NOVEL APPLICATIONS OF ELEVATED TEMPERATURE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: ANALYSES OF TRIAZOLE FUNGICIDES AND VITAMIN E ISOMERS

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Specially dedicated to my beloved mother, sister and brother.

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ABSTRACT

Control of column temperature has become increasingly accepted as a separation parameter in high performance liquid chromatography (HPLC). In this study, high-temperature reversed-phase (RP) HPLC using water-rich and superheated water eluents is evaluated as a new approach for the separation of selected triazole fungicides. Using a polybutadiene-coated zirconia column at temperatures of 100°C to 150°C, clear separations were achieved when 100% purified water was utilized as organic-free eluent. Excellent limits of detection down to pg level were obtained under optimum conditions. Nevertheless, poorer separation efficiency was observed when the triazole fungicides were separated on carbon-clad zirconia column using water-rich eluents. Novel separation of eight vitamin E isomers (α-, β-, γ-, δ-tocopherol, and α-, β-, γ-, δ-tocotrienol) and α-tocopherol acetate on both normal-phase (NP) and reversed-phase (RP) HPLC were also examined. All the isomers were successfully separated using NP-HPLC on amino and silica columns. By simply increasing the temperature for silica column, excellent separation efficiencies and shorter analysis times were achieved. Seven vitamin E isomers were successfully separated using RP-HPLC at high temperatures. Both developed separation methods are rapid, showed excellent repeatability, and suitable to be used as a quantitative method in analyzing vitamin E isomers. Pressurized liquid extraction (PLE) along with elevated temperature NP-HPLC is evaluated as a new approach for the determination of β-carotene and vitamin E isomers in residue oil obtained from palm pressed fiber (PPF). The new developed method demonstrated an outstanding performance with excellent efficiency in terms of total extraction time, total solvent usage, total carotene and vitamin E isomers contents as well as the exceptional method repeatability.

ABSTRAK

Pengawalan suhu turus telah semakin diterima sebagai parameter pemisahan dalam kromatografi cecair prestasi tinggi (HPLC). Dalam kajian ini, HPLC fasa terbalik bersuhu tinggi dengan turus zirkonia tersalut polibutadiena menggunakan pengelusi kaya-air dan air lampau panas telah dikaji sebagai suatu pendekatan baru bagi pemisahan fungisid triazola terpilih. Dengan menggunakan turus zirkonia tersalut polibutadiena pada suhu 100°C hingga 150°C, pemisahan lengkap telah tercapai apabila menggunakan 100% air tulen sebagai pengelusi bebas organik. Had pengesanan yang rendah sehingga tahap pg telah tercapai pada keadaan optimum. Walau bagaimanapun, kecekapan pemisahan yang lemah telah dicerap apabila racun rumpai fungisid triazola dipisahkan dengan turus zirkonia tersalut karbon menggunakan pengelusi kaya-air. Pemisahan baru lapan jenis isomer vitamin E (α-, β-, γ-, δ-tokoferol, and α-, β-, γ-, δ-tokotrienol) and α-tokoferol asetat dengan HPLC fasa normal (NP) dan fasa terbalik (RP) juga telah dikaji. Semua isomer telah berjaya dipisahkan menggunakan NP-HPLC dengan turus amino dan silika. Dengan hanya meningkatkan suhu bagi turus slika, kecekapan pemisahan yang baik dan masa analisis yang lebih pendek telah tercapai. Tujuh jenis isomer vitamin E telah berjaya dipisahkan menggunakan RP-HPLC pada suhu tinggi. Kedua-dua kaedah pemisahan yang dibangunkan adalah cepat, menunjukkan kebolehulangan yang tinggi, dan sesuai digunakan sebagai kaedah kuantitatif dalam menganalisis isomer vitamin E. pengekstrakan cecair bertekanan (PLE) bersama dengan NP-HPLC pada suhu tertingkat telah dikaji sebagai pendekatan baru dalam menentukan β-karotin dan isomer vitamin E dalam sisa minyak diperoleh daripada sabut kelapa sawit (PPF). Kaedah baru yang dibangunkan menunjukkan prestasi cemerlang dengan kecekapan yang baik dari segi masa pengekstrakan, jumlah penggunaan pelarut, jumlah kandungan karotin dan isomer vitamin E dan juga kebolehulangan kaedah yang tinggi.

