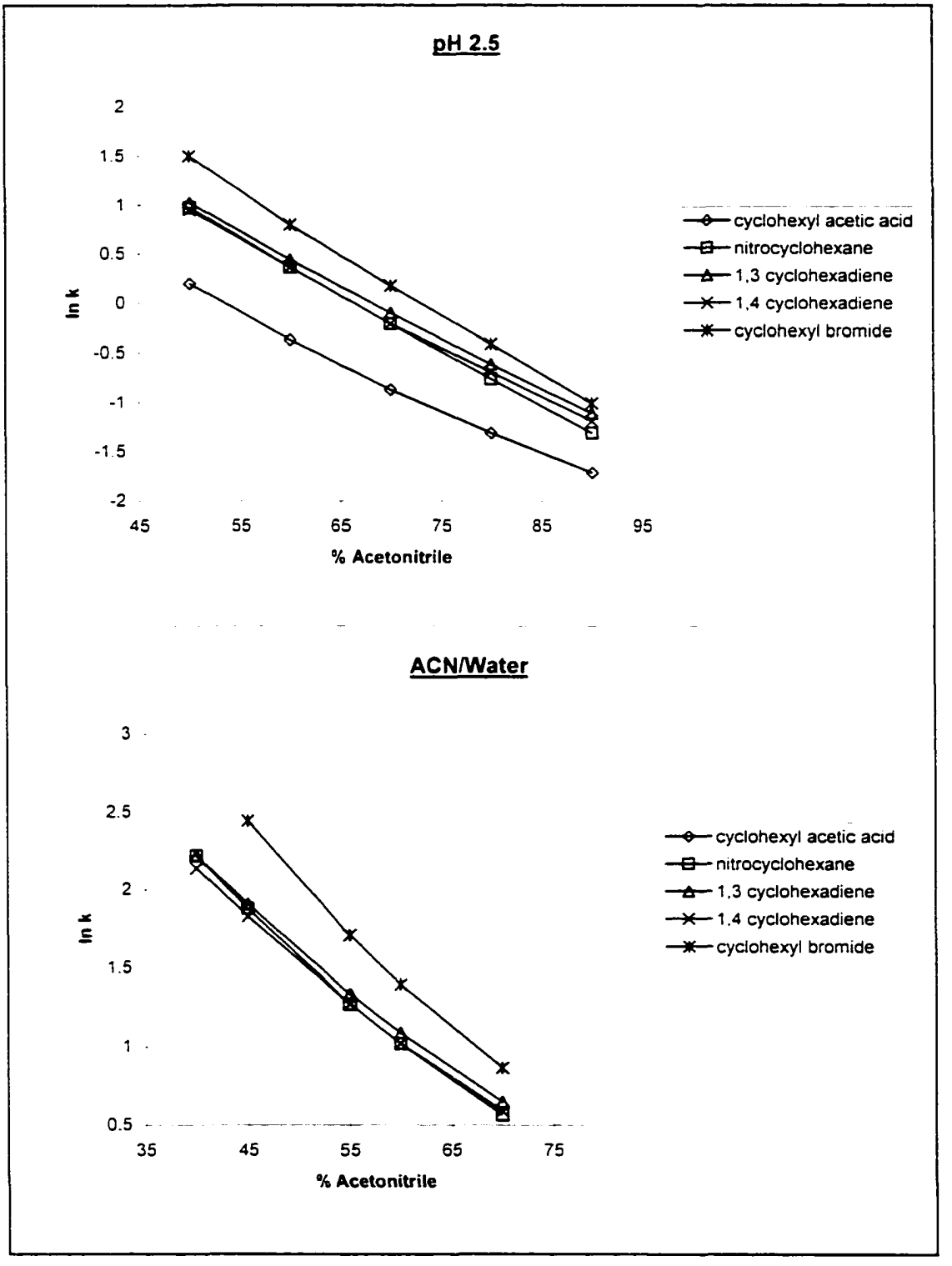


Figure 39. Elution Behavior of Cyclohexyl Derivatives on RP-100



4.3.3 Aromatic versus Non-Aromatic Compounds

The elution order of three aromatic, one cyclic and one aliphatic compound was compared on hydrocarbonaceous and fluorinated phases using aqueous ACN as the mobile phase. The focus of this study was to identify differences in selectivity towards aromatic compounds caused by the fluorination of the bonded phase. Variations in the elution order of model compounds between the octadecyl phase and most of the other fluorinated phases would be hard to interpret due to the considerable differences in chemical structure. The experiment was therefore only performed on Betasil C8 and its fluorinated analog RP-100. In this experiment the elution order at roughly isoelutropic mobile phase compositions was compared. It was confirmed through preliminary experiments that no change of elution order occurs with these compounds with variation of mobile phase composition so this simplified experiment is sufficient. The chromatograms on Betasil C8 and RP-100 are shown in Figures 40 and 41, respectively.

The separation of the compounds is almost identical on the two columns with respect to peak spacing and resolution. Betasil C8 shows slight resolution of toluene and 1,3-cyclohexadiene, which coelute on RP-100.

There is no indication of a difference in the retention of aromatic versus non-aromatic solutes as shown by the comparison of the resolution of ethyl acetate and benzene which is virtually identical on the two phases. Equally comparable is the resolution of the non-aromatic cyclohexadiene from the two aromatic compounds benzene and ethyl benzene. These results further suggest that the special selectivities observed for polycyclic aromatics [85] can not be attributed to chemical interactions with the aromatic π-systems but are more likely a result of the better shape selectivity of fluorinated phases as explained in section 4.3.2.

Figure 40. Separation of Aromatic and Non-Aromatic Compounds on Betasil C8

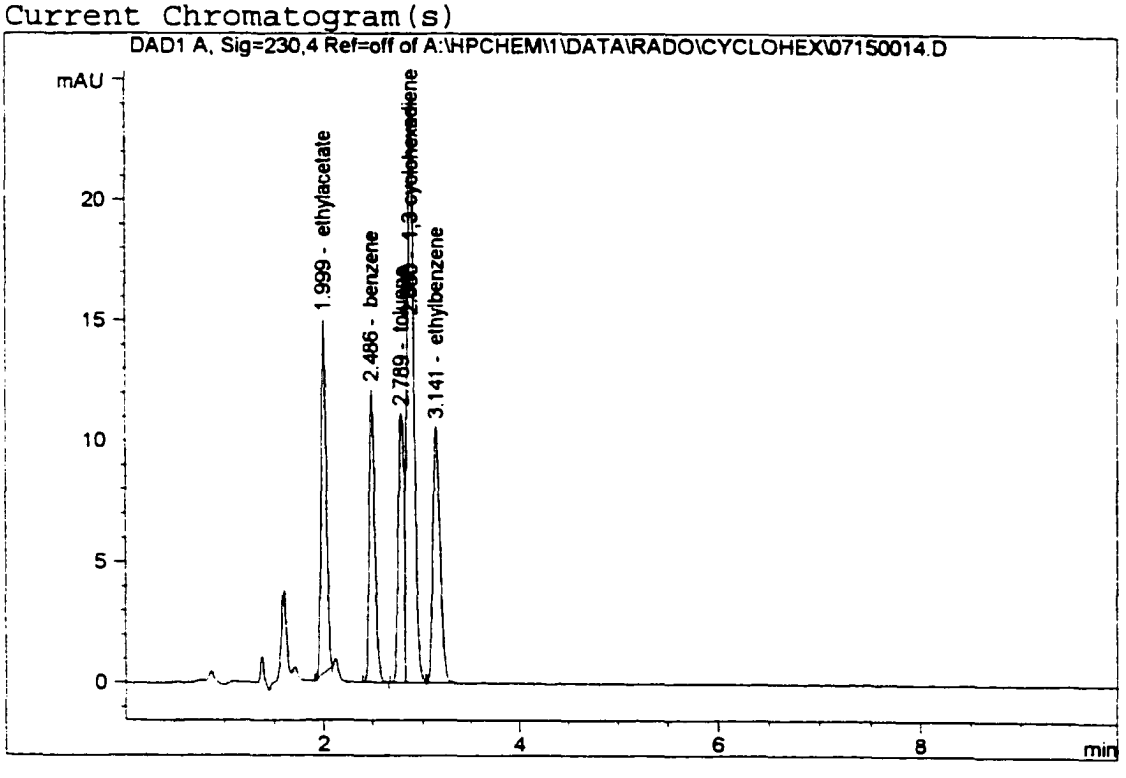
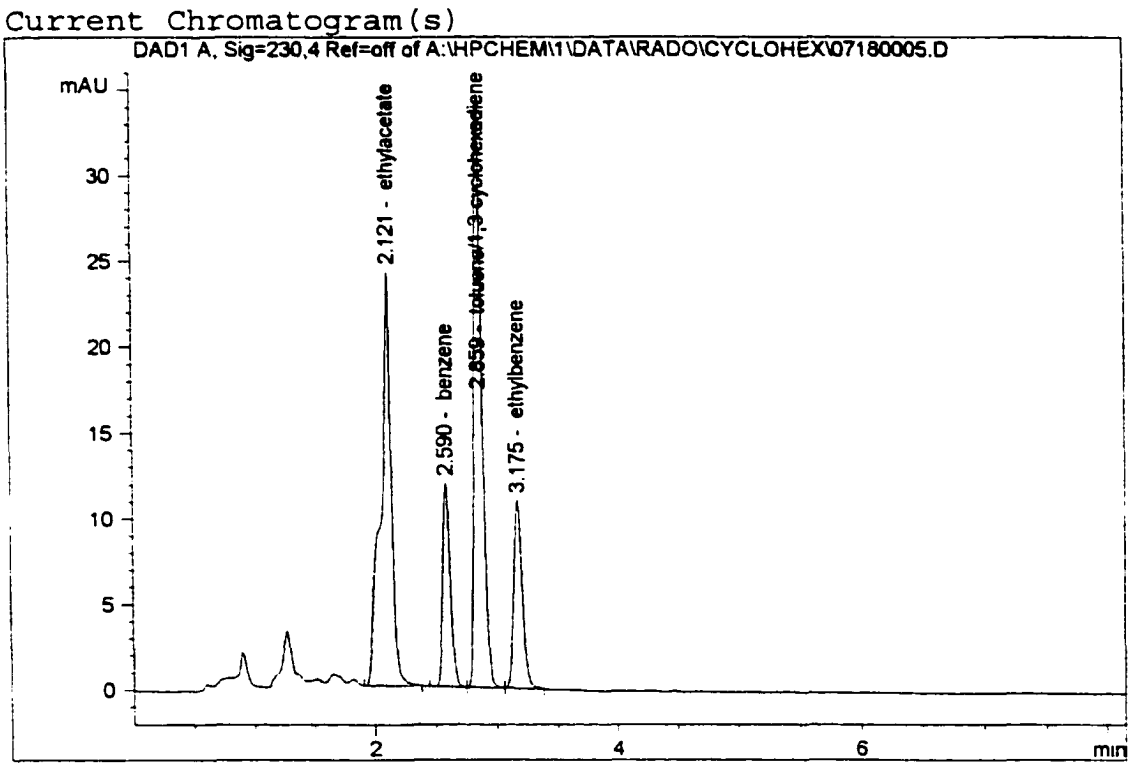


Figure 41. Separation of Aromatic and Non-Aromatic Compounds on RP-100



4.4 Characterization of the Elution Behavior of Taxanes

As explained in section 4.3, investigating the elution behavior of compound mixtures is superior to the simple comparison of elution order to identify the chromatographic characteristics of different stationary phases. Since the focus of this study is to find the differences in the chromatographic separation of taxanes on hydrocarbonaceous and fluorinated phases, the elution behavior of the taxane compounds on the different stationary phases was investigated.

It was stated in section 4.1 that the taxane separations were found to be strongly temperature sensitive. For this reason the elution behavior of the taxane compounds was investigated not only with changes in mobile phase composition but also with changes in temperature.

4.4.1 Elution Behavior of Taxane Compounds with Changing Mobile Phase Composition

The elution behavior of all fifteen taxane compounds was examined on Betasil C8 and its fluorinated analog Ethyl-PFH. Plots of ln k' versus %ACN on Betasil C8 and Ethyl-PFH are shown in Figure 42 and 43, respectively. The results are very similar on the two stationary phases and reveal a lot about the problematic nature of the taxane separation. According to this study, the fifteen taxane compounds can be divided into two major groups that are distinguished by the slopes they exhibit in the ln k' versus %ACN plots. There are four compounds with distinctly steeper slopes than the other ones. These compounds all belong to the xylosyl taxol group distinguished by the sugar group attached to the C-7 carbon. Another compound, 10-deacetyl taxol, has a slope in between the xylosyl taxol group and the remaining taxanes. The elution order amongst the xylosyl taxol compounds and amongst the remaining taxanes is the same on both stationary phases. The positions at which the xylosyl taxol compounds elute within the non-xylosyl taxanes clearly depend primarily on the mobile phase composition and not on the stationary phase. The last six eluting taxanes show very regular retention behavior with virtually parallel slopes. Selectivity

within this group does not depend strongly on the ACN concentration and separation can readily be accomplished through lowering ACN concentration until sufficient resolution of the peaks is achieved. The simplicity of the separation of these six compounds was noted during the method development described in section 4.1.

The practical meaning of the different slopes amongst the first nine taxanes in the ln k' versus %ACN plots is that the selectivity of the separation changes dramatically with changing mobile phase composition. Crosspoints of two graphs indicate coeluting compounds. 10-Deacetyl-7-xylosyl taxol, for example, exchanges elution order with (or "moves" through) three different taxanes with a change in ACN concentration of just 20% as can be seen in both Figures 42 and 43. The complex crossing pattern shows that mobile phase composition has to be carefully adjusted to achieve separation for all the eight compounds in this group. 10-Deacetyl baccatin III elutes first and is well separated from all the other taxanes. On careful examination, however, it can be seen that this compound shows a steeper slope than the non-xylosyl taxanes.

A detailed discussion of the phenomenon of different slopes and changes of elution order with changing mobile phase composition will be provided in section 4.5.

Figure 42. Elution Behavior of Taxane Compounds with Changing Mobile Phase Composition

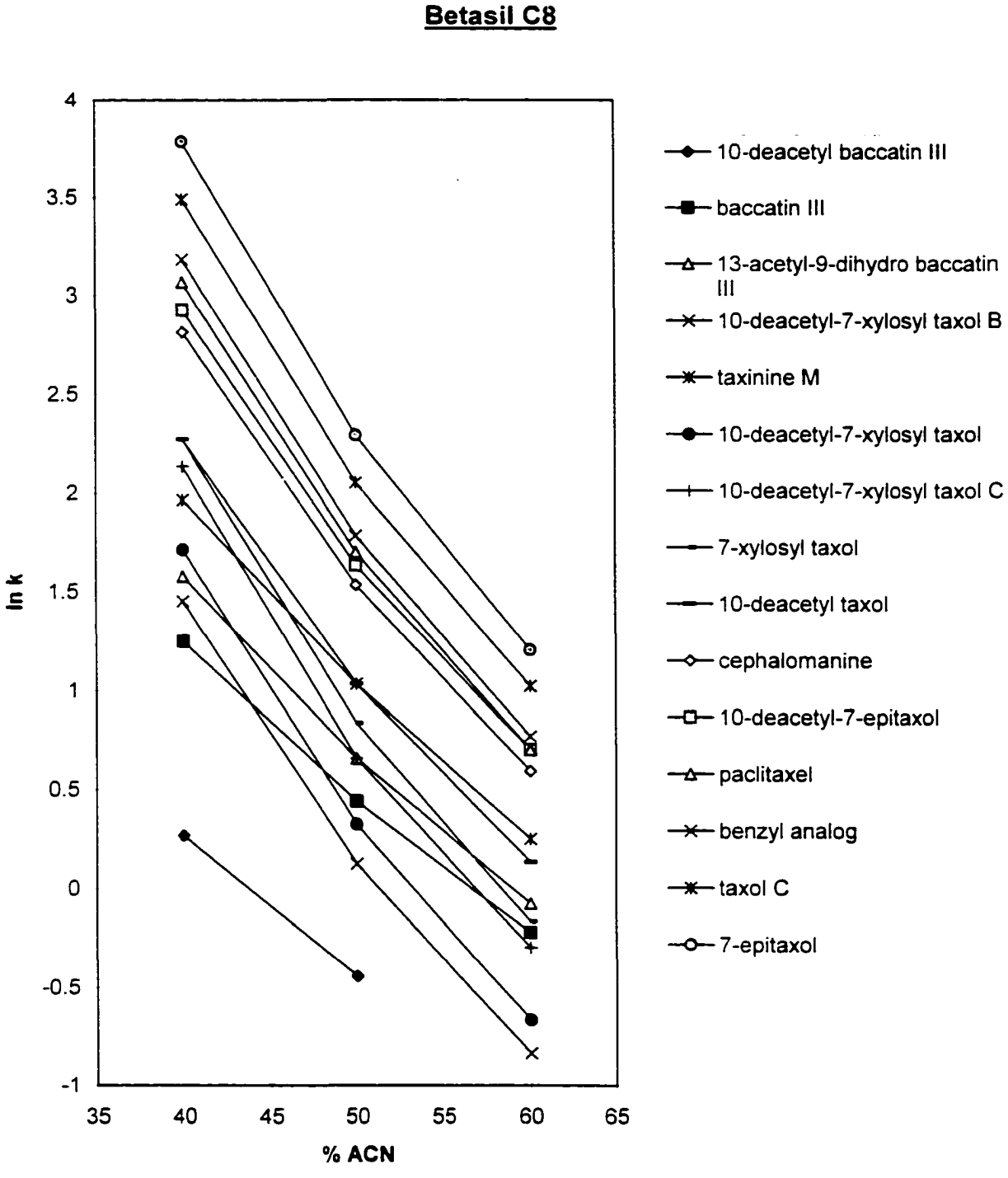
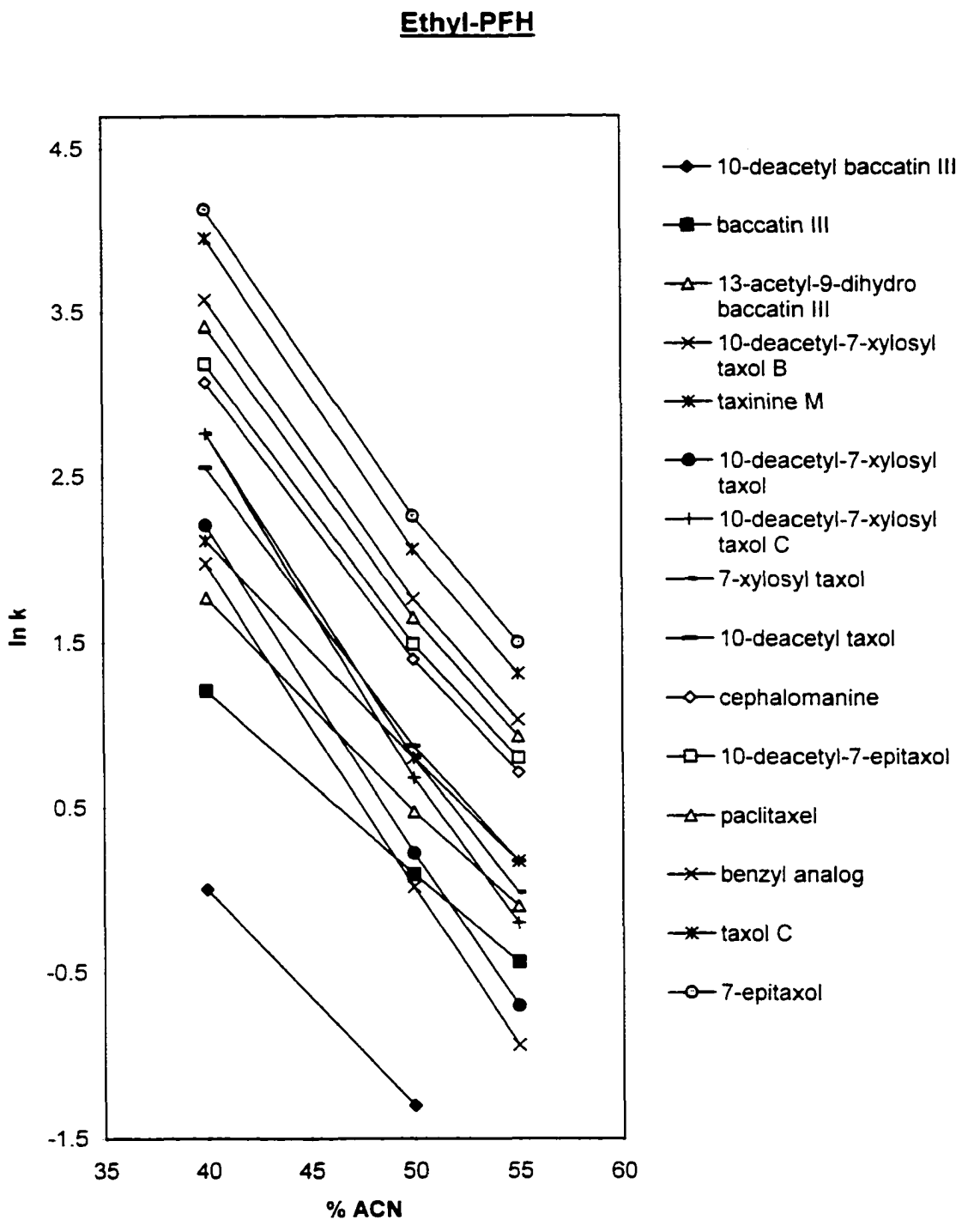


