chemical derivatization in liquid chromatography

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CHEMICAL DERIVATIZATION IN LIQUID CHROMATOGRAPHY

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Preface

The concept of derivatization in liquid chromatography is relatively new. The introductory chapter is therefore intended to familiarize the novice in this field with the basic technique of using chemical reactions and labeling procedures to enhance the sensitivity, specificity and separation properties of liquid chromatography.

It is not our aim to produce another general book on liquid chromatography. The chapter on background is therefore rather brief and it touches on many areas which are not necessarily directly related to separation techniques but which are relevant to derivatization. This chapter should enable the practical worker to recollect some of the fundamental principles involved. The third chapter is concerned with instrumentation. While its scope may not be complete, this chapter enables the investigator to enter the area without the need for extensive library facilities. The final chapter is considered the most important one. It is practically oriented and permits the worker to solve some concrete problems.

The content of the application chapter has been limited essentially to the new aspects of derivatization in liquid chromatography. An account of reactions carried out in thin-layer chromatography (TLC) in order to render the zones visible has been kept to a minimum since the literature is abundant and most of these spraying, dipping or vapour-treatment techniques are not of quantitative analytical interest. We have also been quite brief on pre-separation reaction techniques used for TLC in bioanalytical studies (amino acids and peptides) during the past decade, since several reviews have appeared on this subject. For these reasons, this chapter does not cover all of the pertinent areas, but it should permit the user to generalize the principles and to extend the concept of derivatization for chromatography to groups of compounds and to problems of immediate interest to him and to become familiar with the literature. Many of the practical examples are given with sufficient detail to permit the investigator to reproduce a method without the need to resort to the original literature.

The selection of problem areas, and the level of treatment of fundamental principles, should render this book useful in many areas such as biochemistry, pharmaceutical and medicinal chemistry, geochemistry and also for interdisciplinary studies connected with environmental problem solving, *i.e.*, for all investigators concerned with the use of physical separation techniques for solving complex analytical problems.

We would like to thank all those colleagues who have contributed to this book through helpful discussions. Our thanks also goes to the many companies for supplying photographs and technical information. J.F.L. expresses his appreciation of the Bureau of Chemical Safety, Food Directorate of the Health Protection Branch, Department of National Health and Welfare, Canada, and R.W.F. of Sandoz Ltd., Switzerland, for supporting the effort of preparing the book. Miss Charlotte Laperrière and her staff and Miss Falge are thanked sincerely for their effort in handling the typing and correspondence. Finally, we would like to thank our wives for accepting so patiently our overtime work load.

Ottawa, 1975 J.F. LAWRENCE
Basel, 1975 R.W. FREI

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Chapter 1

Introduction

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The idea of chemically modifying a molecule in order to make it more suitable for a particular analytical procedure is certainly not new. Derivatization techniques have been used in mass spectroscopy (MS), nuclear magnetic resonance (NMR), ultraviolet (UV)–visible and fluorescence spectroscopy in solution, electroanalytical and radiochemical techniques, etc. Numerous reactions of varied selectivity have been adapted for the automatic analysis systems widely used in medical and pharmaceutical sciences. Gas chromatography (GC) has profited most from such procedures. In thin-layer chromatography (TLC), spraying and dipping techniques are used extensively for the detection of the chromatographic zones. Pre-separation reactions have been utilized to a lesser extent in liquid chromatography, although the usefulness of adopting UV and fluorescence derivatization for TLC in biochemical analysis was demonstrated more than a decade ago, *e.g.* for amino acids. Biochemical analysts were also the first to explore continuous reaction procedures for monitoring column effluents in classical or low-pressure liquid column chromatography.

Nevertheless, all these activities have been at a relatively low level in liquid chromatography (with the exception of visualization techniques in TLC), and it is with the emergence of modern techniques such as rapid TLC and particularly high-pressure liquid column chromatography (HPLC) that derivatization methods have become of increased interest to the scientific community. HPLC, which had its renaissance during the past few years, owes much of its success to the fact that it is complementary to GC. It enables the separation of compounds which are not sufficiently volatile and temperature stable to be analyzed by GC. HPLC often offers added selectivity because of the possibility of widely varying the mobile phase. From the foregoing it is clear that derivatization in liquid chromatography usually serves quite a different purpose from that in GC, where compounds are modified primarily to improve their volatility and/or temperature stability.