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LIST OF SYMBOLS/ABBREVIATIONS/NOTATION/TERMINOLOGY

IUPAC	-	International Union of Pure and Applied Chemistry
RP-HPLC	-	Reversed-phase High Performance Liquid Chromatography
HPLC	-	High Performance Liquid Chromatography
η	-	Mobile phase viscosity
T	-	Absolute Temperature
ΔP	-	Pressure drop
ϕ	-	Flow resistant factor
L	-	Column length
u	-	Linear flow rate
d_P	-	Particle diameter
$D_{m,25}$	-	Molecular diffusivity at 25^{o}C
H	-	Theoretical plate height
I.D.	-	Internal diameter
UV	-	Ultra violet
PS-DVB	-	Poly(styrene-divinylbenzene)
ODS	-	Octadecylsilyl
PAHs	-	Polycyclic aromatic hydrocarbons
UV-Vis	-	Ultra-violet visible
LC	-	Liquid chromatography
MS	-	Mass Spectrometry
PBD	-	Polybutadiene
CARB	-	Carbon
Zr	-	Zirconia
GC	-	Gas chromatography
CE	-	Capillary electrophoresis
C	-	Carbon number
HT-HPLC	-	High temperature high performance liquid chromatography
MEKC	-	Micellar electrokinetic chromatography

TLC	-	Thin layer chromatography
NP-HPLC	-	Normal phase high performance liquid chromatography
PFPS	-	Pentafluorophenylsilica
ODPVA	-	Octadecanoyl polyvinyl alcohol
PPF	-	Palm pressed fiber
CPO	-	Crude palm oil
PFAD	-	Palm fatty acid distillate
PLE	-	Pressurized liquid extraction
MAE	-	Microwave assisted extraction
Log P	-	Logarithm of partition coefficient *n*-octanol/water
a_s	-	Absorbance of the sample
a_b	-	Cuvette error
W	-	Weight of sample in gram
R_s	-	Resolution
w	-	Peak width
$w_{A1/2}$	-	Peak width at half height
t	-	Retention time
N/m	-	Theoretical number of separation plates per column length
RSD	-	Relative standard deviation
α	-	Separation factor
k	-	Retention factor
t_o	-	Column void volume values
MeCN	-	Acetonitrile
R	-	Gas constant
ΔH^o	-	Standard enthalpy
ΔS^o	-	Standard entropy
Φ	-	Column phase ratio
THF	-	Tetrahydrofuran

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CHAPTER 1

INTRODUCTION

1.1 Preamble

The originator of chromatography as it is practiced today was Michael Tswett (1872-1919) [1]. In 1906 Tswett, a Russian botanist used the term *chromatography* to describe his work on the separation of colored plant pigments into bands on a column of chalk and other materials and stated " Chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system" [2].

Chromatography is a separation method in which a mixture is applied initially as a narrow zone to a stationary, porous sorbent and the components are caused to undergo differential migration by the flow of the mobile phase, a liquid or a gas. According to IUPAC, chromatography can be defined as a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in a definite direction [2,3].

1.2 Principles of High Temperature Operation in Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

Control of column temperature has become increasingly accepted as a separation parameter in RP-HPLC. Besides, high temperature as an optimization parameter in the separation process of the RP-HPLC system has been widely studied.

This is due to the recent findings of the alternative stationary phase, which has high thermal stability at high temperatures. Recently, the use of higher operating temperatures in RP-HPLC has been demonstrated to be viable and useful for both polymer and pharmaceutical application [4-9].

The advantages of doing RP-HPLC at high temperatures (>100^{o}C) are obvious. High temperature operation in RP-HPLC provides the opportunity to reduce the quantity of organic solvent used in mixed organic-water mobile phase, decreased total analysis time and column back pressure significantly. Elevated temperatures can also increase analyte mass transfer rates and thereby decrease peak width [10-14].

The use of ambient operating temperature in conventional RP-HPLC system with higher flow-rates to reduce analysis time is not recommended. The applicability of high flow rates is limited by the back pressure that different parts of the chromatographic system (pump, injector, and column) can withstand [10]. High flow rates in conventional RP-HPLC system might cause a significant loss in resolution, sacrifice the ruggedness of the separation, shorten the column lifetime and damaged the pump seal [15].