Figure 43. Elution Behavior of Taxane Compounds with Changing Mobile Phase Composition



4.4.2 Elution Behavior of Taxane Compounds with Changing Temperature

Temperature has long been a neglected factor in RPLC. Only lately it has been recognized as a secondary parameter after mobile phase composition to optimize separations [55-61]. In general, however, the effects of increased temperature are believed to be small and to result in a decrease of retention times [35] and increase in column efficiency.

During the optimization of the separation conditions for the taxane mixture discussed in section 4.1 there were strong indications that temperature had a significant effect on the resolution of the taxane compounds. This was observed primarily for taxanes #1-#9. The last six eluting taxane compounds were easy to separate and did not suggest any aberrant behavior. The elution behavior of only the first nine taxanes with respect to temperature was therefore investigated.

The retention of solutes in RPLC with respect to temperature is usually described by plots of ln k' versus the reciprocal absolute temperature. This relationship was found to be linear over moderate temperature ranges for most compounds. This graphic approach is therefore practical to describe the elution

behavior of a compound mixture for characterization and comparison of different stationary phases.

Plots of ln k' versus 1/T of the taxane separations on Betasil C8, Ethyl-PFH, Fluofix 120E and Propyl-PFP are shown in Figures 44 to 47, respectively. The experiments covered a temperature range of about 20 to 60 °C and were performed at mobile phase compositions equal or close to the ones stated for the respective columns in section 4.1 to ensure sufficient peak separation.

The results show dramatic differences of the temperature sensitivity on the various stationary phases. On Ethyl-PFH, shown in Figure 45, the temperature behavior is as generally expected. All compounds show decreasing retention times with increasing temperature in a virtually linear fashion. The graphs are roughly parallell, which means that selectivity changes only slightly with temperature. On Fluofix 120E and Propyl-PFP the results look similar but two compounds show slightly bent graphs leading to crossovers with other compounds. This means that in each of these separations there are two peak pairs that change elution order with changing temperature. A very unexpected temperature behavior was found for Betasil C8. Four of the compounds show distinct, non-linear negative slopes that become more negative with decreasing temperature. The negative slopes indicate that the retention times of these

compounds actually increase with increasing temperature. This aberrant temperature behavior results in multiple crossovers with the other taxanes, for example with 10-deacetyl-7-xylosyl taxol C, which exchanges elution order with three compounds within a temperature range of less than 25 °C. This means that in this separation, temperature has a significant effect on selectivity, comparable even to the effect of mobile phase composition. The four compounds that exhibit the aberrant temperature behavior all belong to the xylosyl taxol compounds whose common feature is the sugar group attached to the C-7 carbon as described in section 4.1. There are two more compounds that show interesting graphs on the ln k' versus 1/T plot of Betasil C8: 10-deacetyl baccatin III and 10-deacetyl taxol. Both compounds show positive slopes in the high temperature range of about 55 °C but negative slopes in the low temperature range of about 24 °C. Only one other study by Sisco and Gilpin [90] could be found that reported this kind of phenomenon and that only for the case of normal phase chromatography. In that case, however the phenomenon occurred to all solutes, not just a part of them, suggesting that in that case it was rather a stationary phase than a solute effect.

The results of this study provide very important clues as to why previous studies reported problems with the separation of taxanes on hydrocarbonaceous

stationary phases. As mentioned in section 2.1.4, none of the previous studies used temperature as a parameter to optimize the separation of taxanes. Figure 44, however, clearly shows that for a hydrocarbonaceous stationary phase temperature optimization is essential. The nine taxane compounds show no less than seven changes of elution order between 25 °C and 55 °C. Considering such strong dependence of selectivity choosing an arbitrary temperature for the separation becomes a gamble. It is unlikely that one would pick a suitable temperature just by chance. As can be seen in Figures 45 to 47 the separations on the fluorinated phases are much less sensitive and arbitrary choice of temperature is much more likely to result in sufficient selectivity of the system.

The important question at this point is what is the cause of the aberrant temperature behavior on the octyl stationary phase? In order to answer this question one has to change the way to look at the results of the temperature study. If the graphs are not compared on the basis of differences in stationary phase but differences in mobile phase, a trend seems to emerge. Ethyl-PFH with the lowest concentration of ACN has regular temperature behavior for all taxanes. At slightly higher concentration of ACN on Fluofix 120E and Propyl-PFP two of the compounds start exhibiting non-linear temperature dependencies and less positive slopes at lower temperatures. At the highest ACN

concentration used on Betasil C8 the aberrant behavior is fully developed with four compounds showing distinct negative slopes. The hypothesis is supported by the fact that the very compounds that show the most negative slopes on Betasil C8 are the ones that start showing non-linear behavior at the intermediate ACN concentration on Fluofix and Propyl-PFP. To test this theory the elution behavior of the taxane compounds with changing temperature was determined at varying mobile phase compositions. Mobile phase ranges were selected for each column on the basis of practical retention times for the late eluting compounds.

The results for Betasil C8 are shown in Figures 48 to 50. As can be seen in Figure 48 a, the temperature behavior of all taxanes is totally normal at 90% ACN. All graphs show positive slopes and are almost parallel. At 70% ACN shown in Figure 48 b, three of the four xylosyl taxol compounds show distinctly negative slopes. The fourth xylosyl taxol compound shows a negative slope only in the low temperature region. At 47.5 % ACN, shown in Figure 49 a, the aberrant temperature behavior of all the xylosyl taxanes has fully developed showing non-linear graphs with negative slopes. In Figures 49 b and 50 a, it can be seen that the aberrant temperature behavior decreases sharply with further increases in the ACN concentration to 30%. At this concentration only 10-

deacetyl baccatin III still shows a negative slope in the low temperature range, but at 27% ACN shown in Figure 50 b, this compound changes to a positive slope as well.

The results of Betasil C8 were compared with one of the chemically most different columns, the perfluorinated phenyl phase. Plots of ln k' versus 1/T for Propyl-PFP are shown in Figure 51 to 53. The results on Propyl-PFP are virtually identical to the ones described for the octyl phase above. At high and low ACN concentrations all taxanes show "regular" temperature behavior. At intermediate ACN concentrations of about 50% the xylosyl taxol group shows graphs with non-linear, negative slopes. Under this condition 10-deacetyl baccatin III also exhibits a negative slope at around 25 °C as described for Betasil C8. 10-Deacetyl taxol does not show a negative slope at the low temperature range as on Betasil C8, but it shows a less positive slope than the other taxanes with "regular" behavior.

Another effect that is interesting is that the xylosyl taxol group strongly changes its retention relative to the other taxanes with changing mobile phase composition. At 90% ACN all xylosyl taxols elute before any other compound in the separation as can be seen in Figures 48 a and 51 a. At 30% ACN, however, the xylosyl taxol group elutes at the end of the separation in an overlap

with the last two eluting taxanes. This observation agrees with the results of the mobile phase composition dependence discussed in section 4.4.1 where it was found that the xylosyl taxol group shows lower retention at low %ACN and higher retention at high %ACN as compared with the other taxane compounds.

The temperature study was repeated on Ethyl-PFH for comparison. Plots of ln k' versus 1/T at varying mobile phase compositions are shown in Figures 54 to 56. The results are similar to the ones described above with "regular" temperature behavior at low and high concentrations of ACN and aberrant behavior at about 50% ACN. But the extent of the aberrant behavior on Ethyl-PFH is decreased as compared to the other two stationary phases. Only two xylosyl taxol compounds show obvious non-linear graphs at 43% ACN and only one of them has a negative slope in the low temperature range. Both these compounds are identical to the ones that show the most aberrant temperature behavior on the other two columns. A likely reason for the more modest aberrant temperature behavior on Ethyl-PFH is the considerably lower retentivity of this stationary phase as compared with the other two. Betasil C8 and Propyl-PFP both retain compounds much more strongly than Ethyl-PFH, which is expressed by the higher ln k' values of the compounds at comparable mobile phase compositions. All the results indicate that the aberrant temperature

behavior decreases strongly with increasing concentration of ACN, meaning with decreased retention. It seems reasonable then to assume that if retention is decreased by the retentivity of the stationary phase the aberrant temperature behavior should be less pronounced, too. This can also be considered with respect to the retention mechanism. The aberrant temperature behavior is likely caused by an effect interfering with the penetration of the solute into the bonded phase layer at lower temperatures. Low retention signals that the solute does not penetrate the stationary phase effectively, therefore any effect interfering with this process should be less pronounced, as well.

The similarity of the results on the hydrocarbonacous, aliphatic Betasil C8 and the aromatic, fluorinated Propyl PFP strongly suggests that the observed aberrant temperature behavior is not a stationary phase effect but rather a solute or mobile phase effect.

An important point of the above results is that the compounds showing the aberrant temperature behavior are the same ones that showed the more negative slopes in the mobile phase composition study in section 4.4.1. The xylosyl taxol group shows the most pronounced effects in both studies while 10-deacetyl taxol and 10-deacetyl baccatin III show more modest differences in elution behavior as compared to the other taxanes. This similarity suggests that

the chemical reason for the different behavior of these compounds with changes in mobile phase composition and temperature is the same. Poole et al. [91] also reported similar correlations in changes of selectivity with variations in temperature and mobile phase compositions, but did not offer any explanations to the mechanism relating the two parameters. A detailed discussion of the possible mechanism of the aberrant behavior and the resulting changes in elution order will be provided in section 4.5.