One of the major disadvantages of HPLC, despite all of the progress in the past years, is a serious lack of detectors, particularly universal detectors, which can match the sensitivity of GC detectors. The best HPLC detectors currently available are (spectro) photometric and (spectro) fluorimetric detectors. It seems logical, therefore, to solve the immediate detection problems at least partially by UV- and fluorescence-derivatization techniques. Recent activities by several research groups show an increased interest in such an approach. Derivatization techniques are not restricted to these two detection modes. Reaction products which yield good MS signals (a favourable fractionation pattern) are

already being considered. With the appearance of new detectors, for example radioanalytical, electroanalytical or electron-capture detectors, these techniques will also become important, *e.g.* in the oxidation of compounds to yield better polarographic signals at a dropping mercury electrode.

Some of the essential advantages of derivatization in liquid chromatography are summarized below:

(1) By labeling poorly detectable compounds with suitable chromophores, fluorophores or other activity enhancing groups, the detectability can be improved often to the level of GC.

(2) The selectivity that can be gained by using labeling reagents of varied reactivity and perhaps also with favourable spectral qualities. This becomes important in the analysis of a complex matrix such as encountered in polluted water samples, biological specimens, pharmaceutical preparations, etc. The derivatization step can then also serve as a clean-up procedure.

(3) The modification of chromatographic properties, unlike in GC, is usually of secondary importance. Often, however, a lowering of the polarity of certain molecules (*e.g.*, sugars) is observed which enables the separation on adsorption chromatographic systems with more convenient solvent systems. This in turn may decrease retention times and consequently improve detection limits.

(4) Improved chromatographic resolution (a better selectivity factor, α) can also result from the chemical modification step, since labeling with large molecules can, for example, enhance steric factors and facilitate separation of homologues.

In principle, two major classes of derivatization can be distinguished in liquid chromatography. The first is derivatization *in vitro* prior to chromatographic separation, which may be called "pre-separation techniques". For column chromatography this would be "pre-column derivatization". The second class is "post-separation techniques". For TLC, this includes all of the reactions carried out on the plate after development. In HPLC the reagents are added to the eluent stream in a continuous flow-through mode. This can be termed "post-column derivatization". In the following section the advantages and limitations of these two techniques are discussed briefly.

1.1 PRE-SEPARATION TECHNIQUES

The advantages of such techniques are summarized below:

(1) Unlike post-column derivatization, no restrictions are imposed by the solvent system used as mobile phase. The reaction conditions can therefore be chosen freely.

(2) The rates of the reactions are not usually limiting for *in vitro* reactions, whereas slow reactions can cause serious mixing and reaction-volume problems in post-column procedures. This effect is not very serious in TLC.

(3) Derivatization prior to chromatography can be used as a pre-clean-up step; selective reagents and extraction procedures can result in the elimination of much interference.

(4) Excesses of reagents can be usually eliminated easily in contrast to reactions after separation.

(5) Chromatographic properties such as retention time and resolution can be improved.

(6) The choice of appropriate labeling reagents can result in suitable fractionation patterns in MS evaluation of the derivatives.

On the other hand, the limitations are quite obvious:

(1) Formation of artifacts or of several derivatives of one compound can occasionally occur.

(2) The reactions must be quantitative, or at least reproducible, in both standard and analysis assays.

Reactions such as the well known dansylation for fluorescence labeling of amino acids, or benzoylation for UV derivatization of steroids, are easily classified under this heading. The formation of agglomerates such as ion pairs (charge-transfer complexes) or metal chelates should also be classified under pre-separation techniques, particularly when these species are formed *in vitro*, then extracted and injected or spotted as such in the chromatographic system. The advantages, particularly of ion-pair chromatography, include the possibility of selective extraction procedures. This can enhance the specificity and serve as a pre-clean-up step. Ion-pair formation is complementary to true pre-column derivatization, in cases where reactive sites for substitution are lacking but where Lewis-acid or -base activity is apparent. The possibility of the formation of artifacts is small. By choosing counter ions with suitable chromophores, fluorophores or polarographic activity, one can improve the detectability of systems. Separation systems can be tailor-made to fit a particular separation problem, since with the appropriate choice of counter ion one can improve resolution properties. One final but very important point is the non-destructiveness of the approach. This renders ion-pair chromatography particularly useful in preparative separations, where the intact compound of interest can be recovered for further studies (structure elucidation, toxicology, pharmacology, etc.).