Elevated column temperature operation in RP-HPLC can be used as a tool to overcome the flow rate problem associated with high back pressure, allowing the use of higher flow rates that otherwise could not be applied. The pressure reduction is due to a decrease in eluent viscosity with increasing temperature. The relationship between viscosity, η, and absolute temperature, T, is given by the empirical expression [10]:

$$\ln \eta = a + \frac{b}{T} \qquad (1.1)$$

Where a and b are empirically determined constants. The lower viscosity decreases the pressure drop across the column and allows higher linear velocities as the limit of pump pressure is approached [16]. The pressure drop across a packed column can be approximated by equation 1.2 [10]:

$$\Delta P = \frac{\phi L \eta u}{d_p^{\,2}} \tag{1.2}$$

where ΔP is the pressure drop, ϕ is the flow resistance factor, L is the column length, η is the mobile phase viscosity, u is the linear flow rate, and d_p is the particle diameter. The viscosity (η) is proportional to the inverse of the temperature (T), therefore, higher temperature can significantly decrease mobile phase viscosity (η) and at the same time decrease the pressure drop (ΔP) across a packed column where all others parameter remain constant.

The advantage of lower pressure drop across the system is that it allows higher flow rates to be applied without decreasing the efficiency of the separation. Increase in the mobile phase flow rate can assist in stabilizing the pressure across the column. Pressure stabilization across the column is extremely important in avoiding the thermal mismatch and temperature gradient that might occur.

Carr and Li [17] in their paper described the rapid analyses of polycyclic aromatic hydrocarbons and typical reversed-phase test mixtures at elevated temperatures and high flow rates. The results showed that analysis time could be decreased about 18-fold at high temperatures and flow rates without any significant loss in resolution relative to that at the conventional temperatures and normal flow rates.

An increase in temperature also increases the diffusion coefficients of the mobile phase and the analytes. According to the Stokes–Einstein relationship, the diffusion coefficient is directly proportional to the absolute temperature and inversely proportional to the viscosity [13]:

$$D_m = D_{m,25}\left(\frac{T}{298}\right)\left(\frac{\eta_{25}}{\eta}\right) \tag{1.3}$$

where $D_{m,25}$ and η_{25} are the molecular diffusivity and the viscosity of the eluent at 25^{o}C, respectively. High-temperature separation has been shown to improve analyte

resolution by decreasing mobile phase viscosity and by increasing the diffusion rate of the sample species, thus increasing mass transfer of the analyte to the stationary phase and thereby decreasing the peak width [13].

1.3 Instrumental Consideration and Performance in High Temperature RP-HPLC System

Elevated temperature as an optimization parameter in the separation process of the RP-HPLC system is less popular among the researchers. This is due to few reasons. Firstly, alternative stationary phases that have high thermal stability force at high temperature are generally inadequate. The traditional silica-based stationary phases are less stable at high temperatures. Secondly, the design of a chromatographic system could not minimize thermal mismatch broadening and balance heat transfer in the heater effectively. Thirdly, not all the analytes are thermally stable on the time scale of the chromatographic run. Therefore, analyte stability at high temperatures should be well considered [16].

To avoid the problems during the operation of the RP-HPLC system at high temperature, a few modifications should be considered. Carr and Thompson [16] suggested that one of the methods to solve the main problem was to minimize the thermal mismatch broadening in high-temperature RP-HPLC. The temperature mismatch between incoming mobile phase and the column must be minimized because such a mismatch is a very serious cause of peak broadening of high-temperature RP-HPLC.

Schrenker [18] has implemented the study on the effect of mobile phase pre-heating on HPLC column performance. The results showed that control of constant column temperature using conventional temperature-controlled devices such as “air-bath” would lead to significantly axial and radial temperature gradients at temperature different from ambient if the mobile phase enters the column at ambient temperature. The use of conventional temperature-controller always lead to

insufficient heat transfer from air to the column wall and through the column wall into the mobile phase.