Figure 44. Elution Behavior of Taxane Compounds with Changing Temperature

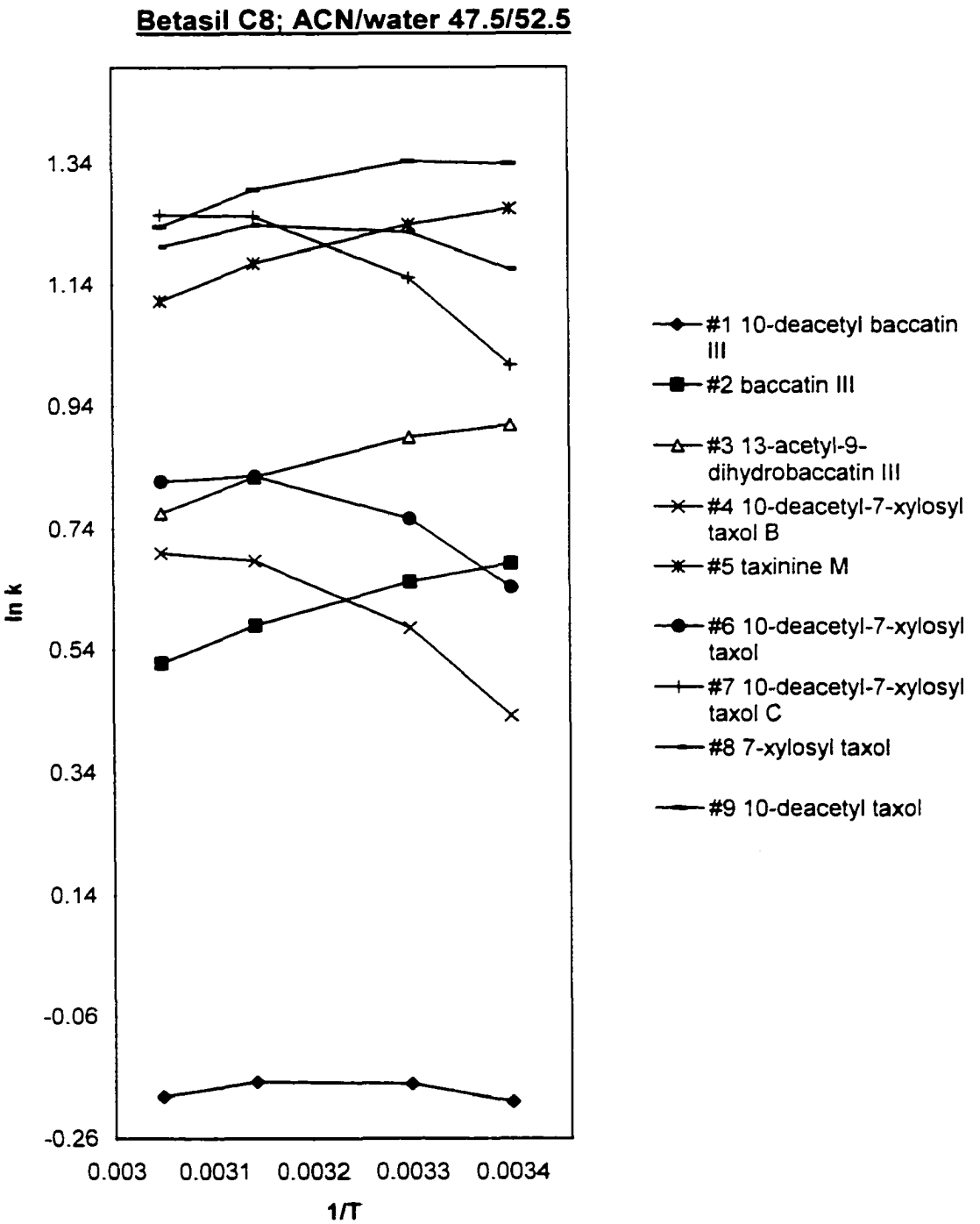


Figure 45. Elution Behavior of Tanxane Compounds with Changing Temperature

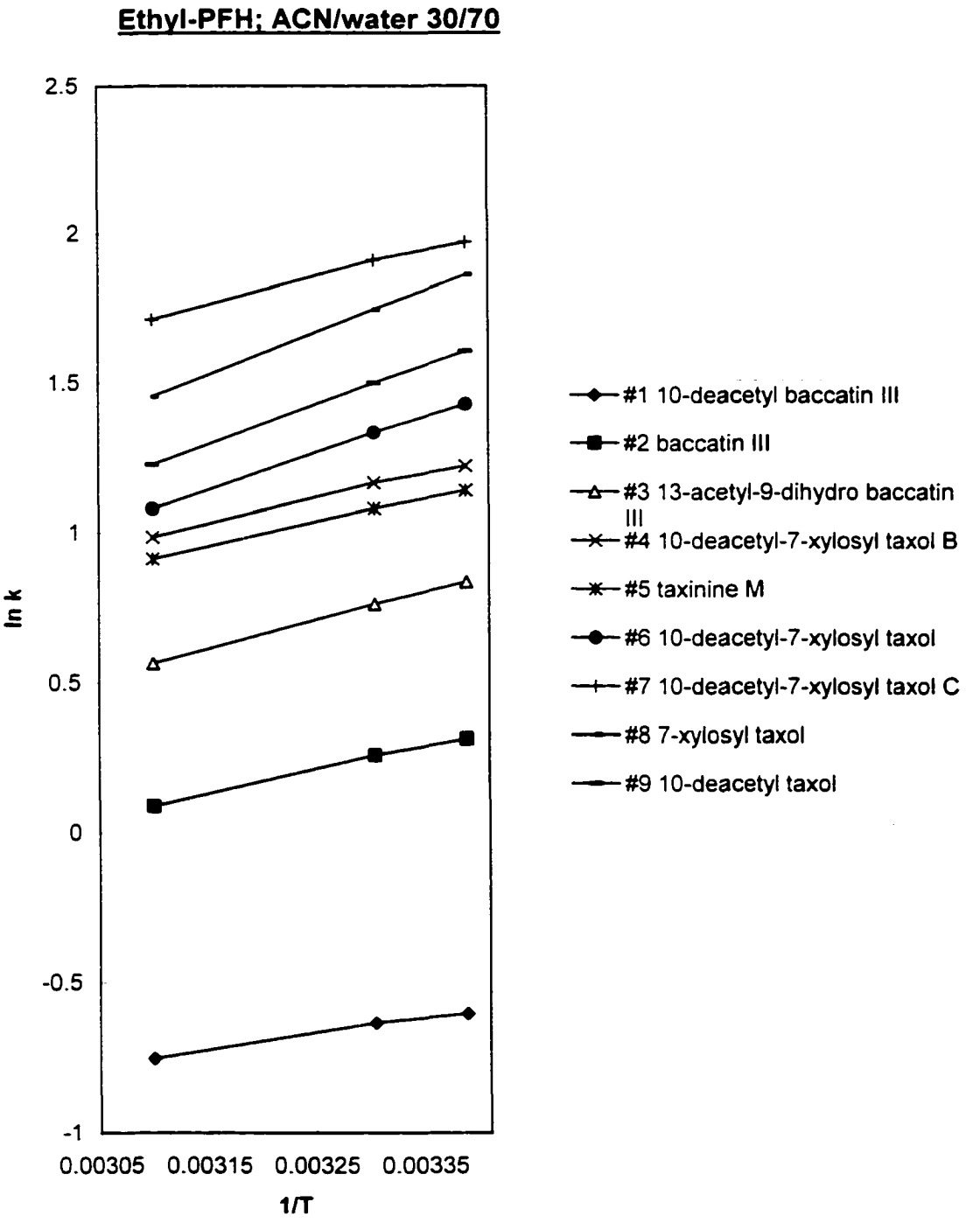


Figure 46. Elution Behavior of Taxane Compounds with Changing Temperature

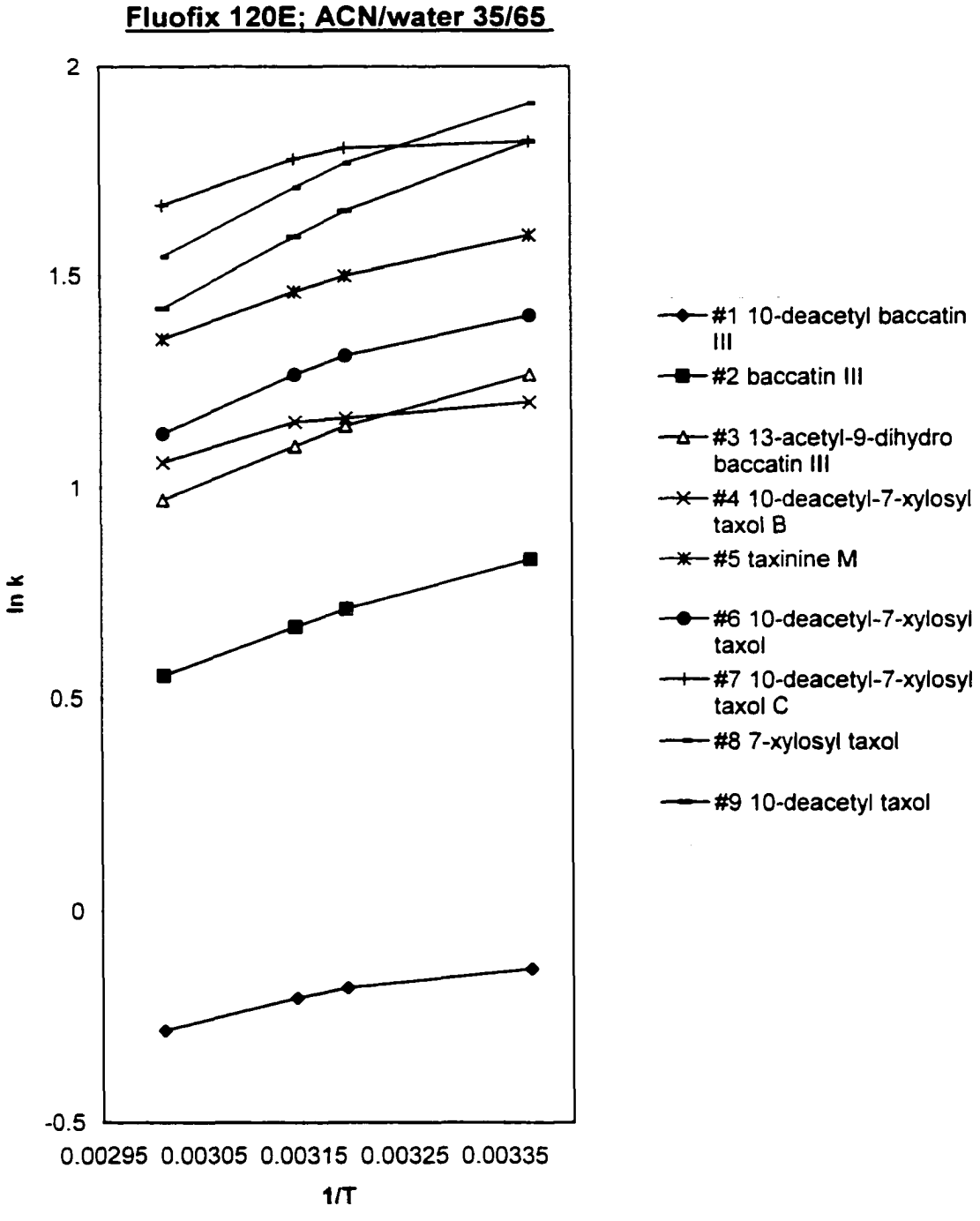


Figure 47. Elution Behavior of Taxane Compounds with Changing Temperature

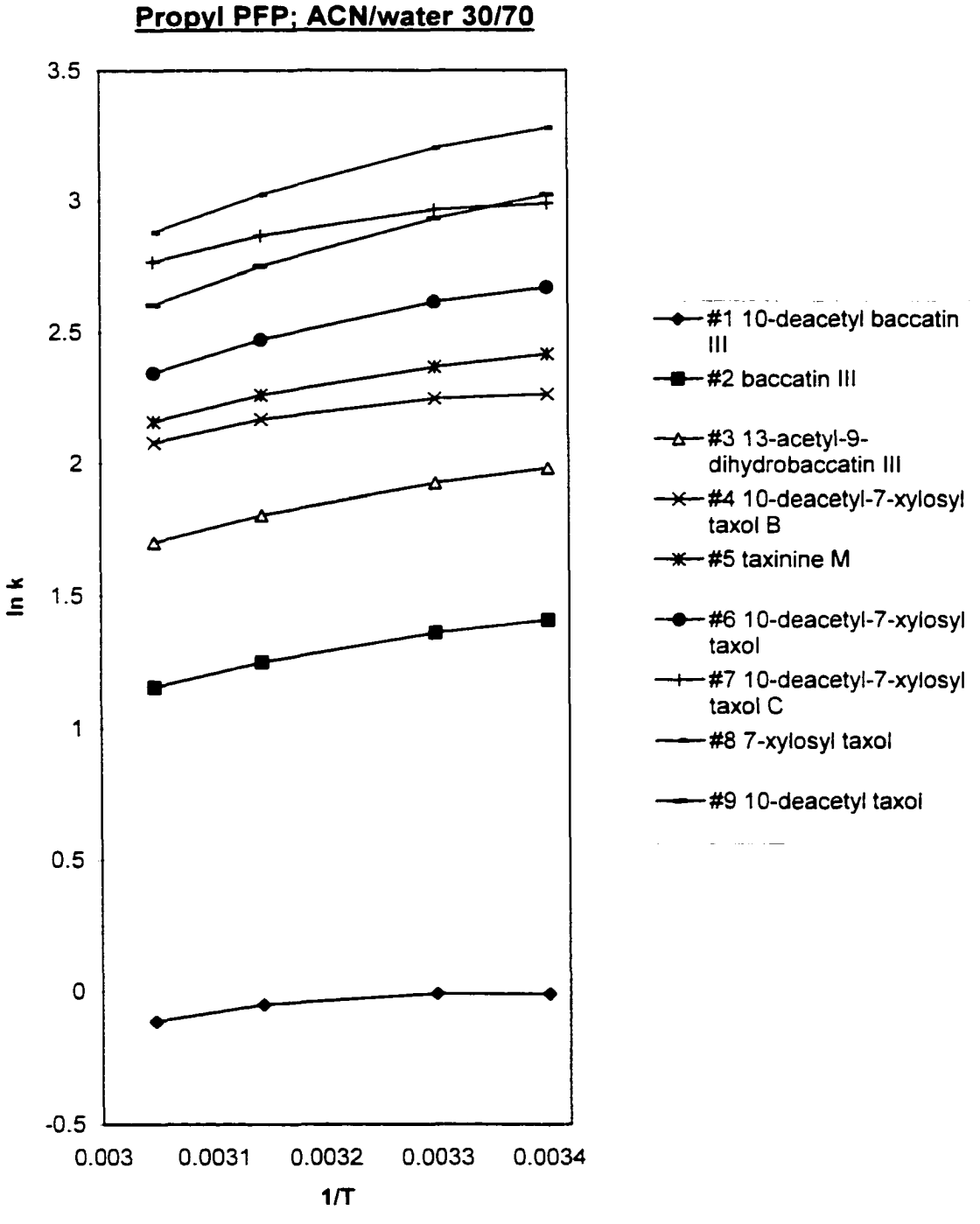


Figure 48. Temperature Behavior of Taxanes at Varying Mobile Phase Composition

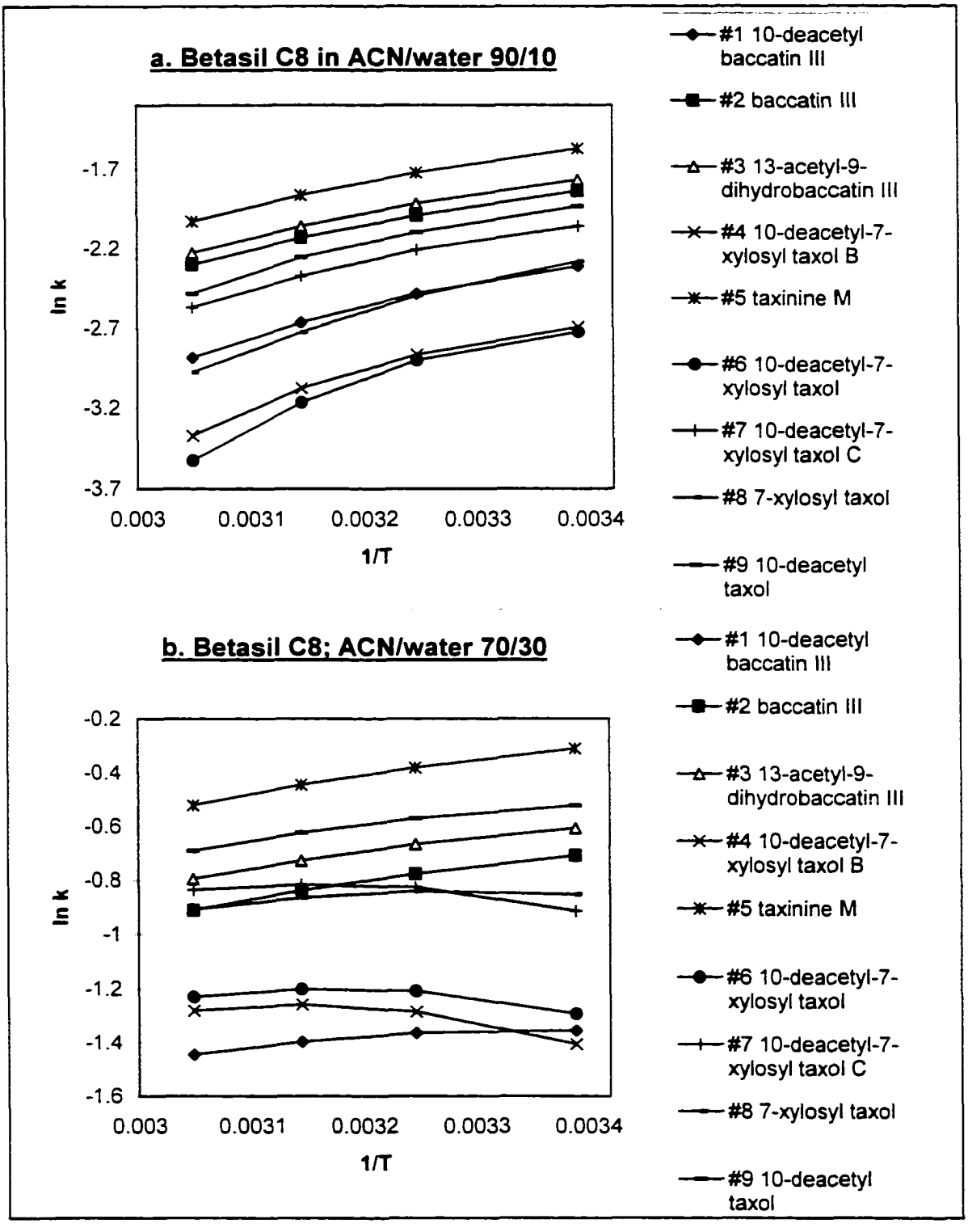


Figure 49. Temperature Behavior of Taxanes at Varying Mobile Phase Composition

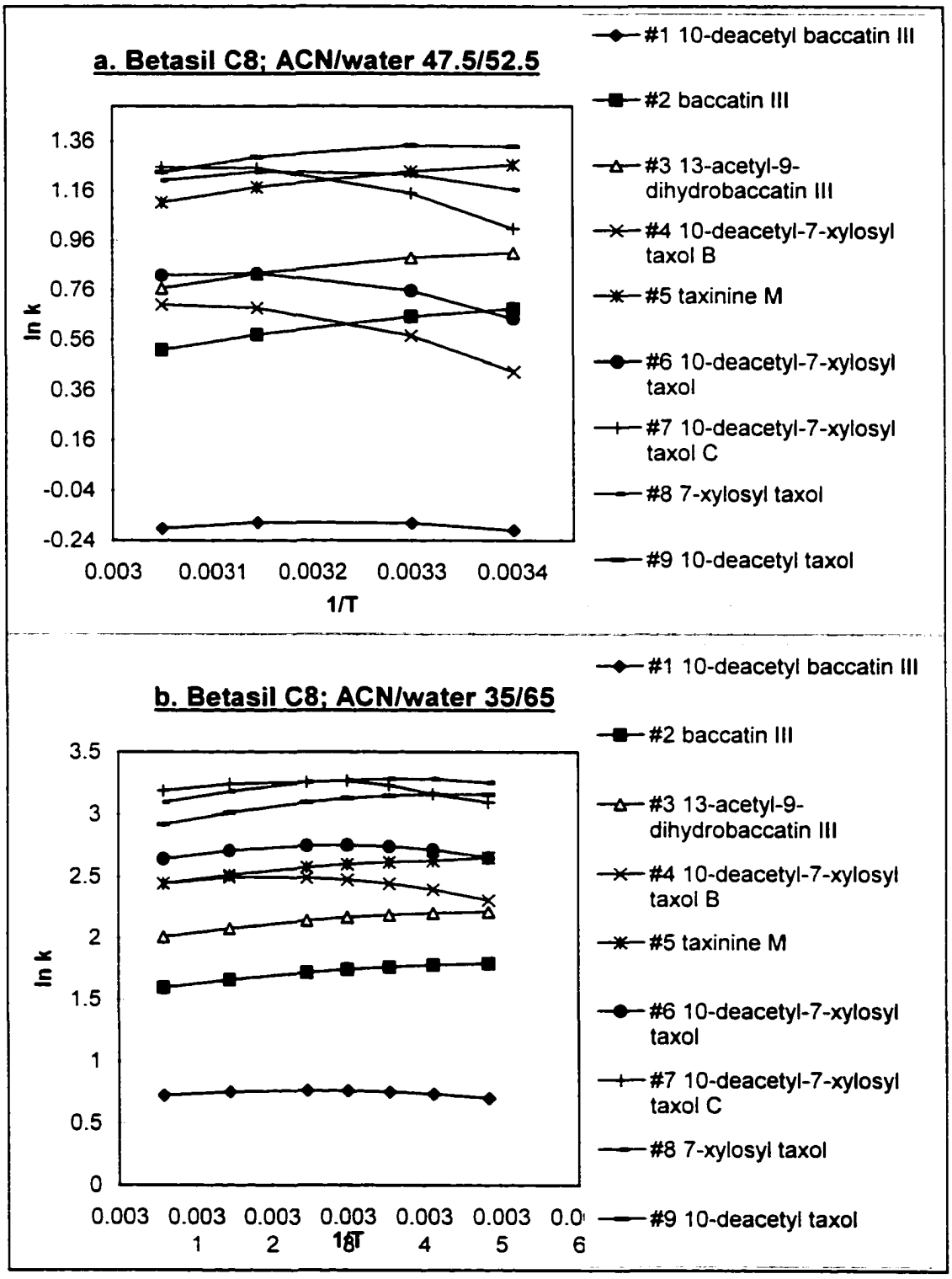


Figure 50. Temperature Behavior of Taxanes at Varying Mobile Phase Composition

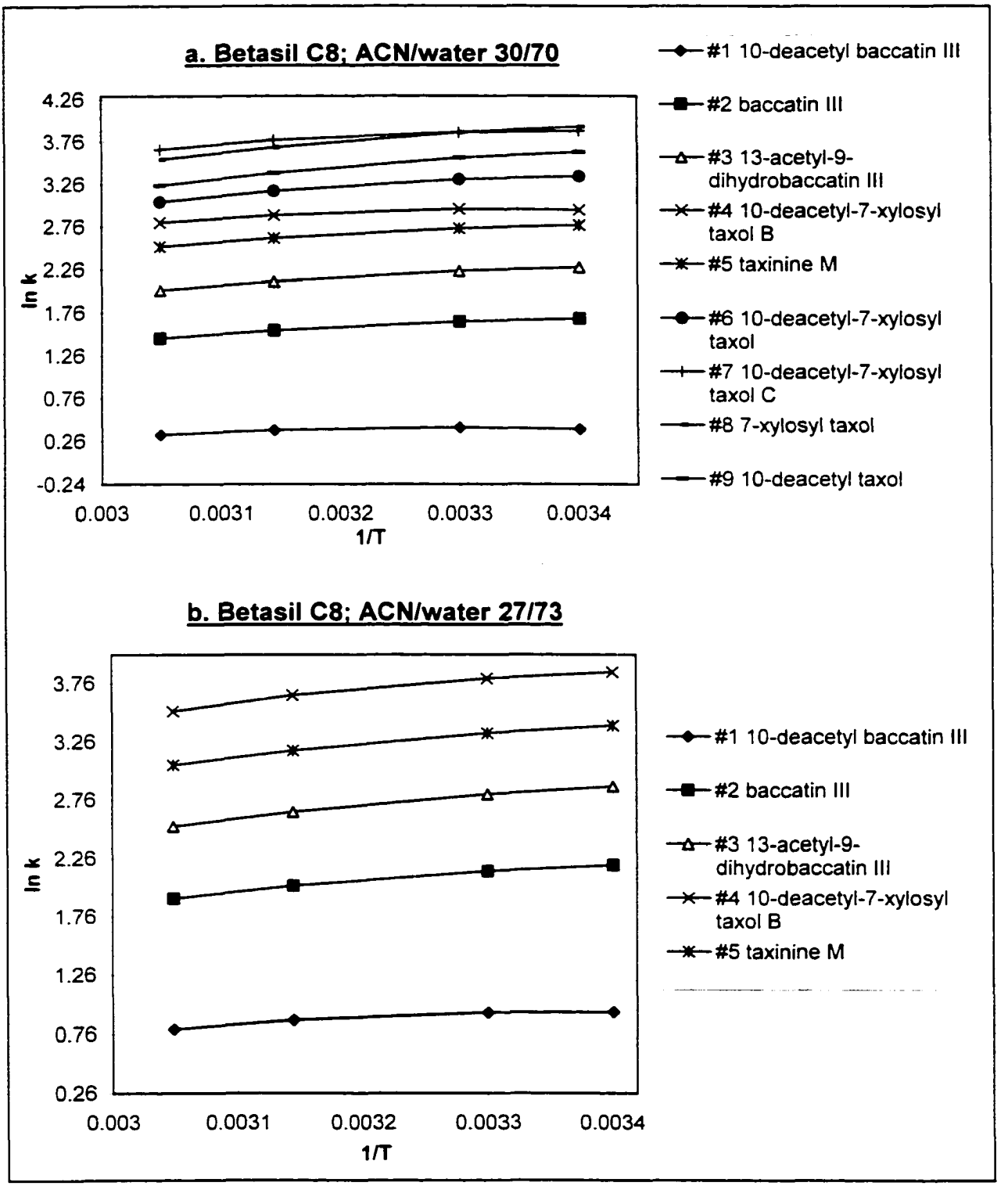


Figure 51. Temperature Behavior of Taxanes at Varying Mobile Phase Composition

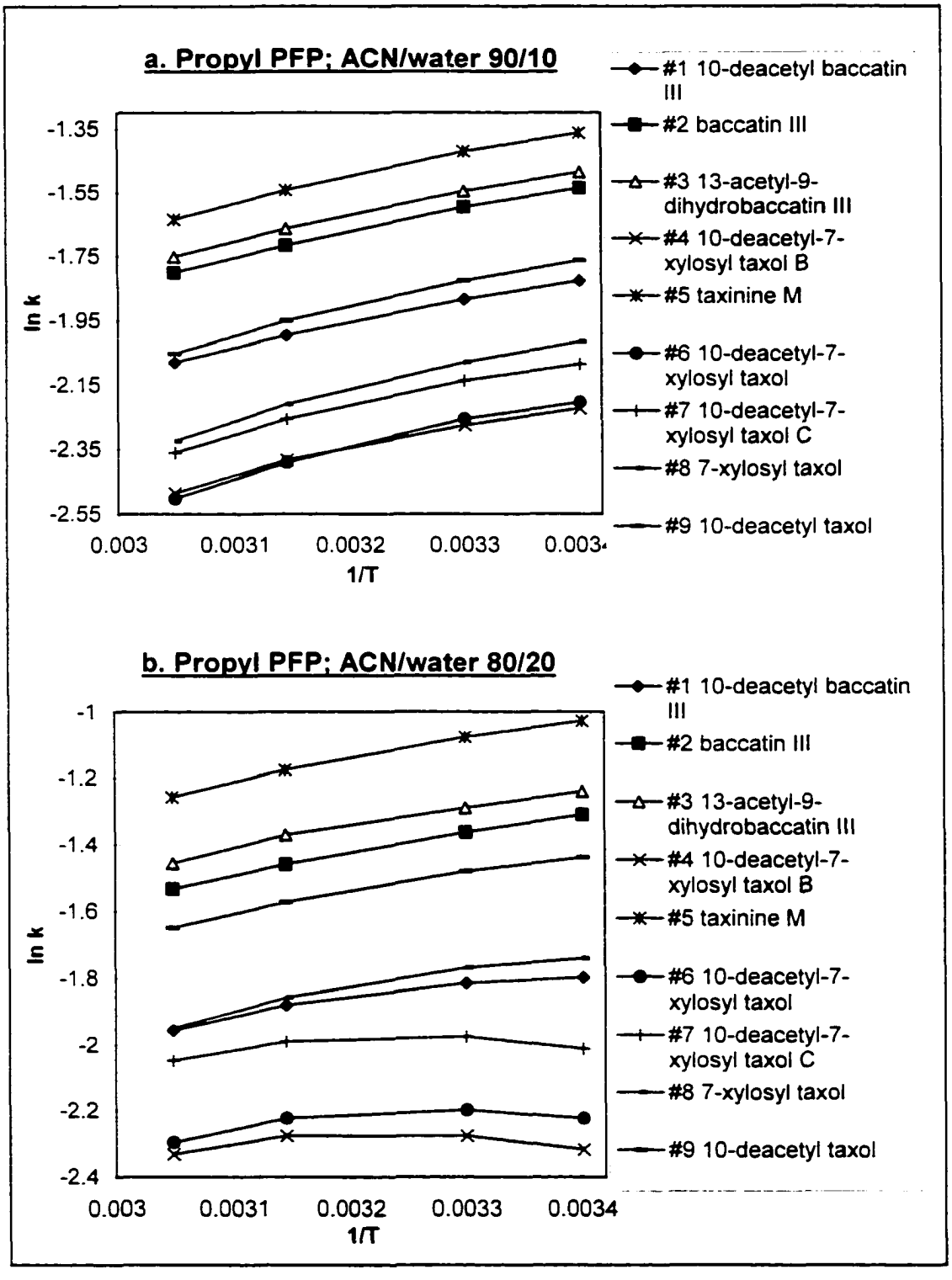


Figure 52. Temperature Behavior of Taxanes at Varying Mobile Phase Composition

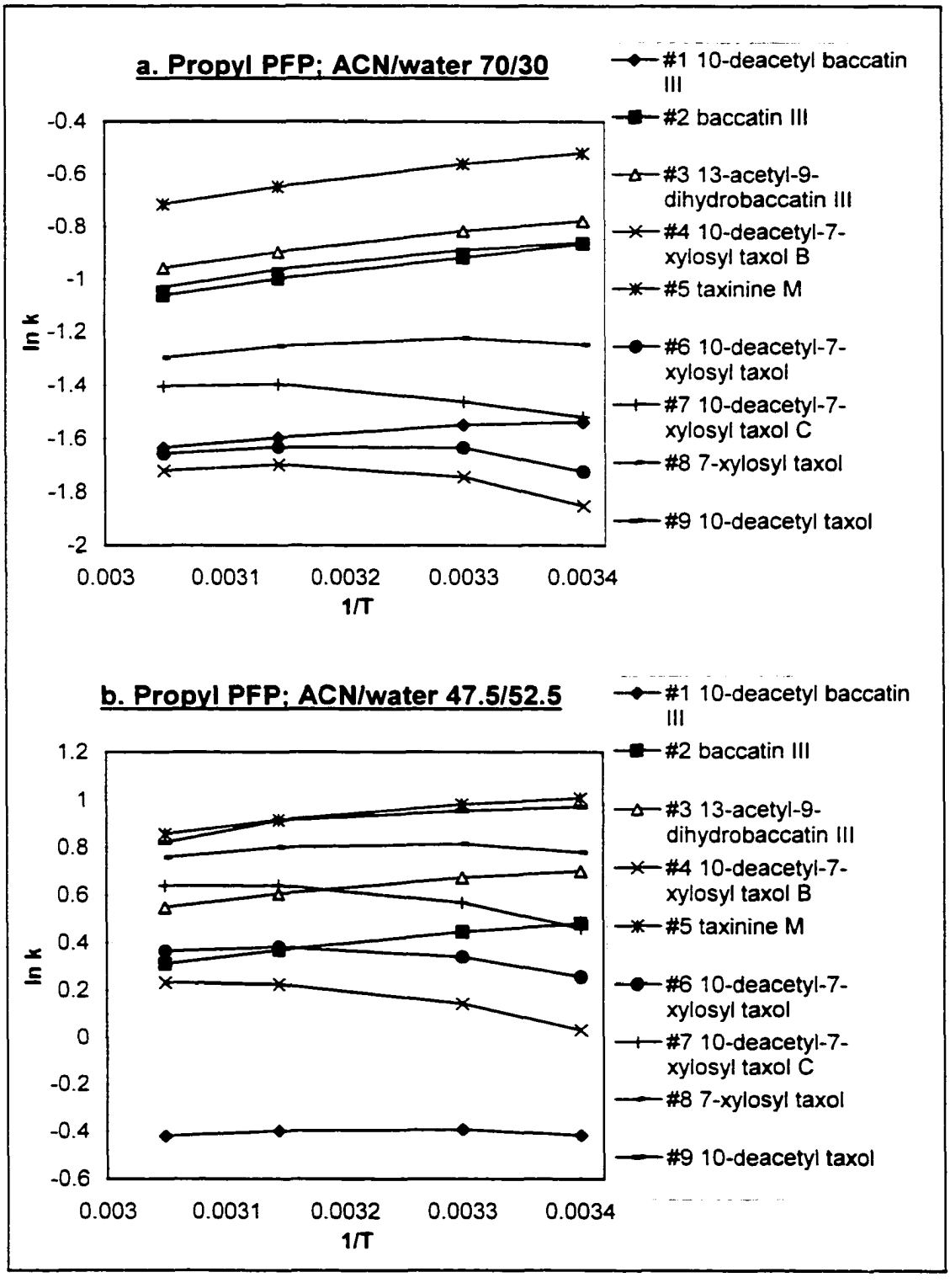


Figure 53. Temperature Behavior of Taxanes at Varying Mobile Phase Composition

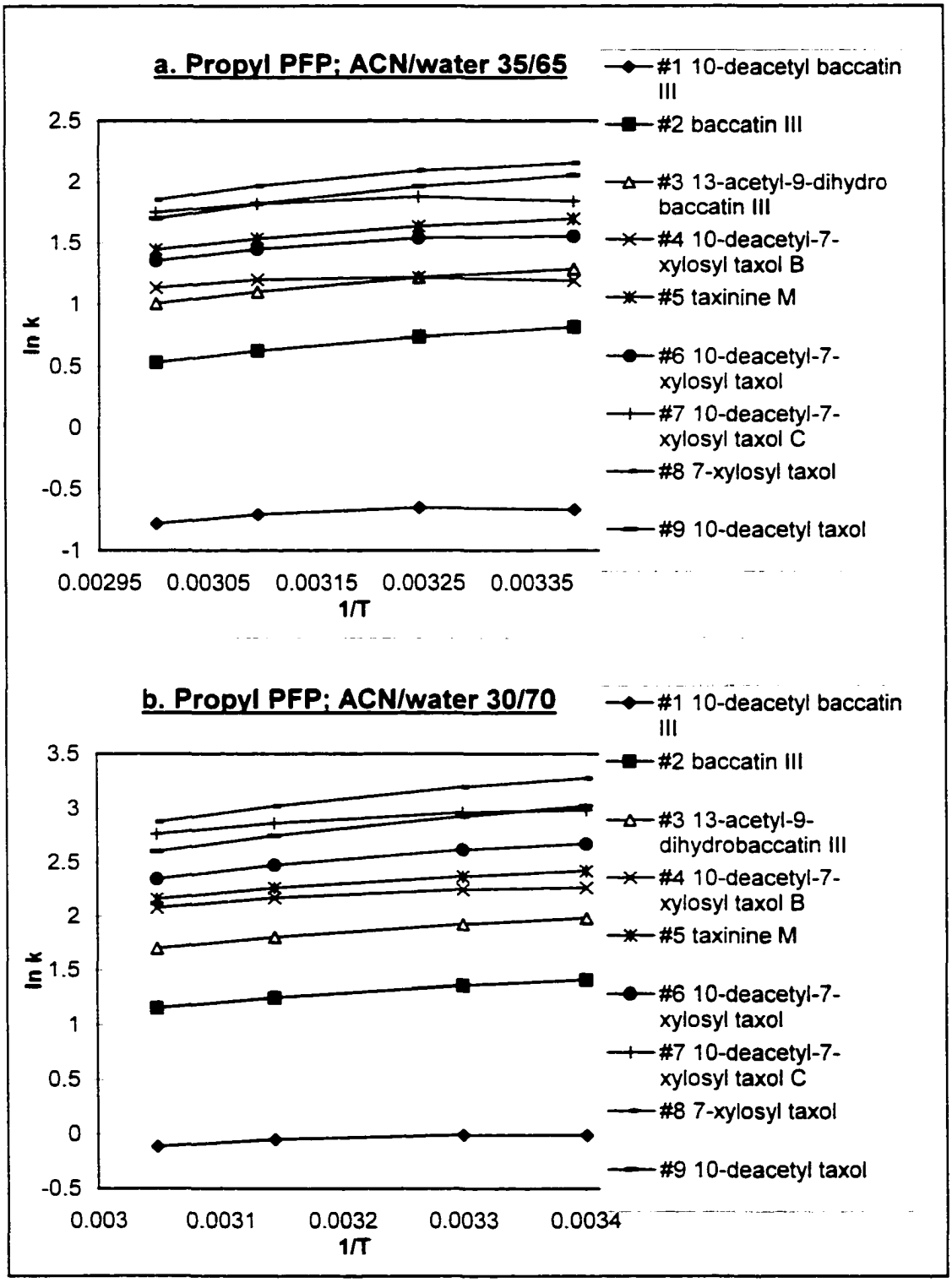


Figure 54. Temperature Behavior of Taxanes at Varying Mobile Phase Composition

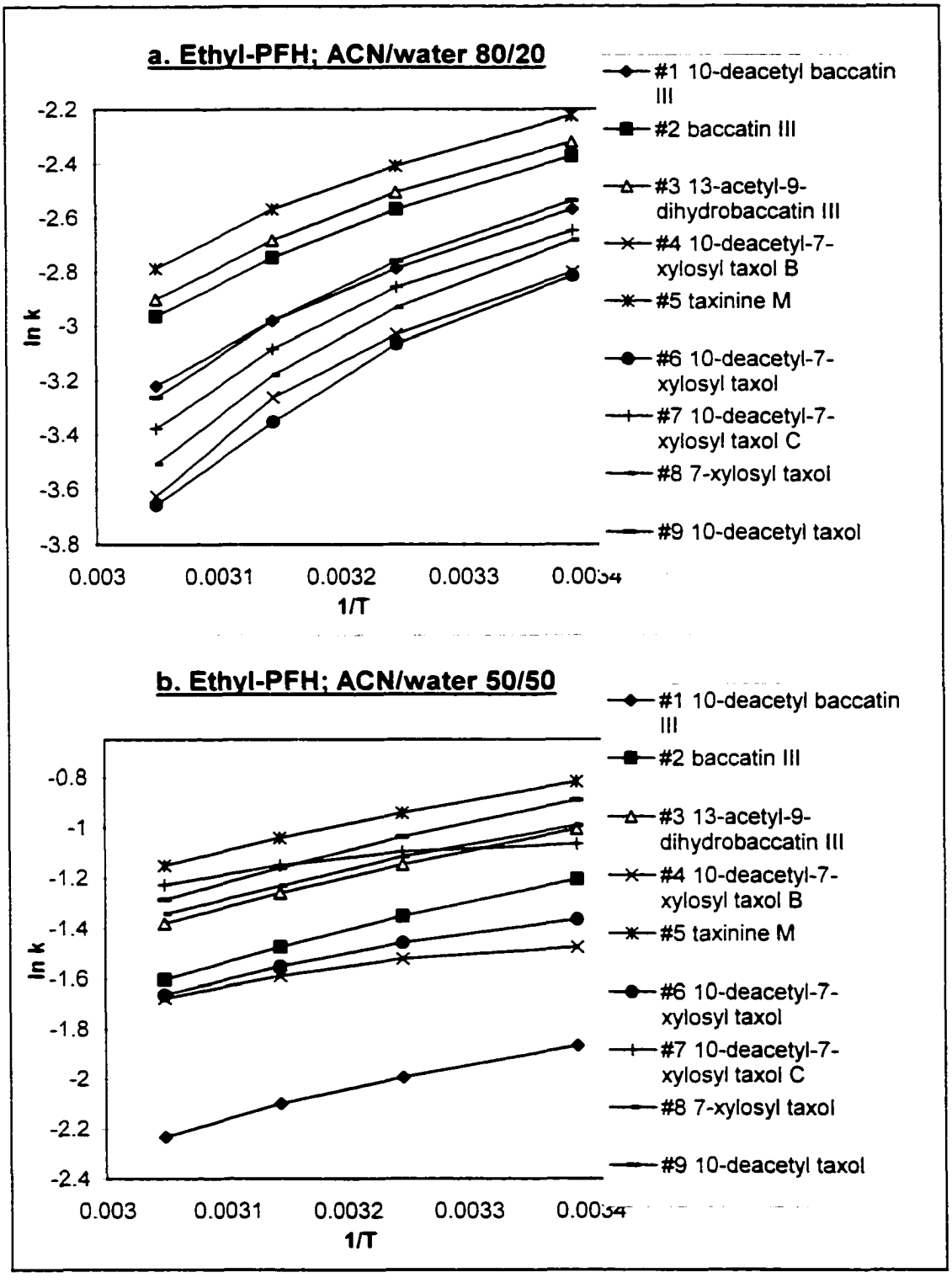


Figure 55. Temperature Behavior of Taxanes at Varying Mobile Phase Composition

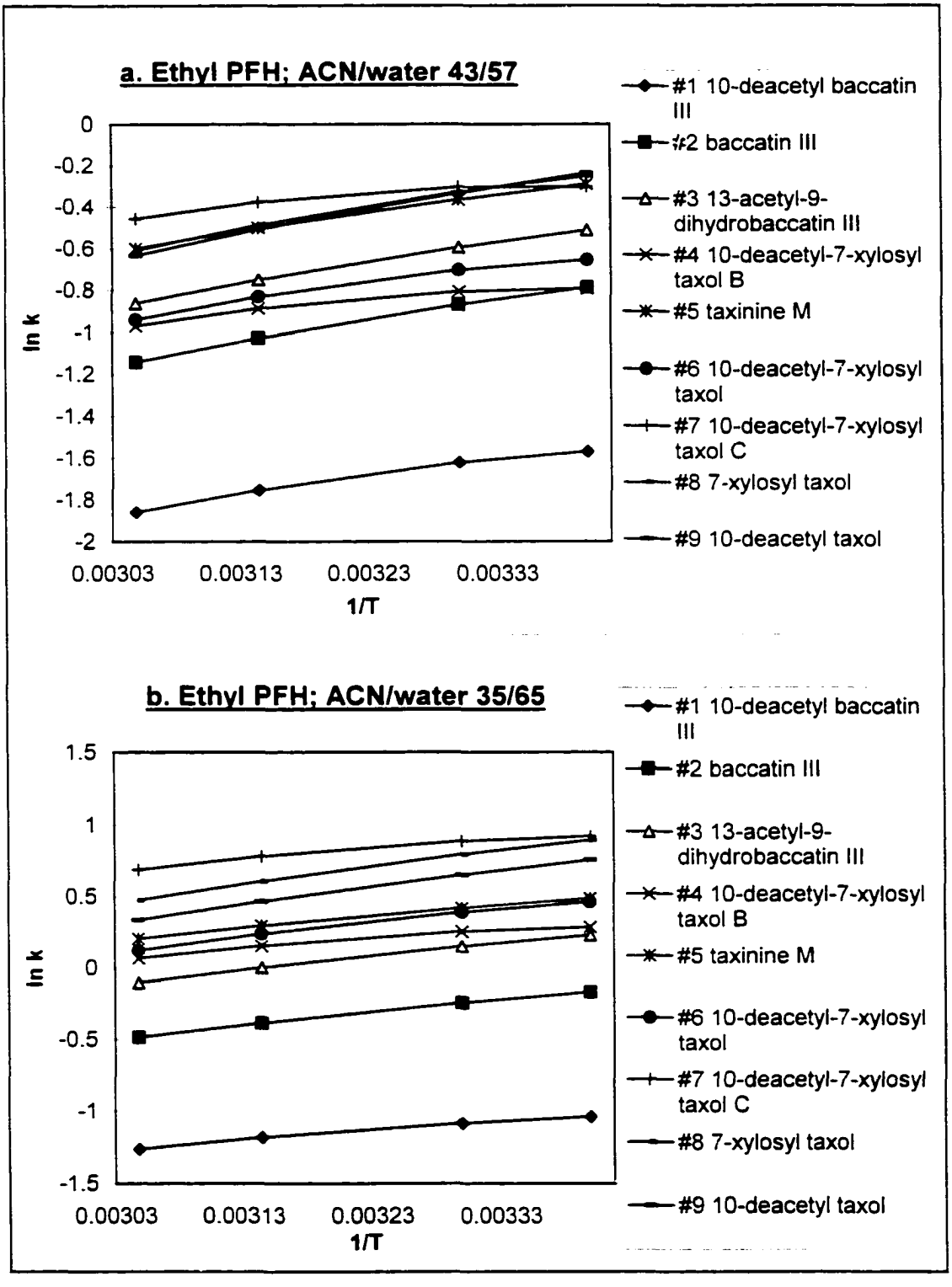
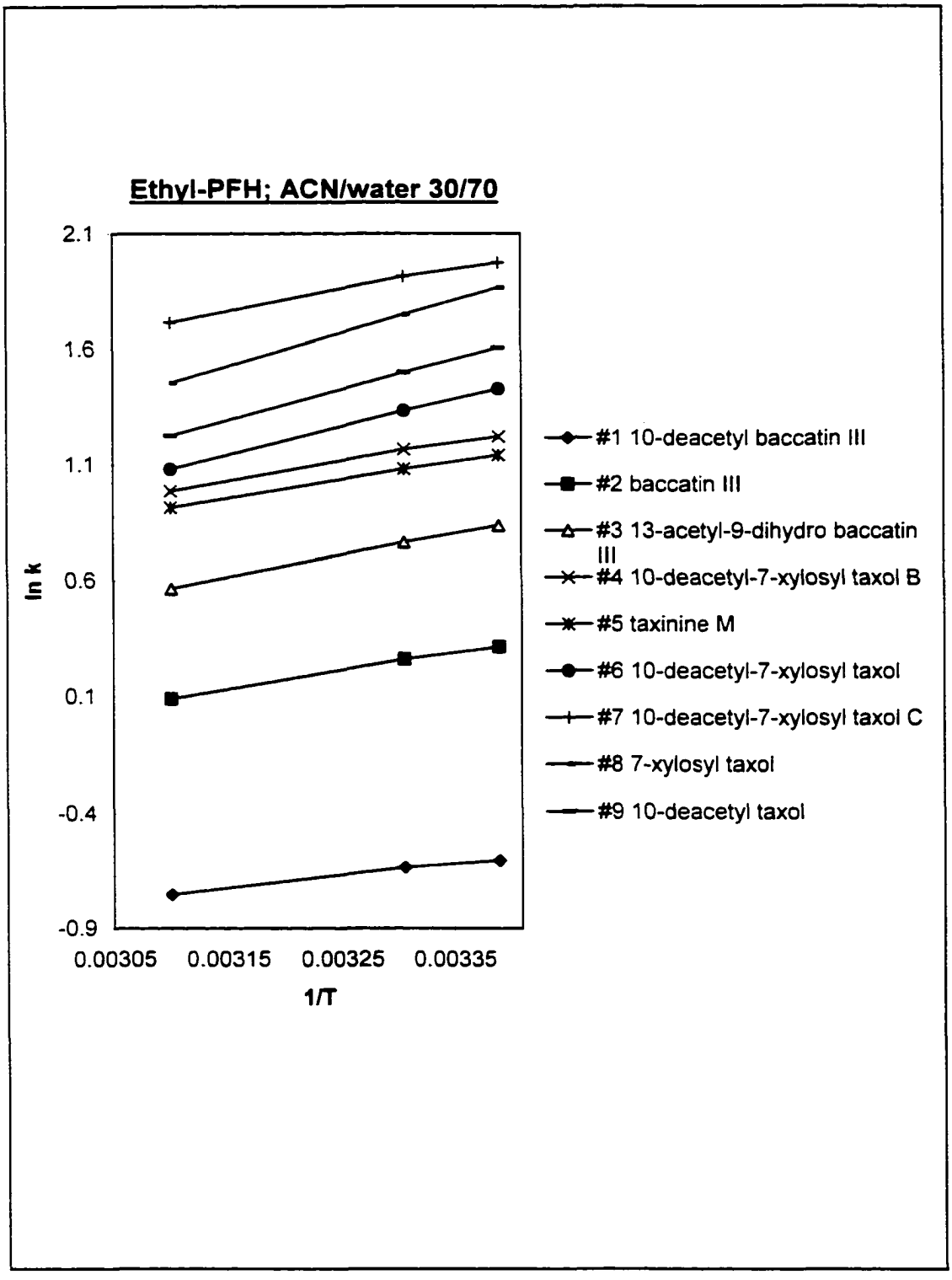


Figure 56. Temperature Behavior of Taxanes at Varying Mobil Phase Composition



4.5 Mechanism of Elution Order Changes with Variation of Mobile Phase Composition and Temperature

It has been known for a long time that changes in mobile phase composition, such as variations of organic modifier concentration or use of different organic modifiers, can result in selectivity changes or change of elution order. Most books about RPLC mention this fact [33,35,54] and many studies have investigated the use of mobile phase composition to optimize separations. In recent years it has been found that temperature can be used as a parameter to affect selectivity as well, but its influence was believed to be small [55-61]. Very few investigations reported changes of elution order with variations in temperature [102]. Obviously, many studies have investigated the effect of mobile phase composition and temperature. In fact, the vast majority of work deals with changes of elution order in one way or another. It is hard to understand, therefore, that despite very thorough literature research not even a suggested mechanism could be found explaining the change of elution order in RPLC. At most the phenomenon is described, but no explanations are ever provided. Studies on the effect of temperature on retention use Van't Hoff Plots to attribute the change in selectivity to variations in the enthalpy of solute

transfer from the mobile to the stationary phase as described in section 4.4.3. But this way of argumentation omits the real question: what are the chemical processes that cause the variations in the enthalpy of solute transfer? There are a multitude of chemical processes in RPLC that are influenced by temperature and mobile phase composition including:

1. phase changes of the stationary phase
2. formation of dimers, clusters and aqueous-organic complexes
3. adsorption effects between solute and bonded phase
4. adsorption effects between solute and residual silanols
5. preferential solvation of the stationary phase (bonded phase and/or residual silanols) with any of the mobile phase species described in 2.
6. preferential solvation of the solute with any of the mobile phase species described in 2.

While points 1 to 5 have been widely explored and used to explain retention in RPLC point 6 has been virtually ignored. This is surprising since point 6 should have a considerable effect on selectivity. While the effects in points 1 to 5 act on all solutes, preferential solvation of the solute is specific to each compound

separated by RPLC. This study will try to find evidence to support the theory that preferential solute solvation is a major cause of the change of elution order with variation of mobile phase composition and temperature.

4.5.1 Preferential Solute Solvation

Whenever a chemical compound is dissolved in a binary solvent mixture, the compound will preferentially be solvated with the chemically more compatible component of the solvent. The preferential solvation of the entire solute might not be homogenous due to local solvation effects of functional groups on the dissolved compound. This phenomenon has been explored by physical chemists since the mid 1960's [92-97]. Most of these studies present theoretical, mathematical models [92-97] describing the change in preferential solvation with changes in solvent composition [92-93,95-96] and temperature [92], some of them supporting their theories with experimental evidence.

