Reversed phase chromatographic separations of cationic and anionic metal complexes

by

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ABSTRACT

Separation of a variety of mixtures of cationic chromium(III) complexes and anionic cobalt(III) complexes by Ion-pair Reversed Phase HPLC is reported. Achieving separation, or rather improving the resolution of mixtures of highly charged anionic Co complexes, was of major concern.

To improve resolution, a number of mobile phase parameters are manipulated. They include changing the organic solvent composition, the length and the number of alkyl chains of the ion interaction reagent and the concentration of the ion interaction reagent. A higher concentration of organic solvent improves the resolution by narrowing the peaks, but the other two parameters do not change the resolution significantly.

Resolution is further improved by minimising peak tailing through masking of the active sites on the stationary phase. For this, a competing ion is incorporated in the above method. In this way a dramatic improvement in resolution is achieved within very short elution times and with very low amounts of organic solvent in the eluent. This method gives complete separation of

anionic Co complex mixtures but has failed so far to separate geometrical isomers.

The effect of the nature and the concentration of the competing ion on resolution is also investigated. Peak splitting is observed with higher concentrations of the competing ion in the eluent. Finally, the effect of the temperature and the sample size on peak splitting is studied.

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LIST OF ABBREVIATIONS

AA	bidentate ligand
CRT	cathode ray tube
en	ethylene diamine
HPLC	high performance liquid chromatography
IEC	ion exchange chromatography
IIR	ion interaction reagent
ODS	octadecylsilane
pn	1,2-diaminopropane
RP	reversed phase
tn	1,3-diaminopropane
UV/Vis	ultraviolet/ visible

To My Parents

Introduction

Separation of an analyte from potential interferences is an important step in analytical procedures. One of the most widely used means of analytical separations is Chromatography.

Chromatography is a process by which different types of molecules can be separated from one another. A sample mixture is introduced onto a bed of finely divided "stationary phase" which can be either a solid or an immobilized liquid, and swept through at an optimum rate by a fluid gas or liquid which is referred to as the "mobile phase". In this way the sample components are made to interact with the two phases. The interactions of sample components with the stationary phase and the mobile phase differ in magnitude for different sample components, so that their rate of passage through the stationary phase bed differs and separation is thus achieved. The mobile phase introduced on to the stationary phase bed is referred to as the "eluent" and the fluid which leaves the end of the

stationary phase bed, with eluent plus solutes is called the eluate. Solutes in the eluate can be detected by different means depending on the sample components and can be subsequently characterized by a variety of analytical techniques. In a given chromatographic system, the time taken to elute a specific substance from the stationary phase is in principle a constant, and is called the retention time(t_R) of that substance.

In early work, chromatography was restricted to the separation of colored substances on a column (the word chromatography is derived from the Greek word "chromatos" meaning color). The polish botanist Tswett used this method in 1907 to separate the pigments in green leaves. He passed extracts of plant pigments through a chalk column, using petroleum ether as a solvent, and was able to isolate two distinct "chlorophyllins" which he designated Chlorophyllin α and β.

Liquid Chromatography(LC) is a version of Chromatography, in which the mobile phase is a liquid. Early Liquid Chromatography, including Tswett's original work, was carried out in large diameter glass columns using the pressure difference due to gravity to facilitate the flow of the eluent. The analysis times were long and the procedure was tedious. Since the 1960's, attempts to speed up the classic procedure resulted in the evolution of "High Performance Liquid Chromatography" (HPLC).