Foong [19] carried out a study on the column efficiency differences of separation process by comparing the high temperature RP-HPLC system with a mobile phase pre-heating coil and one without it. The results showed that thermal mismatch problem between incoming mobile phase and the column can be overcome by simply modifying the conventional RP-HPLC system with an additional mobile phase pre-heating coils. The column efficiency of the RP-HPLC system with a mobile phase pre-heating coils showed higher values of plate number compared with the system without the modification.

Several journals have described the instrumentation structure of RP-HPLC system at high temperatures. Generally, instrumentation system for RP-HPLC at high temperatures is almost the same with a commercial RP-HPLC system in the market. The most obvious difference is that for RP-HPLC system at high temperature, both mobile phase and column will be placed inside the temperature controller or simply inside a heater.

Commercial temperature controller or column thermostat such as "water-bath" and "block heater" are less popular among the researchers. New type of column thermostat such as gas oven is well accepted by most of the researchers because of it low heat capacity which allows sufficient heat transfer from air to the column wall and through the column into the mobile phase. According to Schrenker [18], a good column thermostat can save analysis time and improve detection limits, because at higher column temperatures lower plate height (H) are usually observed and the optimum of the Van-Deemter curve shifts to higher mobile phase velocities which allowed the use of a higher flow rate in the separation.

The use of high operating temperature in conventional RP-HPLC system is strongly dependent on maintaining the mobile phase in the liquid state. As we know, the boiling point for mobile phases (water~100^{o}C and acetonitrile~81.6^{o}C) are usually lower than the instrument operating temperature [20]. Therefore, an extra piece of equipment is needed to overcome this problem. A pressure regulator is

usually attached to the detector outlet to provide back pressure to eliminate the formation of bubbles in the mobile phase and thereby stabilize the baseline. Besides pressure regulator, small I.D. restriction tubing (~ 0.10 mm) also can be used to maintain a constant back pressure (~20 bar) at the outlet of the detector. Because the pressure regulator is attached behind the detector, it would not cause extra column broadening [2,10,19,21].

In our studies, the mobile phase is preheated to the same temperature as the column oven temperature and then passes through injection valve and reaches the column. The hot mobile phase exiting the column will be immediately cooled by ice water before it reaches the detector.

1.4 Water-Rich and Superheated water Eluents on High Temperature RP-HPLC

The mobile phase is one of the important parameters that need to be considered in RP-HPLC. Peak shape, specification of functional group, and other operating system parameters are strongly dependent on the nature of the mobile phases that are used [20].

Organic solvents such as acetonitrile, methanol, and tetrahydrofuran are commonly mixed with water and used as mobile phase in conventional RP-HPLC system. Acetonitrile is widely used because of its high elution strength compared with methanol and low UV transparency value. However, it is highly toxic and expensive. The control of organic material waste disposal and its implication towards chemist's health were the problems that should be overcome [22-25].

In order to reduce the usage of organic solvents in RP-HPLC system, attention was paid to new substitutes, for instance pure water. Water is always characterized as a unique solvent because of its highly hydrogen-bonded structure, and at ambient temperature it has disproportionately high boiling point for its mass, a high dielectric constant, and high polarity [26]. It is readily available, relatively

cheap, non-toxic, and causes no significant problems with disposal. However, water at ambient condition is too polar to solvate most organic pollutants. The polarity of liquid water can be controlled over a wide range by changing temperature under moderate pressures to maintain water in the liquid state [24]. Increasing the water temperature to 200°C–250°C causes a similar change in solvent polarity (measured by dielectric constant), as achieved by the common HPLC method of mixing methanol or acetonitrile with the water to a liquid concentration of 100% (Figure 1.1).