1.2 POST-SEPARATION TECHNIQUES

This approach has been used extensively for amino acid analysis using low-pressure ion-exchange chromatography and post-column ninhydrin reaction. Spraying, dipping and vapour-treatment techniques are well known as post-separation reactions in TLC, but these are considered only briefly since the majority of them are not quantitative. While the problems of pre-separation techniques are quite similar for TLC and HPLC, they differ considerably for post-separation reactions.

The advantages of post-column procedures are summarized below:

(1) The formation of artifacts is not very likely.

(2) Different detection principles can be utilized simultaneously. It is possible, for example, to use a UV detector immediately after the column. A fluorescence-generating reagent can then be added followed by fluorescence detection. This permits detection of substances with a poor UV chromophore or may discriminate interferences.

The limitations of this mode of operation can be quite numerous:

(1) The eluent can strongly influence the reaction medium. In many cases this can lead to a serious limitation in the choice of mobile phase due, for example, to solubility problems with the reagents or reaction products or to possible side reactions with the solvent itself.

(2) In HPLC, high flow-rates occur through small columns. Therefore the rates of post-column reactions must be relatively rapid (< 30 sec), otherwise considerable dead-volume and reaction-volume problems ensue. The use of heating devices or of catalytic effects can reduce this problem in some instances.

(3) A strict condition is that the derivatization reagent must not be detectable under the conditions used for detection of the derivative.

These and other technological difficulties may explain why only few applications of this derivatization mode have appeared in the literature to date. However, a substantial increase in work in this area may be expected in the near future arising from the knowledge of automatic analysis systems.

Chapter 2

Background

CONTENTS

2.1 CHROMATOGRAPHIC PRINCIPLES

2.1.1 Adsorption

The major factor in the chromatographic separation of molecules by an adsorption process is intermolecular forces. These may be divided into Van der Waals and London forces, which exist between the surface and the adsorbed molecules, and electrostatic forces resulting from molecular polarity. Charge-transfer forces between electron donors and acceptors, and hydrogen bonding, are also considered to be adsorption (chemisorption) processes but these are normally dealt with separately as charge-transfer chromatography and ion-exchange chromatography. An important feature of physical adsorption is that it is a rapid reversible process which is necessary for fast mass transfer between the mobile phase and the stationary phase thus permitting efficient separations. Such reversibility is very dependent on the choice of solvents and adsorbent surfaces.

The separation of compounds is achieved by their relative difference in adsorptive strength for the given adsorbent and solvent system. This is often referred to as a difference in adsorption isotherm. An adsorption isotherm is defined as an isothermal plot of the equilibrium quantity of compound taken up per unit weight of the adsorbent *versus* the concentration of compound in the mobile phase. The greater the equilibrium quantity of sample adsorbed for a given concentration of sample in the mobile phase, the slower is the migration through the chromatographic system. Fig. 2.1 shows examples of adsorption isotherms and the corresponding solute band shapes [1]. Isotherm B is considered ideal and a true gaussian distribution of solute exists in the linear portion of the isotherm. Isotherm A is the most commonly encountered type in actual situations. The solute band shape is a slightly skewed gaussian distribution with the tailing following the peak as it passes through the chromatographic system. Modern high-speed liquid chromatography

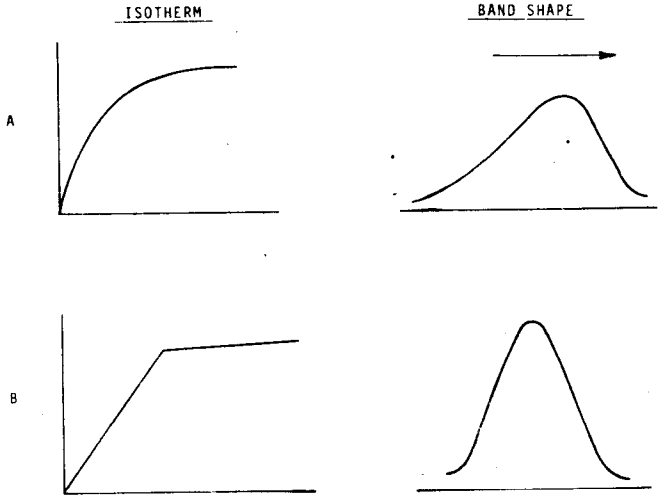

Fig. 2.1. Adsorption isotherms and the resulting band shape.

with its high efficiencies and specially designed solid support materials enables isotherm B to be approximated.