There is surprisingly little literature on the effect of solute solvation in liquid chromatography. Very few research publications can be found and books on RPLC seem to all but ignore the topic. Berek et al. attributed ghost peaks in gel permeation chromatography to mobile phase concentration disturbances caused by preferential solvation of the dissolved polymer [98]. Jaroniec et al. did extensive work on developing thermodynamic models of the effect of solute solvation in liquid chromatography [76-78, 99] but the studies lack experimental evidence and conceptional treatment of the topic. Guillaume et al. found

evidence for the solvation of ionizable solutes with ACN clusters in RPLC [100], through comparison of a thermodynamic model with experimental data. Boehm and Martire used a mathematical model called the Bethe-Guggenheim approach to include solute solvation in the theoretical retention in RPLC and claim a better correlation with experimental data than models that omit solute solvation [74].

4.5.2 Effect of Solute Solvation in RPLC

A possible reason why this research has not been picked up by main stream chromatography is that each of the studies fails to supply a conceptional description of solute solvation and its effect on solute retention in RPLC. To approach this problem it is necessary to go back to the most fundamental reasoning about retention. According to the fundamental understanding of the reversed phase mechanism, more polar compounds elute before less polar ones. Countless studies of homologous series and compounds with various functional groups support this theory. This means that if two compounds change elution order they also change their relative polarity. In other words, one of the compounds either becomes more polar or the other one becomes less polar, or both. This seems to be impossible since solutes are not known, and are not supposed to undergo chemical reactions during the chromatographic process. Obviously, to make this theory work a new picture of the solute species is needed, which allows for changing polarity with changing mobile phase composition and temperature. A possible description of such a species is a solute covered by a solvation shell. This species is a complex consisting of the solute compound and a solvation shell consisting of mobile phase components

bound by hydrogen bonds, dipole-dipole interaction, etc. Changes in mobile phase composition and/or temperature will affect the total and/or relative amounts of mobile phase components in the solvation shell as proven by the research on preferential solvation described above. This in turn will change the polarity of the solute-solvent complex resulting in changes in retention. In theory this explanation looks very reasonable and is suitable to explain changes of elution order without dropping basic perceptions about RPLC. There is, however, one fundamental question to make this theory work: "Is the solvation shell stable enough to be retained throughout the retention process or does it disintegrate during adsorption or partition". In a study about solute-solvent interactions on the surface of silica gel, Scott and Kucera found that hydrogen bonded surface solvation is more stable than solvation formed by other intermolecular forces [101]. The adsorption of non-hydrogen bonding species does not result in displacement of the hydrogen bonded solvation layer on the surface of silica gel. Only solutes that can engage in hydrogen bonding, will displace the solvation layer on the stationary phase [101]. These results can be used directly to form a theory about the dynamics of solvation in RPLC. If the solute can engage in hydrogen bonding it will solvate with the water component of the mobile phase. According to the results from Scott and Kucera this

solvation shell will remain on the solute throughout the retention process since there are no hydrogen bonding groups present or accessible (supposedly) on the stationary phases in RPLC. The only exception is amino phases, but they are rarely used and were therefore not considered in this study. The solvation of the non-polar part of the solute can be expected to negligible in its effect on retention since it is likely to disintegrate during partitioning of the solute into the non-polar bonded phase. One can also reasonably assume that the solvation of the solute with water is an exothermic process. A schematic description of the solvation process and its effect on RPLC retention is shown in Figure 57.

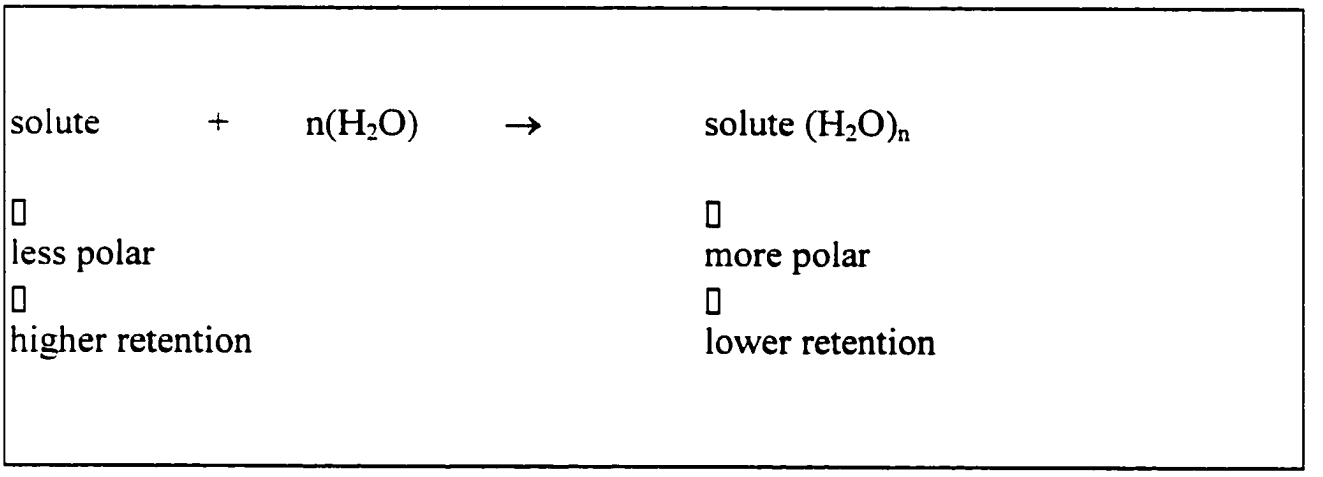


Figure 57. Theoretical Effect of Solute Solvation on Retention in RPLC

4.5.2.1 Retention of Hydroxyl Compounds at Varying Mobile Phase Composition

The theory suggested in section 4.5.2 implies an interesting effect. The retention of a solute in RPLC is normally expected to increase as the amount of water in the mobile phase is increased. According to the scheme above, however, the retention of a solute able to engage in hydrogen bonding (preferentially with water versus organic modifier) should initially decrease from 0% water to >0% water due to the onset of solute solvation. To test this hypothesis, the retention of the first nine taxane compounds was investigated at mobile phase compositions at and close to 100%ACN. The results are shown in Figure 58 a. The results support the scheme in Figure 57. The taxane compounds show initial decreases of retention time with increasing % water in the mobile phase to varying degrees. Comparison of the extent of initial decrease of retention with the molecular structure of the taxanes leads to the following correlation. The larger the number of hydroxyl groups with respect to the total size of the structure, the more extensive the initial decrease of retention time. The xylosyl taxols with the hydroxyl-rich sugar group have a total of 5 or 6 hydroxyl groups and show the strongest effect. Within this group