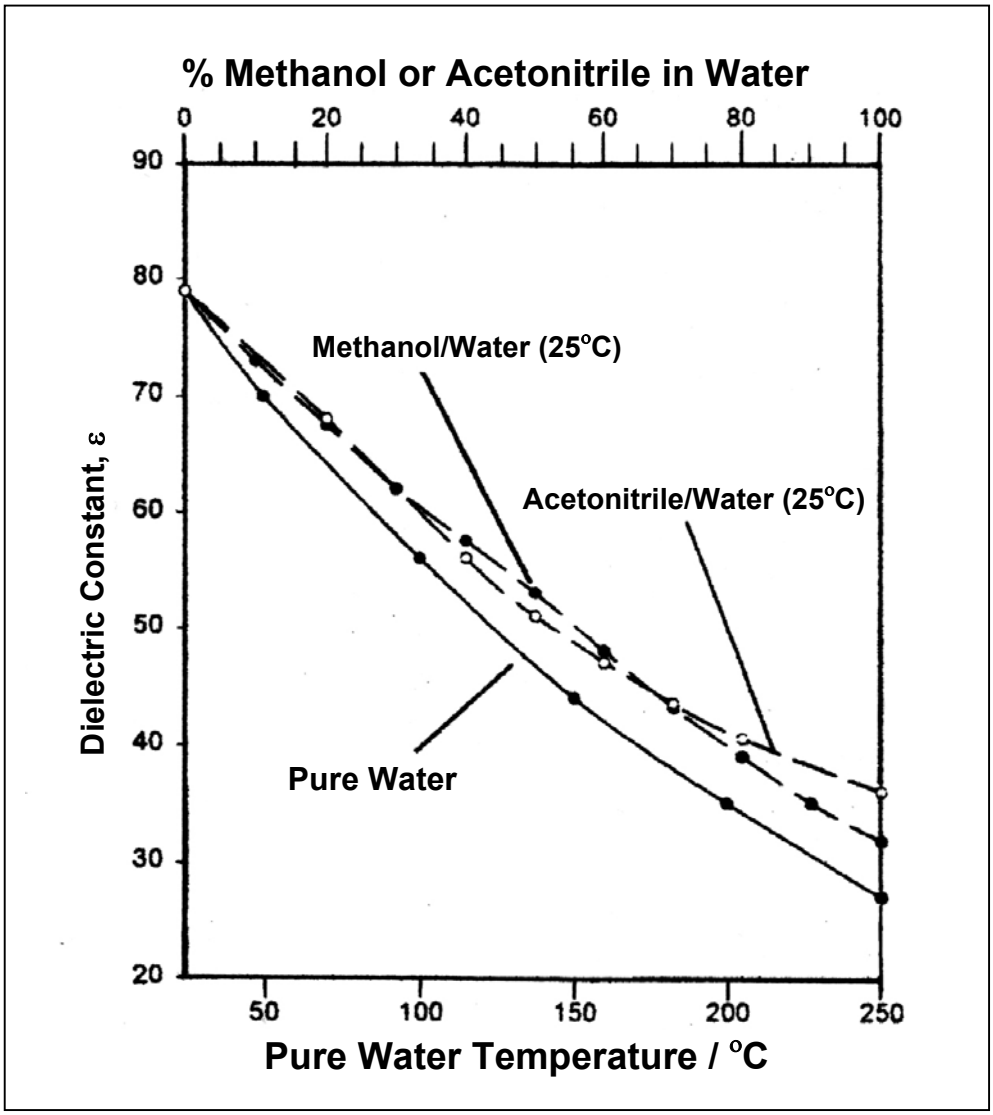

Figure 1.1: Control of solvent polarity (dielectric constant) by changing temperature (at 50 bar) with pure water compared to methanol- or acetonitrile-water mixtures at 25°C and ambient pressure [27-28].

Yang *et al*. [24] described that besides the polarity of water, two additional mobile phase parameters which control liquid phase separation are the solvent surface tension and viscosity. Low surface tension can significantly reduces the retention for reversed-phase separation and low viscosity can results in better mass transfer, thus achieving better chromatographic efficiencies. Water, heated at high temperature has low viscosity and the relationship between the viscosity factor and temperature has been explained previously (section 1.2).

A number of researches that use pure water as mobile phase at high temperature liquid chromatography were recently reported. Mixtures of phenols, parabens, barbiturates, and other analytes have been separated on poly(styrene-divinylbenzene) (PS-DVB) and ODS-bonded silica columns at temperatures up to 210^{o}C by Smith and Burgess [25] using superheated water as an eluent. Dasgupta and Kephart [21] described the application of superheated water eluent in a capillary scale reversed-phase liquid chromatography system.