If the isotherm is linear, the migration-rate of the solute through the system is directly related to the gradient of the isotherm, S. The total volume, R, required to carry the solute through the system may be described as

$$R = WS + V \tag{2.1}$$

where W is the weight of the adsorbent in the column and V is the void volume of the column (equal to the volume of the mobile phase in the column). For analytical work it is necessary that R is constant over the range of sample concentration considered. Thus, in a specific system, reproducible migration rates can be obtained. If the linear portion of the isotherm is exceeded, overloading occurs which adversely affects sample migration, band shape and chromatographic separation.

The most common adsorption systems consist of silica gel or alumina adsorbents in association with an organic solvent system. The adsorbent can exert a considerable influence on the separation of compounds. Alumina and silica gel, for example, have significantly different properties and can result in quite different separations. Activation of the adsorbent also influences sample retention. The presence of water on the adsorbent decreases the adsorbent activity due to blockage of active sites. If large quantities of water are present, a partition system may be set up which may extensively change the retention times due to the different chromatographic principle involved. Table 2.1 compares results obtained for the separation of the insecticide carbaryl (Sevin) and its hydrolysis product 1-naphthol on alumina and silica gel. Comparisons between activation and deactivation are made. The results show that separation of the two components is reversed with the two adsorbents examined. In most cases, activation of the plates caused the R_F values to increase relative

TABLE 2.1

R_F VALUES OF CARBARYL (SEVIN®) AND 1-NAPHTHOL

Solvent systems: A, chloroform; B, chloroform–nitromethane (1:1). The R_F values are the average of those obtained from six spots.

Adsorbent	A		B	
	Carbaryl	1-Naphthol	Carbaryl	1-Naphthol
Alumina + binder*				
activated	0.89	0.08	0.94	0.12
unactivated	0.69	0.09	0.93	0.34
Silica gel + binder (G)				
activated	0.25	0.50	0.87	0.95
unactivated	0.15	0.27	0.84	0.95
Silica gel, no binder (H)				
activated	0.43	0.60	0.84	0.94
unactivated	0.20	0.33	0.80	0.91

*Tailing was observed for 1-naphthol.

to those obtained with unactivated layers. These results seem to indicate that, for the system studied, the presence of water tends to hold back both compounds.

The mobile phase plays an important role in the separation of components. Often multicomponent mixtures are required to achieve the desired separations. Much work has been done on the TLC separation of, for example, amino acids. Numerous solvent systems have been developed for such purposes, and more than one solvent system is usually necessary before separation of all of the components is achieved. Two-dimensional chromatography is often required; such a separation is shown in Fig. 2.2.

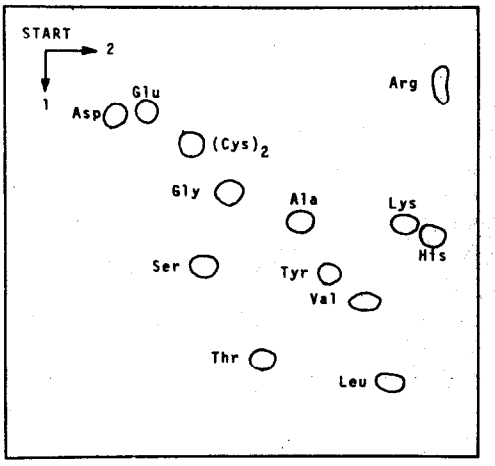

Fig. 2.2. Separation of some amino acids by two-dimensional TLC on cellulose MN-300. Solvent systems: (1) *n*-butanol–acetone–diethylamine–water (10:10:2:5); (2) isopropanol–formic acid–water (20:1:5).

The temperature may influence some separations, since equilibria are involved in the chromatographic process. It is often necessary to immerse tanks in constant-temperature baths in order to reproduce difficult separations.