the deacetyl xylosyl taxols show the most pronounced decrease of retention due to the additional hydroxyl group provided by the deacetylation of the C-10 carbon. Comparable to this group is 10-deacetyl baccatin III. This taxane does not contain the sugar group, but it is overall more polar due to the missing C-13 side chain. This probably accentuates the effect of the additional hydroxyl group provided by the deacetylation of the C-10 carbon. Taxinine M shows almost linear behavior, which agrees with the trend since it has only two hydroxyl groups on the relatively large non-polar structure.

According to the scheme in Figure 57, the extent of solvation and the corresponding extent of initial decrease of retention should be lower at higher temperatures due to the exothermic nature of the solvation process. To test this hypothesis the experiment was repeated at 55 °C. The results are shown in Figure 58 b. The initial decrease of retention is clearly reduced at the higher temperature. The concentration of water in the mobile phase at minimum retention has decreased about 5% as compared with the results at 21 °C indicating a reduced solvation with water. At 21 °C, for example, 10-deacetyl-7-xylosyl taxol B has its lowest retention time at 85% ACN but at 55 °C the point of lowest retention has shifted to 90% ACN.

There are data points that fall out of the trend of the graphs in Figures 58

a and b, especially at mobile phase compositions close to 100% ACN. These apparent jumps in retention time are probably caused by impurities in the taxane standards that start coeluting with the taxane peaks and lead to shifts in the peak maxima.

To ensure that the observed phenomena are not caused or severely affected by the chemistry of the bonded phase, the experiment was repeated with Propyl-PFP at 21 °C, shown in Figure 59. The results are comparable to Betasil C8. The excessively high retention time of the xylosyl taxol compounds at 100 % ACN is most likely caused by a coeluting impurity.

To test the hypothesis that the initial decrease of retention is caused by the hydroxyl groups, the study was repeated with benzene and several hydroxylated derivatives. The results, shown in Figure 60, clearly show the direct correlation of the number of hydroxyl groups and the initial decrease of retention as observed with the taxane compounds. Especially interesting are the results for 1,2-dihydroxy benzene and 1,3-dihydroxy benzene. The spatial distribution of the hydroxyl groups on the compound is obviously of importance. 1,3-Dihydroxy benzene does not only show an overall lower retention but also a stronger initial decrease of retention. This suggests that more even distribution of the hydroxyl groups on the structure leads to lower retention

and possibly more effective solvation. The lower retention is probably caused by the fact that effective penetration into the bonded phase is hindered more effectively if the hydroxyl groups are spread over a wide range of the compound. If they are concentrated to one end of the compound the remaining non-polar part of the molecule would be comparably larger and can more deeply penetrate into the bonded phase resulting in better retention. Additionally, it seems reasonable to assume that solvation of hydroxyl groups that are further apart from one another should be more effective due to a lack of possible steric interference of the respective solvation shells.

The decrease of retention for hydrophilic solutes at high ACN concentration has been reported before for crown ethers [103] and amino acids [104]. Those studies attributed the phenomenon to normal phase behavior caused by residual silanol groups on the stationary phase. This interpretation, however, is contradicted by several studies. Kazachevic argues that the coverage of the bonded phase is so tight that not even ACN can fit in between the bonded chains [75]. It is hard to imagine that a molecule as large and sterically bulky as the taxol compounds could penetrate deeply enough into the bonded phase to interact with silanol groups on the silica surface. Furthermore, the surface excess isotherms experiments described in section 4.2.1 suggested that the

number of residual silanol groups is very low on the Betasil C8 and Propyl-PFP columns making it unlikely that they should have such a pronounced effect on solute retention. It is also hard to picture how the hydrophilic sugar group on the xylosyl taxol compounds could penetrate through the extremely hydrophobic bonded phase layer of octyl and, especially, the pentafluorophenyl phase to interact with surface silanols. This view is supported by Berendsen and Galan [66], who found evidence that polar, hydrophilic groups hinder the retention of alkyl chain solutes since they can not penetrate into the bonded phase layer. Contact between the surface silanols and the hydroxyl groups is therefore unlikely.