The properties and characteristics of water at high temperatures had been studied. The capability of the system at high temperature was demonstrated with the separation of benzene derivatives on polybutadiene and elemental carbon modified zirconia packing. Carr *et al.* [16,17,29] who introduced polymer coated zirconia column performed a complete study. He succeeded in applying the superheated water as mobile phase to the separation of polycyclic aromatic hydrocarbon (PAH) compounds by developing a rapid separation at high temperature (200^{o}C).

1.5 High Thermal Stability Stationary Phase in the RP-HPLC

Column plays a very important role in RP-HPLC system because all separation process will occur in a column that is packed with specific stationary phase. Reversed phase packing of alkyl silane-bonded phase is by far the most popular stationary phase in RP-HPLC. However, these alkyl-bonded phases have several shortcomings, the major one being their thermal and chemical instability [30].

The typical conventional alkyl silane-bonded silica phase proved to be unstable at temperatures 20^{o}C-30^{o}C higher than room temperature (>50^{o}C) and higher temperature accelerates the dissolution of silica in aqueous solution [31]. In addition, degradation of the stationary phase occurs outside the pH range of 2.5-8. Thus, the ion suppression method cannot be employed with sample solutes having pKa value less than 2 or more than 7 [30-31].

To overcome the problem faced during the usage of alkyl silane-bonded silica phase, a few alternative stationary phases that have higher thermal stability and extreme acceptable pH range of 1-13 have been introduced (Table 1.1). Knox *et al.* [32] introduced alternative stationary phase, which is called Porous Graphitized Carbon (PGC). Meanwhile, Foong [19] have recently done a comprehensive research about PGC column, which focused on the RP-HPLC operation system at high temperature, using low organic solvent composition.

Table 1.1: General characteristics of the four reversed-phase LC test columns [32].

Description	Types of Stationary Phases			
	Graphitized Carbon-Clad Zirconia	C18 Silica	Polymeric	Polybutadiene-Coated Zirconia
Particle size (μm)	3	3.5	5	3
Pore size (Å)	300	300	100	300
Column size (mm x mm)	150 x 4.6	150 x 4.6	150 x 4.6	150 x 4.6
Low pH limit	0.5	1.8	1	0.5
High pH limit	14	8	14	14
Temperature limit (^{o}C)	200	80	150	200
Carbon loading (% carbon)	1.1	2.8	Not available	3.0

Polystyrene-divinylbenzene stationary phase can be regarded as one of the earliest stationary phase introduced which is able to withstand extended exposure to mobile phase at extreme pHs (1-14) and column temperatures as high as 200^{o}C [30]. Separation mechanism and retention behavior on the PS-DVB stationary phase is

strongly dependent on the neutral non-polar polystyrene surface that function as the active site for reversed phase separation with aqueous eluent [33]. Meanwhile, See [34] has recently done a complete research about PS-DVB column, which focused on the RP-HPLC operation system at high temperature, using water-rich and superheated water as eluents.

1.5.1 Zirconia based stationary phase

Typically, the applicability of the novel stationary phase can be evaluated by comparing to the Unger's specification of the ideal phase. First, the particles must have a narrow size distribution and high surface area. Second, the pores must have a diameter appropriate to the size of the analyte and good connectivity to allow for fast analyte mass transfer and third, the support material should resist thermal, mechanical, and chemical degradation but have a surface that is both energetically homogeneous yet chemically modifiable [35].

Zirconia based stationary phase from liquid chromatography has been introduced by Carr and co-workers [16,17,29]. Zirconia based column have received a great deal of attention recently because of their extraordinary stability under extreme thermal and chemical conditions. The outstanding stability of the zirconia can be explained in detail based on the physical properties of the zirconia structure. In the monoclinic oxide, each zirconium atom has coordinate bonds to seven neighbouring oxygen atom. In contrast, silica has only four bonds to oxygen atoms, which largely accounts for zirconia's superior resistance to chemical degradation, especially by acid and base [36].

In order to examine the stability of the zirconium, it was dissolved in the pH range from 1 to 14 and the results showed that there is no dissolution of zirconium in this wide pH range using inductively coupled plasma MS as the detection method. The ability to adjust the pH over a wide range can be quite critical in developing a good separation. Use of high and low pH is often helpful in improving band spacing