2.1.2 Liquid–liquid partition

The separation of compounds by their differential partition between two immiscible phases is the basis for partition chromatography. The system consists of a stationary liquid phase coated on an inert solid support, and an immiscible mobile phase. Chromatographic separations are based on the different equilibrium distributions of the samples between these two phases. The greater the quantity of substance in the stationary phase at equilibrium the slower is the migration. For analyses, this equilibrium must remain constant over a suitable concentration range. Thus an increase in the concentration of solute results in a linear increase in the concentration of solute in the mobile and stationary phase, respectively. Under these conditions, the retention time, t_R, is independent of the amount of sample chromatographed and a symmetrical peak (gaussian band) is observed.

t_R is related to the retention volume, V_R, via the flow-rate, F ($V_R = t_R F$), and V_R is

directly proportional to the velocity of the mobile phase, v. An important parameter is V_M, the elution volume for the non-retained components in the column. The basic retention equation is

$$V_R = V_M + KV_S \tag{2.2}$$

where V_S is the volume of the stationary phase and K is the equilibrium distribution coefficient of the solute (ratio of concentrations in the stationary and mobile phases). Another important parameter is the capacity factor, k', which is a measure of the chromatographic distribution behaviour. Experimentally, k' is computed from

$$k' = (t_R - t_0)/t_0 \tag{2.3}$$

where t_0 is the elution time of the non-retained solute. This is related to the column length, L, and the velocity of the mobile phase, v, by the equation, $t_0 = L/v$. Substituting in eqn. 2.3, the retention time is defined by:

$$t_R = L\,(1 + k')/v \tag{2.4}$$

Eqn. 2.4 shows the influence of L and v on t_R. Other factors which affect t_R are k', which depends on the separation mechanism, and V_S and V_M which indicate the relative amounts of the stationary and mobile phase.

The resolution, R_s, of two peaks is governed by the distance between the peaks and the width of the bands, and can be defined as

$$R_s = 2\,(t_{R_1} - t_{R_2})/(w_1 + w_2) \tag{2.5}$$

where w_1 and w_2 are the band widths of peaks 1 and 2, respectively. The larger the value of R_s, the better is the separation. Since the separation of peaks is directly proportional to the column length, L, and band broadening only to the square root of L, a longer column can improve separation but at the expense of time.

The "theoretical plate" concept in chromatography is a popular approach to determining column efficiency (relative band broadening in the column). The number of theoretical plates, N, is related to the retention time and to the width of the solute peak by

$$N = 16\,(t_R/w)^2 \tag{2.6}$$

For a column of a given length, N increases if w decreases relative to t_R at a constant flow-rate. This means that columns which produce narrow peaks have large numbers of theoretical plates. Since narrow peaks indicate good efficiency, it is preferable to create as many plates as possible in a given column. The velocity of the mobile phase plays a significant role in column efficiency. Figure 2.3 shows how the velocity of the mobile phase affects the height equivalent to a theoretical plate (defined as HETP = L/N). Thus the column efficiency decreases with increasing HETP.

Partition systems usually consist of polar stationary phases with less polar mobile phases. The two phases must be immiscible since removal of the stationary phase from the adsorbent by the mobile phase would otherwise occur. In HPLC, for example, oxydipropionitrile (OPN) has been used as stationary phase for partition systems [2–4]. These systems

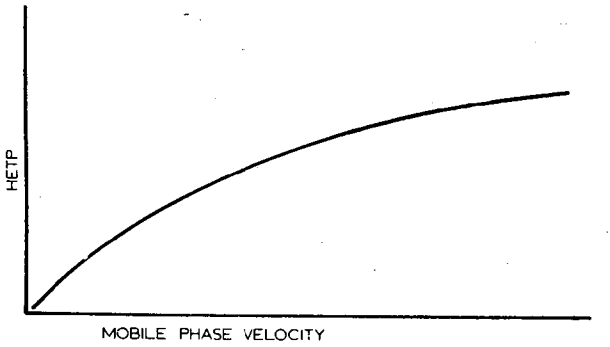

Fig. 2.3. Efficiency (HETP) plotted against the velocity of the mobile phase.

required that the mobile phase was pre-saturated with OPN and that a pre-column containing a high loading of OPN was placed before the analytical column in order to ensure saturation of the mobile phase and the prevention of "bleeding" of the stationary phase.