Figure 58 a. Retention Behavior of Taxanes at High ACN Concentration

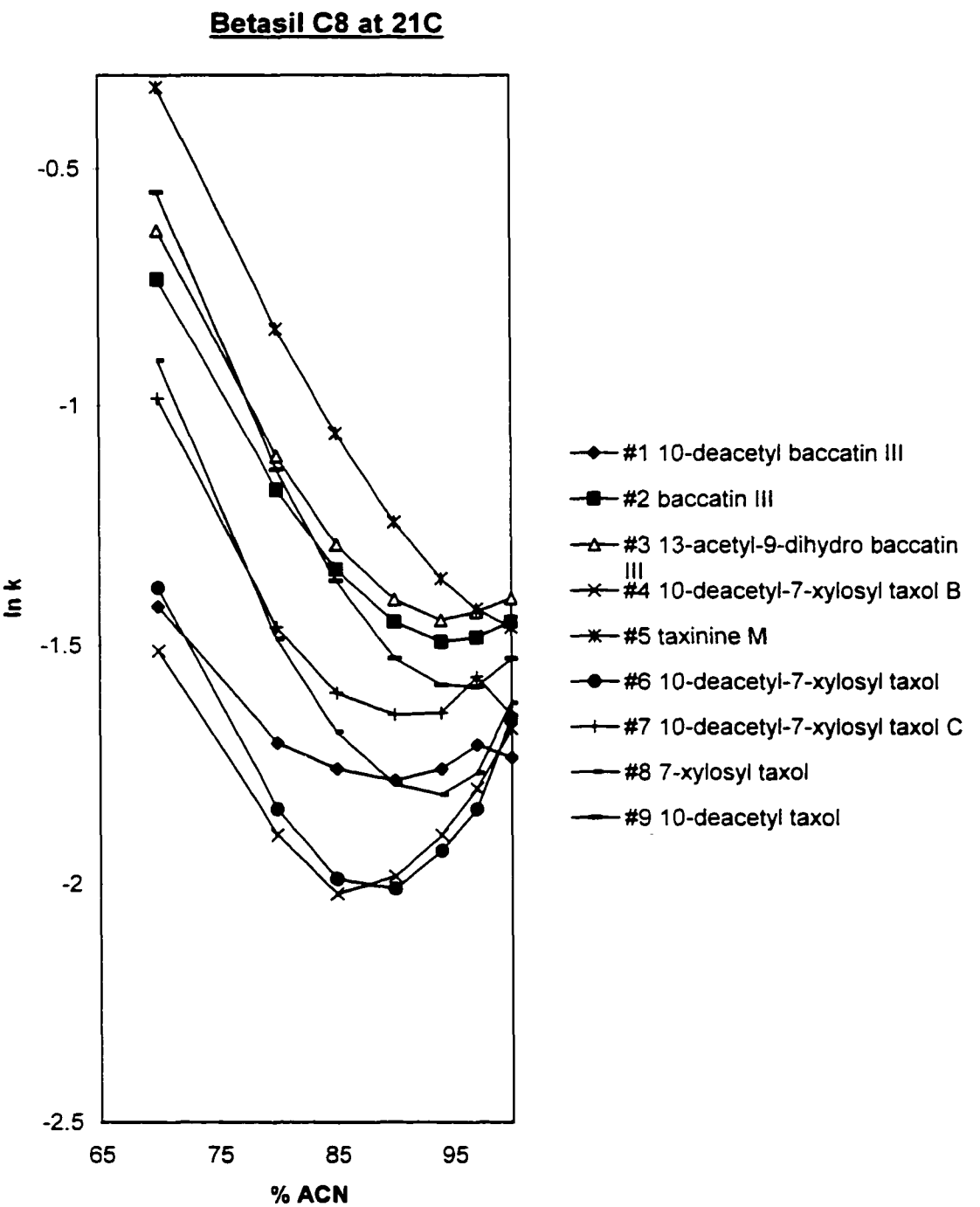


Figure 58 b. Retention Behavior of Taxanes at High ACN Concentration

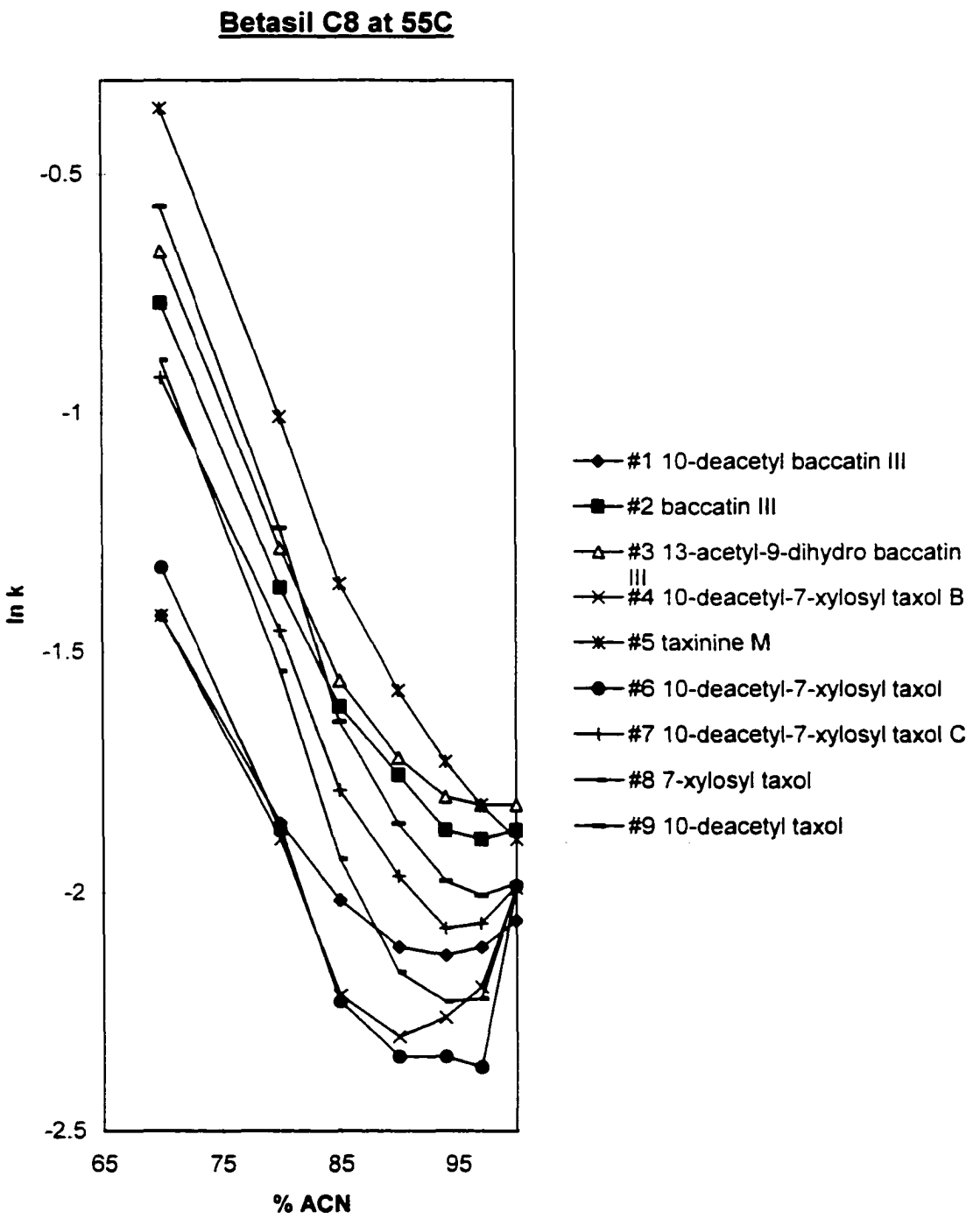


Figure 59. Temperature Behavior of Taxanes at High ACN Concentration

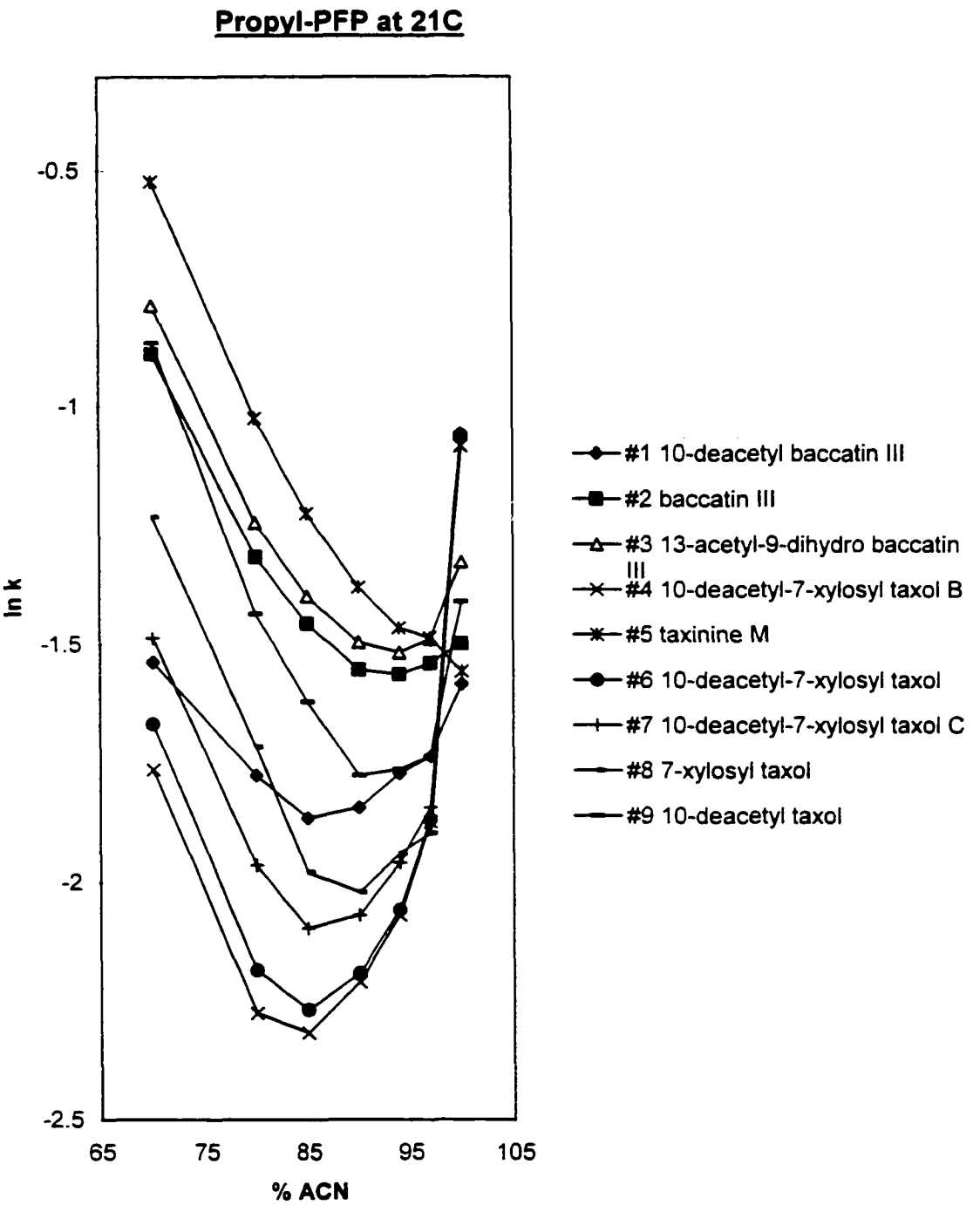
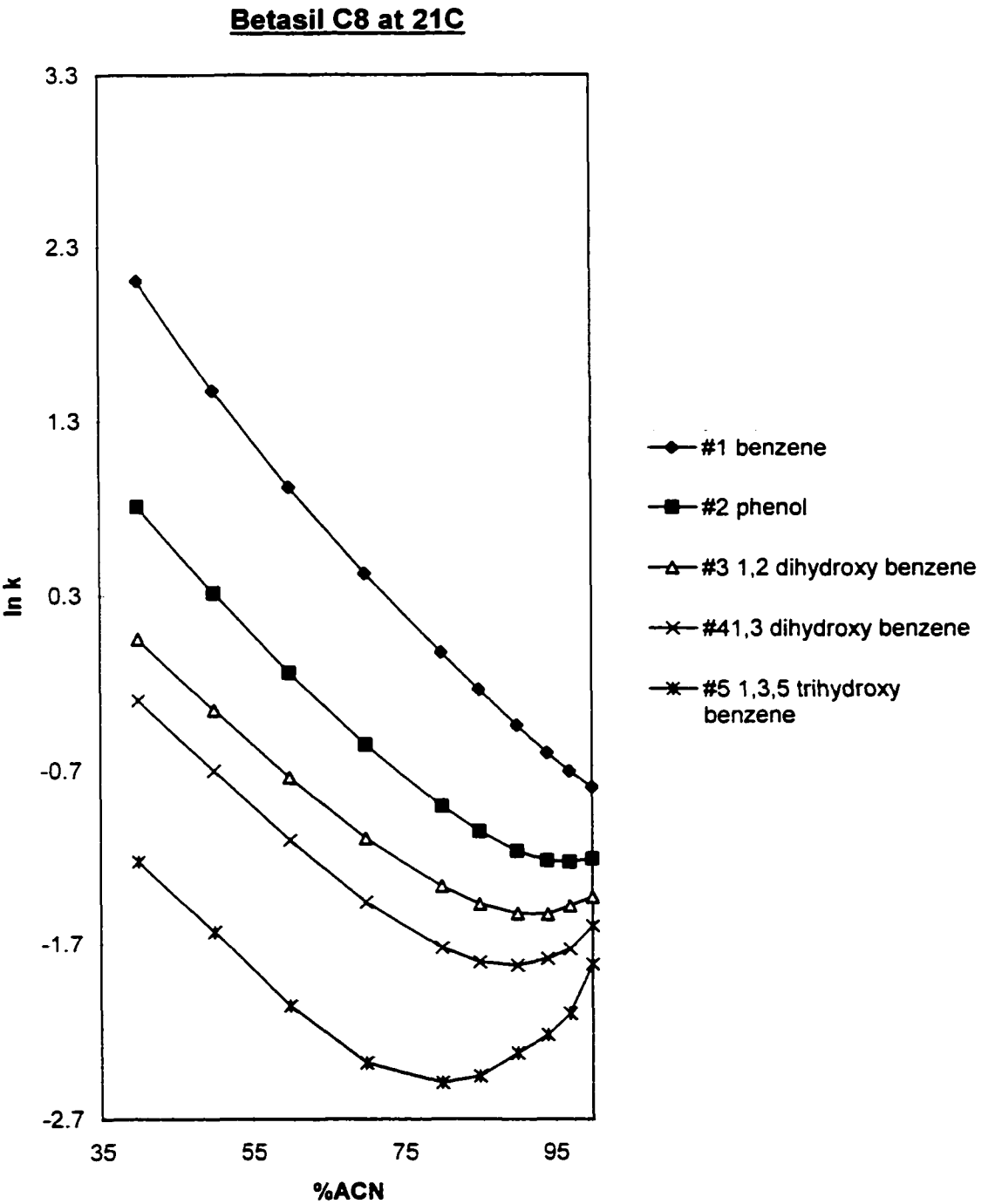


Figure 60. Retention Behavior of Hydroxyl Benzene Compounds at High ACN Concentration



4.5.2.2 Retention Behavior of Hydroxyl Compounds with Varying Temperature

The next step is to find out if the hydroxyl groups are also responsible for the aberrant temperature behavior of the hydroxyl-rich taxane compounds. To answer this question, the temperature dependence of retention times of the hydroxy benzene compounds was investigated at varying mobile phase compositions. To make the experiments time-efficient only the low temperature range between 20 °C and 30 °C, at which the most aberrant behavior was found for the taxane compounds, was investigated in most of the experiments. Plots of ln k versus reciprocal temperature for mobile phase composition from ACN/H_2O 40/60 to 70/30 are shown in Figure 61 and 62, respectively. At all mobile phase compositions the graphs are virtually parallel with positive slopes. The temperature behavior of the hydroxyl benzene compounds is absolutely regular and shows none of the aberrations of the taxane compounds.

The benzene derivatives, however, are not good representatives of the taxane compounds since they are aromatic. On the taxanes, the hydroxyl groups are bound to non-aromatic structures. This could possibly affect the temperature behavior of the solvation since the aromatic π-system might interact with the

hydroxyl groups.

To rule out the interference from π-systems, the experiments were repeated using nine model compounds including alcohols, cyclic ketones and hydroxy ketones. The results of the study of temperature dependence, of retention times of these compounds at mobile phases from 10% to 90% ACN are shown in Figure 63 and 64. The non-hydroxylated ketones, cyclohexanone and γ-butyrolactone, show positive slopes throughout the entire mobile phase range. The various alcohols and hydroxylated ketones, 2-hydroxy cyclohexanone and α-hydroxy-γ-butyrolactone, however, show temperature behavior comparable to the xylosyl taxol compounds. The graphs have positive slopes at ACN concentrations of 90 % shown in Figure 63 a. However, at decreasing ACN concentrations of 85 % to 45 % shown in Figures 63 b and 64 a the alcohols and the hydroxyl ketones, 2-hydroxyl cyclohexanone and α-hydroxy-γ-butyrolactone, change to negative slopes. The extent of the negative slopes is directly correlated to the number of hydroxyl groups. This effect is clearly illustrated for the hexane and cyclohexane derivatives. Cyclohexanone shows a positive slope at 45% CAN, while cyclohexanol, 1,2-hexanediol and 1,2,3-hexanetriol show a successive decrease in slope. Further decrease of the ACN concentration to 10% slowly results in the re-establishment of positive

slopes for almost all compounds as shown in Figure 64 b. Only 1,2,3-hexanetriol retains a slightly negative slope. This study clearly indicates that hydroxyl groups bonded to non-aromatic structures are responsible for the aberrant temperature behavior observed for the highly hydroxylated taxane compounds. There seems to be a competition between two effects when the temperature is increased:

1. decreasing retention with increasing temperature, caused by the reduced partitioning of the solute into the bonded phase due to increased thermal motion
2. increasing retention with increasing temperature implied by the thermodynamics of solute solvation as described in Figure 57.

A greater number of hydroxyl groups in the structure can lead to the dominance of the latter effect causing the negative slopes. At high or low ACN concentrations, the first effect is dominating leading to positive slopes. It looks like very weak or very strong retention both favor positive slopes while at intermediate retention and the appropriate chemical structure negative slopes can dominate. The definite mechanism for this effect, however, can not be

inferred from the data in this study and is subject to further research.

Figure 61. Temperature Behavior of Hydroxyl Benzene Compounds on Betasil C8

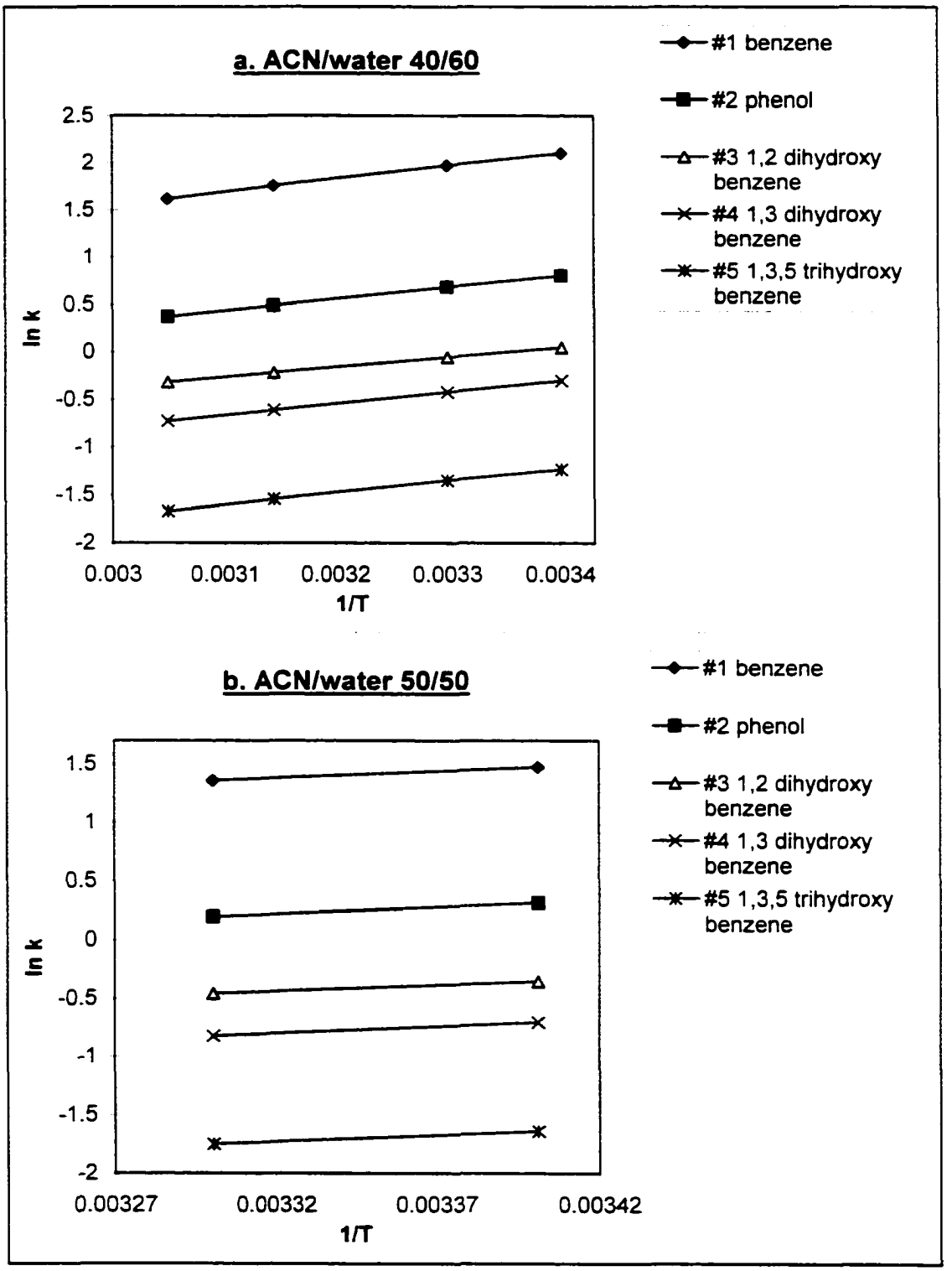


Figure 62. Temperature Behavior of Hydroxyl Benzene Compounds on Betasil C8

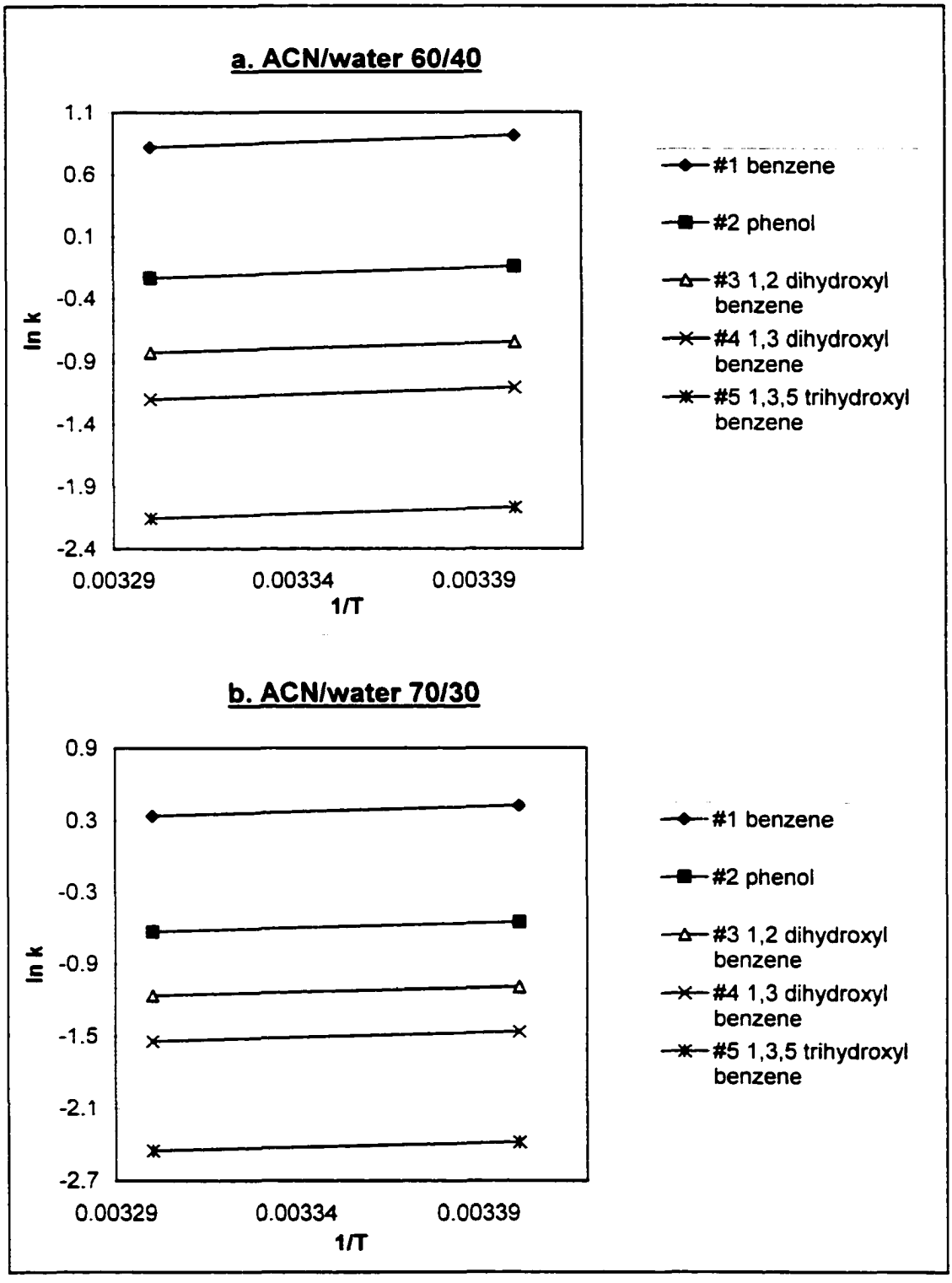


Figure 63. Temperature Behavior of Non-Aromatic Hydroxyl Compounds on Betasil C8

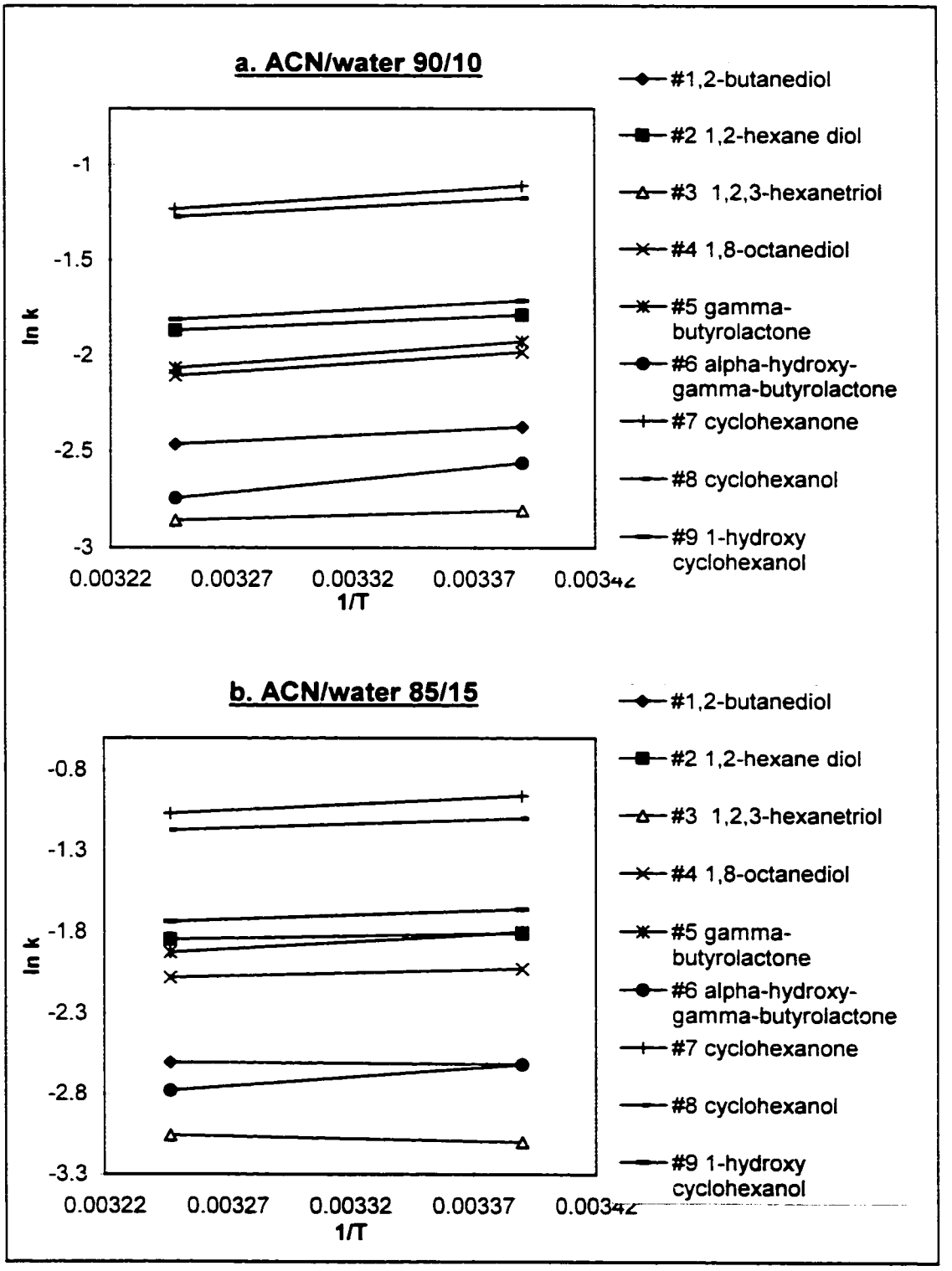
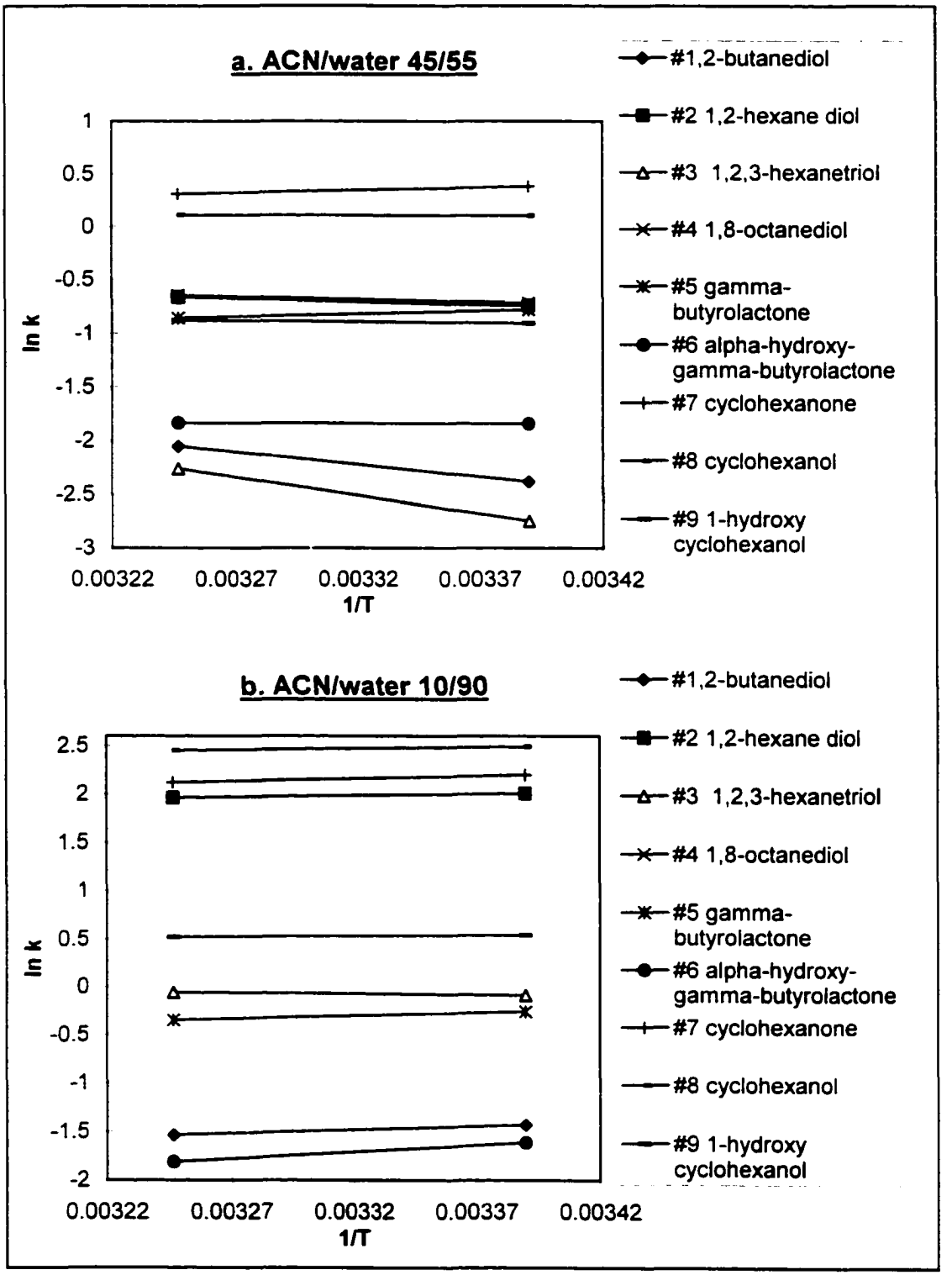


Figure 64. Temperature Behavior of Non-Aromatic Hydroxyl Compounds on Betasil C8



4.5.2.3 Explanation of Changes of Elution Order with Varying Mobile Phase Composition

As mentioned above, the taxanes that show aberrant temperature behavior are the same that show more negative slopes in the ln k versus %ACN studies. It is therefore reasonable to assume that the structural feature that caused the aberrant temperature behavior for these compounds, namely the hydroxyl groups, is also responsible for their special behavior with respect to the mobile phase composition. It has already been shown in Figure 60 that hydroxyl groups cause an initial decrease of retention upon addition of water to the organic mobile phase. This means that the retention of a hydroxylated compound is initially reduced far below the retention of its non-hydroxylated analog. Based on the retention behavior of the taxane compounds in Figures 58 and 59, it looks as though the xylosyl-taxols, after the initial decrease of retention, try to rise to the retention time of their (imagined) non-hydroxylated analogs. The overall retention reducing effect of the hydroxyl groups seems to become less and less effective as the concentration of water in the mobile phase is increased, allowing hydroxylated compounds with large non-polar backbone structures to penetrate more effectively into the bonded phase layer. All the

xylosyl taxol compounds in Figures 58 and 59 have the C-13 side chain giving them more non-polar backbone structures than the other five taxanes in the study which lack the C-13 side chain. To test this hypothesis, the retention behavior of the model compounds of the temperature study in Figure 63 and 64 was investigated at varying mobile phase compositions. The results of the experiment are shown in Figure 65. To clarify particular trends, selected graphs are shown on separate plots in Figures 66a to 66c. As expected, the initial decrease of retention becomes more pronounced with the number of hydroxyl groups in the solute structure, as can be seen for cyclohexanol, 1,2-hexane diol and 1,2,3-hexanetriol in Figure 66 a. After the initial decrease, there is a strong increase of retention that is equally correlated to hydroxylation of the compounds. 1,2,3-hexanetriol shows the strongest increase followed by the diol and cyclohexanol, respectively. After the initial diversion, all three compounds seem to try to converge again at low ACN concentration.

Figure 66 b shows the effect of different backbone structures on the retention behavior. Due to the multiple hydroxylation, the retention of 1,2,3-hexanetriol initially decreases very strongly resulting in its elution well before the butane and cyclobutane structures. Upon further reduction of the ACN concentration, however, the retention of hexanetriol increases much more

sharply than the butane compounds. Consequently, hexanetriol moves through all the butane structures changing from last eluting to first eluting compound. Figure 66 c further illustrates this phenomenon in the comparison of three hexane and three butane compounds. At high and intermediate concentrations of ACN, the elution order of the two groups is mixed. With decreasing ACN concentration, however, the butane compounds converge to a lower retention (ln k: -0.5 to 0.5) while the hexane compounds converge at a higher retention (ln k: 2 to 4). The retention times of the more hydroxylated compounds approaches the retention time of the less and non-hydroxylated analogs. The results of this study are in complete agreement with the hypothesis stated above and explain the changes of elution order with increasing concentration of water in the mobile phase.

A further point of interest is the retention behavior of 1,8-octanediol. As can be seen in Figures 66 a to c, 1,8-octanediol initially elutes before 1,2-hexanediol. The decreased retention is most likely caused by the better spatial distribution of the hydroxyl groups on the structure of octane, which more efficiently prevents partition into the bonded phase layer. As was shown above, the effects of the hydroxyl groups are considerably reduced at low ACN concentration, which explains the change of elution order of 1,8-octanediol with

1,2-hexanediol in this mobile phase range. Again, the two compounds approach the elution order of their non-hydroxylated analog.