Commonly used partition systems for TLC are cellulose, hydroxyapatite, some silica gels and Kieselguhr (diatomaceous earth). Paper chromatography is generally considered to function predominantly as a partition system, although other processes such as adsorption and ion exchange are thought to occur to a small extent. The reason that these solid supports tend to behave as partition systems is that they hold water strongly. Thus chromatography on cellulose is accomplished by partitioning of the solute between the strongly held water layer and the mobile liquid. Activation of silica-gel plates for adsorption chromatography removes the adsorbed water from the surface in order to prevent this partitioning effect, and to make available more active sites for adsorption.

Reversed-phase chromatography is the term commonly applied to a system where a non-polar liquid phase is coated on the solid support and elution carried out with an immiscible polar phase. Such systems are often necessary for separations which cannot be carried out by normal partition or adsorption chromatography. For TLC, the stationary phase is normally a liquid of high boiling point which does not readily evaporate from the adsorbent. Paraffin oil, silicone oil or *n*-tetradecane coated on silica gel or Kieselguhr are frequently used with water-based mobile phases such as acetone–water (3:2) or acetic acid–water (3:1). Reversed-phase chromatography is very useful for the TLC analysis of lipids and related compounds.

Many applications have been found for reversed-phase chromatography in HPLC. The composition of the stationary phase is more easily controlled than with the TLC methods, and thus provides more reproducible separations. The use of bonded non-polar phases enables gradient elution to be carried out in a reversed-phase system. This approach has been useful for the analysis of polar compounds and gives improved separations compared with normal-phase HPLC. These methods usually involve separation with systems consisting of Carbowax, C_{18}-polymer or similar phases bonded or physically coated on the support. (The physically coated support may require a pre-column to ensure establishment of equilibrium, as described above.) The mobile phase is usually water–methanol in various ratios, or, in the case of bonded phases, a gradient proceeding from water to methanol. A list of some chemically bonded reversed phases is given in Chapter 3.

2.1.3 Ion exchange

2.1.3.1 General

The principle of ion exchange is based on the fact that some solid materials exchange ions when in contact with a solution. The most commonly used materials are the resin ion exchangers. These have replaced most of the older aluminosilicate or hydrated oxide materials, and are available in a number of types and particle sizes.

The separation of compounds by ion exchange depends on the differences in electrostatic field strength around the sample ions and on the activity coefficients of the resin. However, these are not the only factors involved since many anomalies have been found. A rigorous mathematical treatment of ion-exchange chromatography would be rather complex and well beyond the scope of this text. A brief description of the principles of ion-exchange chromatography is given below.

The column is pre-saturated with a weakly bound ionic species (say, W). The components to be separated are placed at the top of the column where they replace the W ions of the column. The column is then eluted with a mobile phase containing an excess of W ions. At equilibrium the sample ions are in a constant state of motion between the ion exchanger and the mobile phase. When a sample ion leaves the column material and enters the mobile phase a W ion takes its place, thus returning the ion-exchange site to its original form. Meanwhile, the sample ion is moved down the column by the mobile phase before it returns to the ion exchanger to replace another W ion. In this manner, the mobile phase containing the W ions slowly transports the sample ions down the column at a rate which is dependent on their distribution coefficients between the mobile phase and the ion exchanger. Sample ions which are strongly bound will take longer to migrate than weakly bound species.

Ion-exchange chromatography is well suited to many inorganic salts, chelates and organic complexes. It is also useful for very polar organic compounds which are not effectively separated by adsorption or partition chromatography. Selectivity can be achieved by changing the resin material, or by varying the ion-exchange sites and the types and concentrations of the ions in the mobile phase. Such selection depends on the molecular structure and ion-exchange behavior of the compounds to be analyzed. If the separation of acidic components is desired, the use of strongly basic resins should be avoided since these require large volumes of eluent to remove the sample components. Also, extremely basic ion exchangers are difficult to activate fully and to free from strongly held ionic impurities such as chloride ions.

Ion-exchange resins also have a tendency to swell significantly under certain conditions. Acidic ion exchangers often increase in size when converted to the metal salt form, since bound hydrogen ions cover only a small area of the exchanger in the acidic form, whereas strongly bound metal ions such as sodium which have larger ionic radii than hydrogen ions force the resin to spread. Such swelling can adversely affect column efficiency and result in poor separations. The same is especially true of the reverse process, that of shrinking when ions are exchanged. Shrinking can create voids which may cause excessive channelling and poor mass transfer between the exchanger and the mobile phase. Many ion-exchange chromatographic separations require gradient elution