Figure 65. Temperature Behavior of Non-Aromatic Hydroxyl Compounds on Betasil C8 at 22C

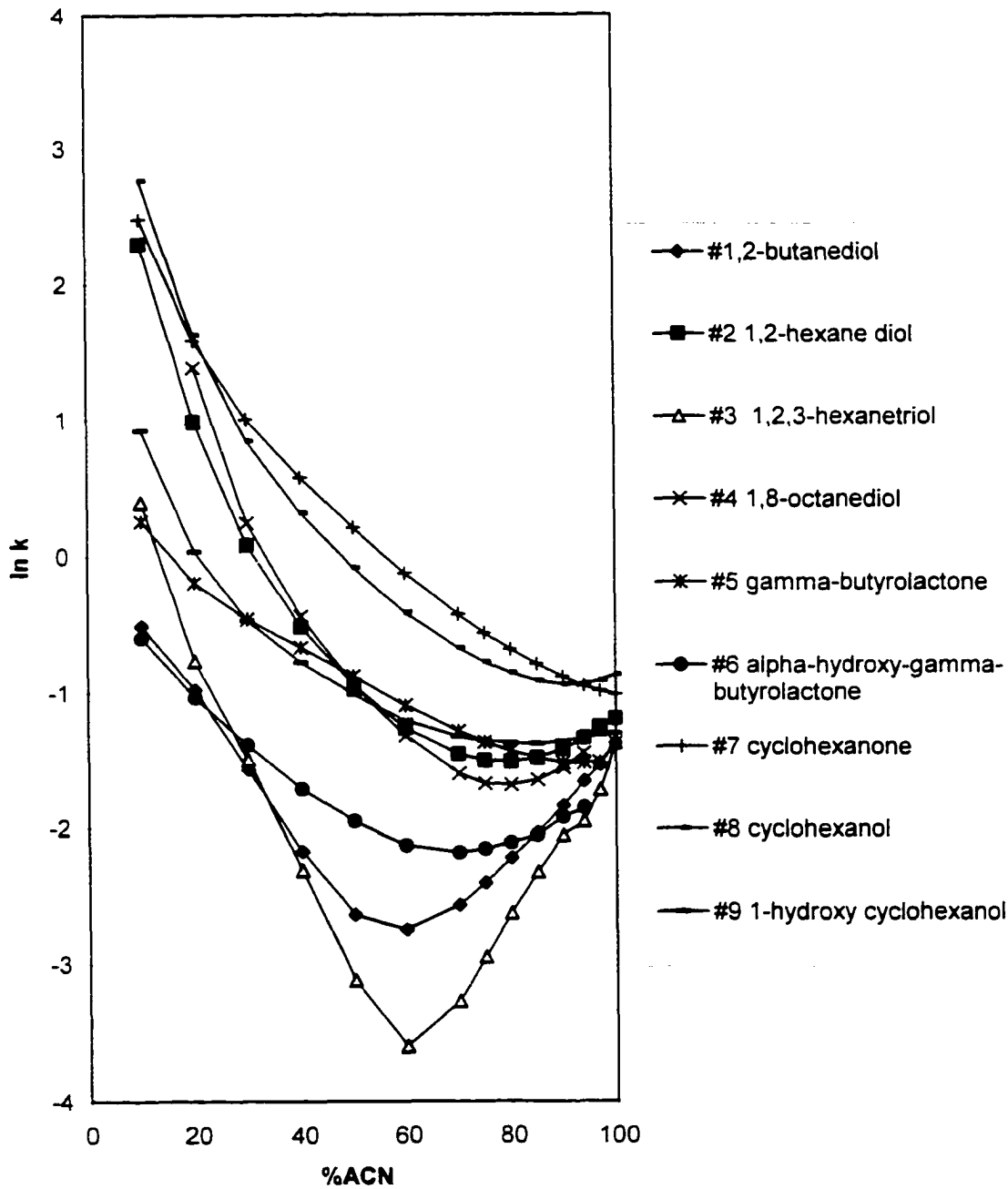


Figure 66 a. Retention Behavior of Non-Aromatic Hydroxyl Compounds on Betasil C8 at 22C

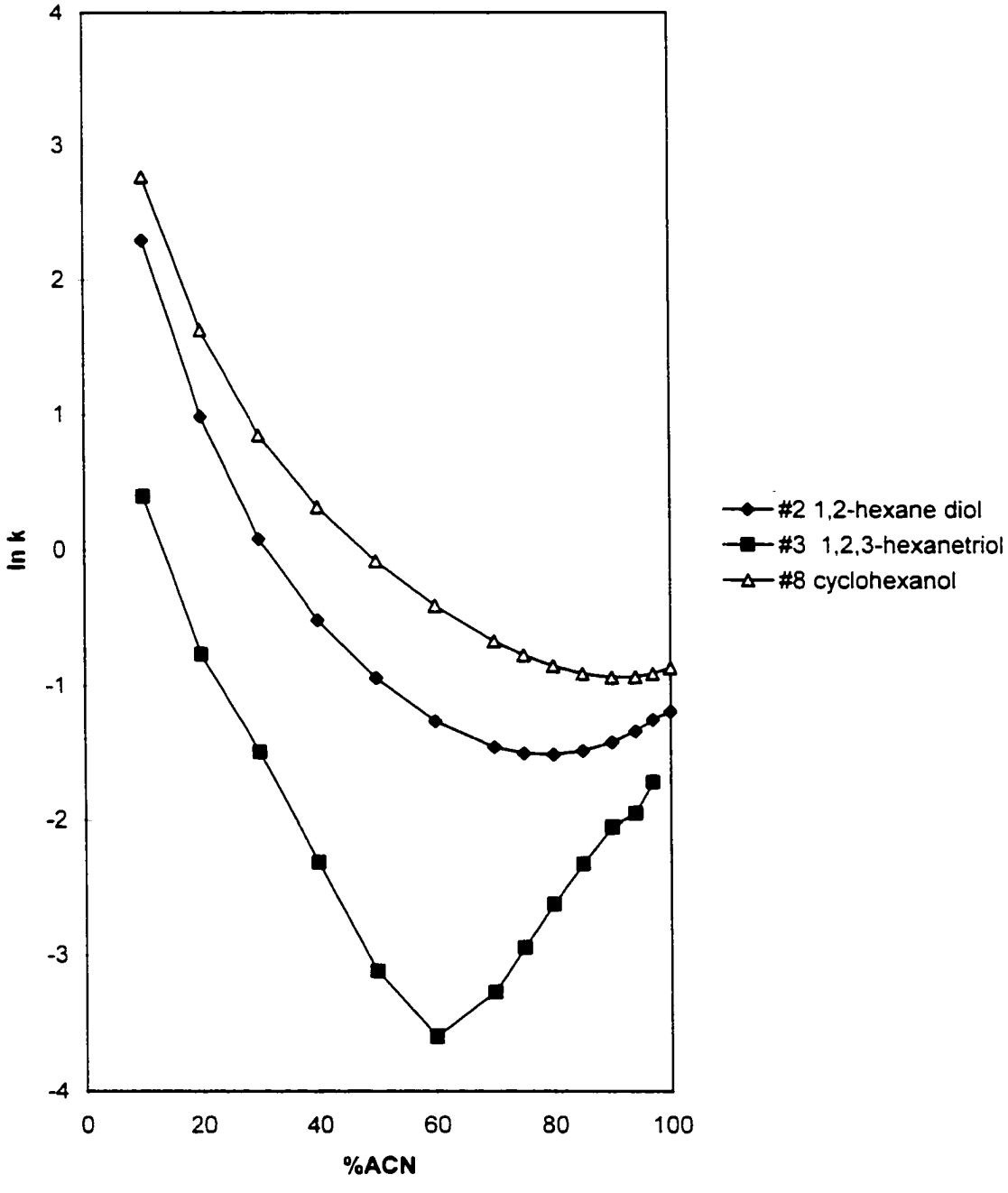


Figure 66 b. Retention Behavior of Non-Aromatic Hydroxyl Compounds on Betasil C8 at 22C

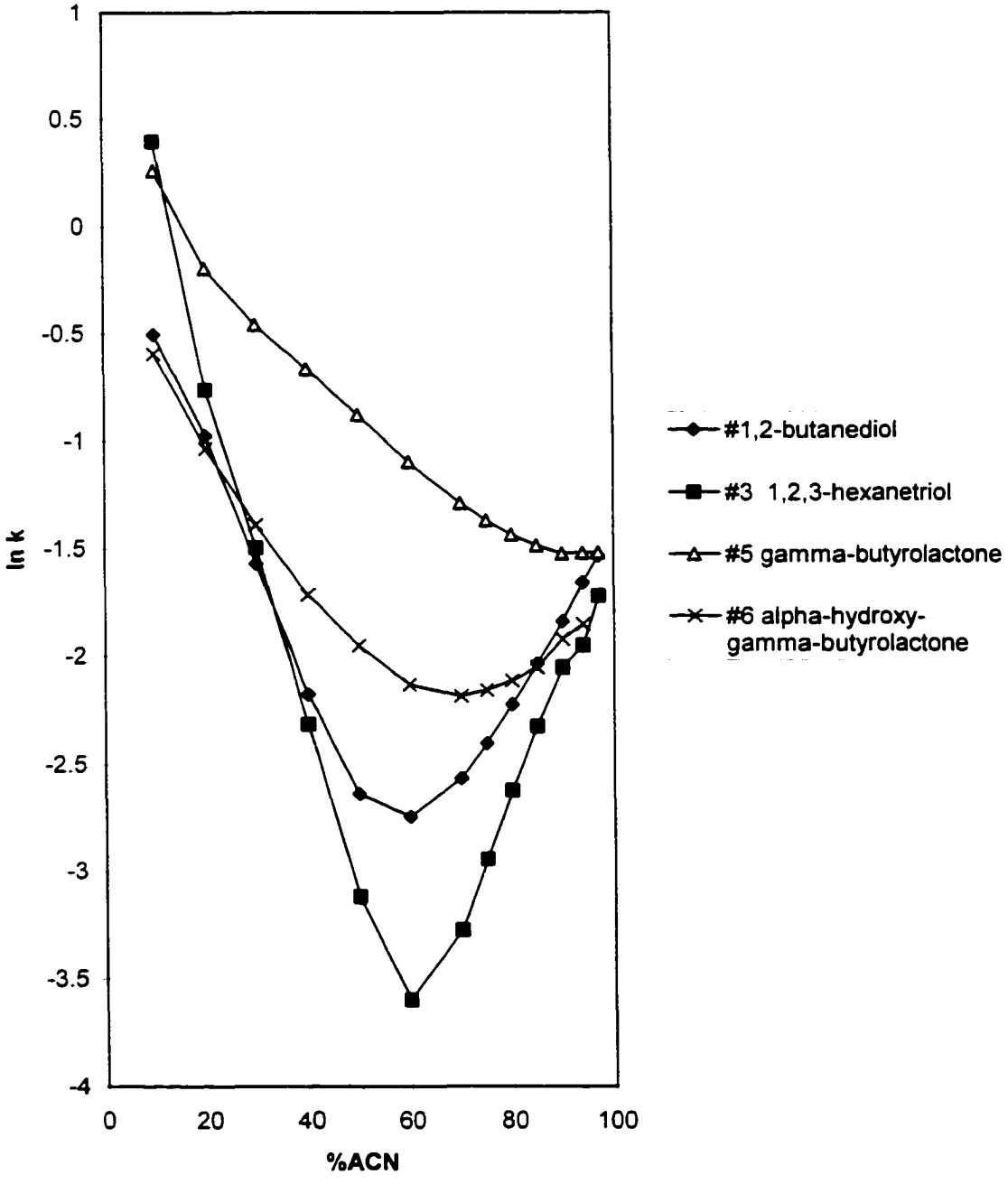
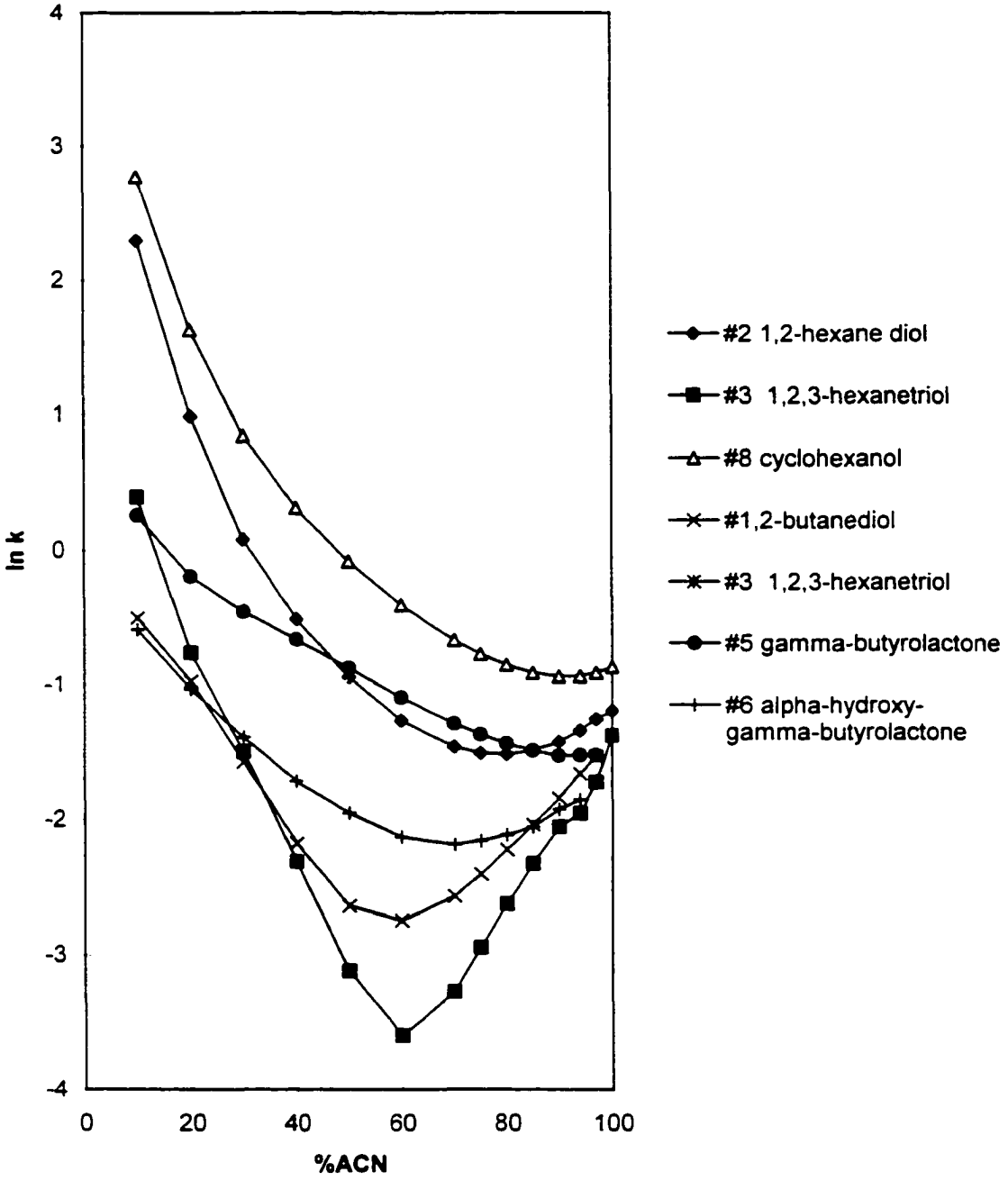


Figure 66 c. Retention Behavior of Non-Aromatic Hydroxyl Compounds on Betasil C8 at 22C



Chapter V

Conclusions

5. Conclusions

Nine reversed phase liquid chromatographic methods for the separation of taxanes were developed on hydrocarbonaceous and several fluorinated stationary phases. Complete separation of all 15 taxanes in the standard mixture was achieved in less than 20 minutes on Propyl-PFP and PFP-100, in less than 12 minutes on Ethyl-PFH and RP-100, and in less than 10 minutes on Fluofix 120E. Three different methods for the complete separation of 14 and 15 taxanes in as little as 13.5 minutes were developed on Betasil C8. This proves that hydrocarbonaceous stationary phases can have sufficient selectivity to separate complex taxane mixtures.

Comparison of the retention behavior of taxanes and various model compound mixtures did not reveal any difference in chemical selectivity of the fluorinated bonded phases as compared to the hydrocarbonaceous phase.

The particular effects described below were observed irrespective of the bonded phase and are therefore not column- but solute-specific. The xylosyl taxol compounds were found to show a much sharper increase of retention time with increasing water concentration in the mobile phase than the other taxanes. This effect shows mobile phase composition has a strong influence on the

selectivity and elution order of the taxane separations and transfer of taxane separation methods from one chromatographic system to another will therefore require precise calibration and control of the gradient mixing modules.

At mobile phase compositions near 50% CAN, the xylosyl taxol compounds and 10-deacetyl baccatin III were found to show increasing retention times with increasing temperature while the remaining taxanes show decreasing retention times. The effect seems to be independent of the bonded phase chemistry but is less pronounced at stationary phases with low retentivity like Ethyl-PFH. At increasing or decreasing ACN concentration the aberrant temperature behavior of the xylosyl taxols slowly disappears. The fluorinated stationary phases are hardly affected by this phenomenon since they have a low retentivity and therefore require low ACN concentrations. On the other hand, Betasil C8 has a higher retentivity requiring mobile phases around 50% ACN. Consequently, taxane separations on this and other hydrocarbonaceous columns are highly temperature sensitive. The lack of temperature control or optimization in all the previously reported taxane separation methods is likely the cause for their failure in achieving maximum selectivity with alkyl bonded phases.

It was determined that the cause of the aberrant temperature behavior is the relatively large number of hydroxyl groups on the respective taxanes.

Experiments with simple model compounds showed that hydroxyl groups bound to non-aromatic backbone structures cause increasing retention with increasing temperature. The effect becomes more pronounced with either and increase of the number of hydroxyl groups or a reduction of the size of the non-polar backbone structure. The xylosyl taxol compounds are mostly affected by this phenomenon due to the hydroxyl rich sugar group attached the C-7 carbon.

It was found that the taxane compounds show an initial decrease of retention upon increase of water concentration in the mobile phase, starting at 100% ACN. The extent of this effect is related to the number of hydroxyl groups in the structure each taxane. Experiments with aromatic and non-aromatic hydroxylated model compounds confirmed this trend. As the concentration of water in the mobile phase is further increased, the effect of the retention reducing hydroxyl groups is more and more diminished and the solute approaches the retention time of its non-hydroxylated analog. This phenomenon is responsible for the sharper increase of retention of the xylosyl taxol compounds with respect to the other taxanes. The xylosyl taxols have a long, non-polar chain attached to the C-13 carbon. The other early eluting taxanes lack this feature. They therefore approach a lower retention maximum corresponding to less non-polar character of their backbone structure.

The mechanism of preferential solute solvation was proposed to explain the temperature and mobile phase effects observed with solutes containing hydroxyl groups. The solvation shell affects the retention of the solute and is responsible for the initial decrease of retention upon addition of water to the mobile phase. Increase of temperature would reduce the solvation of the solute and make it effectively less polar, resulting in the observed increase of retention. This theory explains all the observed effects while remaining consonant with basic concepts of reversed phase chromatography.

This is the first study to provide a detailed explanation of the mechanistic reason for changes of elution order with respect to temperature and mobile phase composition in RPLC. Irrespective of the proposed mechanism, the findings can be used to predict selectivity changes in a separation based on the chemical structure of the solutes. It is reasonable to assume that other functional groups that can engage in hydrogen bonding such as the amine group could exhibit similar behavior. Further research could reveal helpful information on the influence of particular chemical features that affect selectivity in RPLC.

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