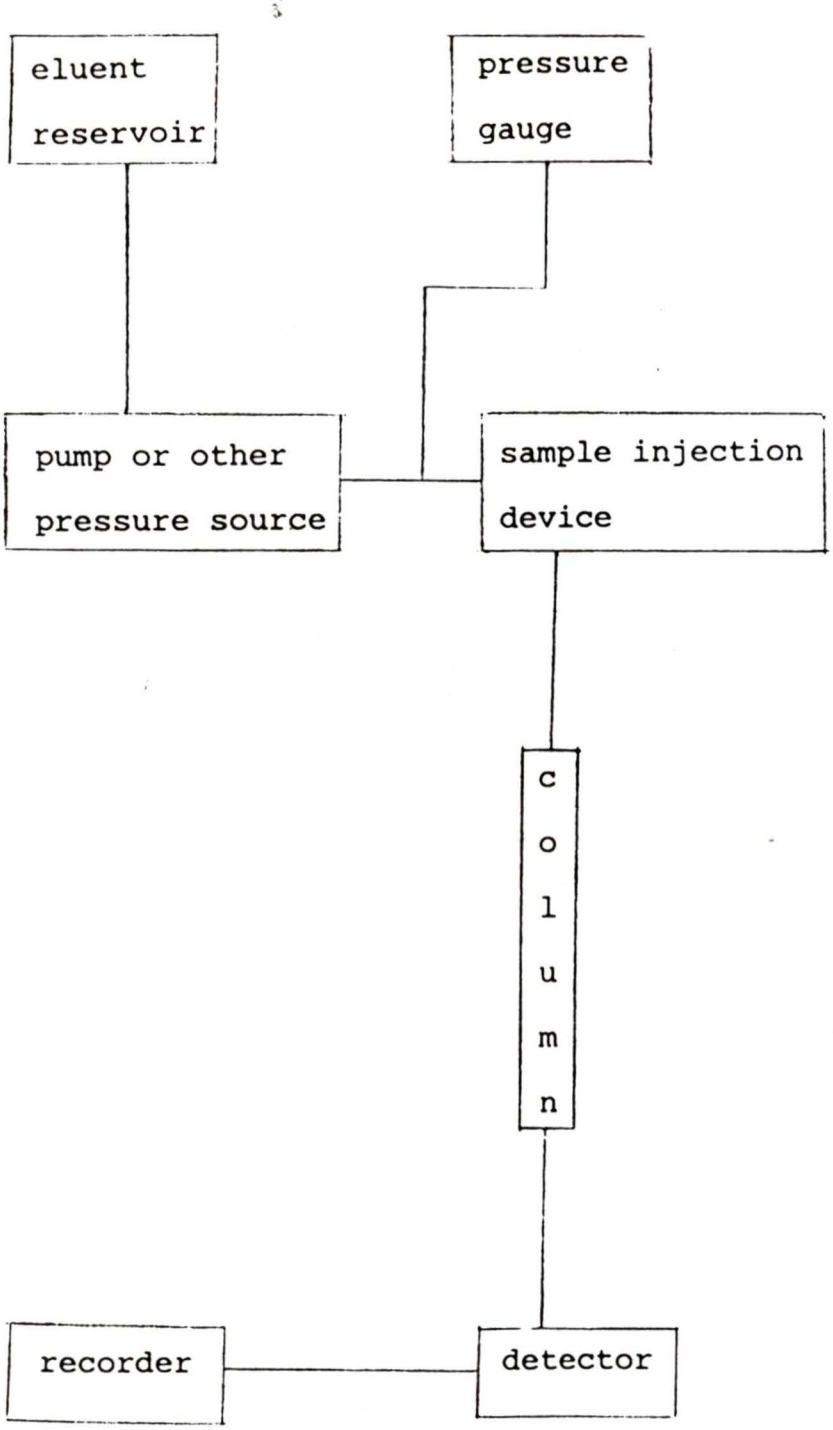


Fig. 1. Diagram of the HPLC apparatus.

The major increase in column efficiency is obtained by a decrease in particle size of the column packing which in turn requires high pressure pumping in order to achieve reasonable eluent flow rates. A typical experimental set up is shown schematically in fig. 1.

HPLC is one of the fastest growing analytical techniques due to its very high sensitivity, ready adaptability to accurate quantitative determination, its ability to separate non-volatile species and the extremely short analysis times obtainable.

A. TYPES OF HPLC

In normal phase HPLC the stationary phase is polar in nature (eg. silica) and the mobile phase is nonpolar, so that polar substances are retained on the column longer than less polar or non polar materials. Reversed-Phase(RP) HPLC is the inverse of this. The stationary phase is nonpolar. It is normally prepared by chemically attaching linear organic molecules or organo silanes on to the surface hydroxyl groups of the silica particles and is therefore called a bonded phase. The most widely used bonded phases include octadecylsilane (ODS/C_{18}) and octylsilane (C_8). The mobile phase in reversed-phase mode is a polar liquid. Thus the more non polar the analyte is, the longer it will be retained.

B.SEPARATION MECHANISM

Although for convenience bonded phases are usually classified under partition chromatography (i.e., the solute dissolves in both the stationary and the mobile phases), the separation mechanism, particularly in the case of reversed phase chromatography with alkyl silyl phases, is not purely partition[1].

It is believed that there are at least three separation mechanisms occurring simultaneously: adsorption, partition and surface tension. Adsorption chromatography depends upon specific interactions of the solute with the surface of the solid stationary phase. The surface tension mechanism has been utilized for separation of moderately and highly polar substances[2]; these separations are accomplished by utilizing the surface tension properties of the mobile phase. In this case the adhesive forces between the sample molecules and the stationary phase can be reduced by using less water in the mobile phase in order to facilitate the elution process.

C.MODES OF ELUTION

In liquid chromatography one can use either a single solvent or a mixture of solvents of constant composition as the mobile phase. This mode of elution is called isocratic. In contrast the gradient mode of elution utilizes a linear

or nonlinear change in the polarity of the mobile phase during analysis. This mode is very useful for shortening the time of analysis if the sample components have widely different polarities.

For reversed phase chromatography, the more polar the solvent, the "weaker" an eluent it is considered to be. Water is therefore the "weakest" solvent commonly used and yields the longest retention times. Where aqueous/organic mixtures are employed, increasing the concentration of the less polar solvent in the eluent decreases the retention time.

D.ION-INTERACTION CHROMATOGRAPHY

Ion-Interaction Chromatography (also called Ion-Pair Chromatography) is a technique which uses a reversed phase packing material in combination with a hydrophilic eluent containing an organic modifier (the less polar organic solvent used in conjunction with water) and a small concentration of an ionic, hydrophobic substance such as a detergent. A hydrophobic substance is chosen which has the potential to form an ion-pair with an ionized form of the solute. Thus for anions, an eluent containing a cationic detergent would generally be used. To ensure that the substance of interest is in the preferred ionic form, the pH of the solution may have to be controlled. This method has become well established as a successful

technique[1-12,14,15,37-52]. It is widely used for organic ions and has the advantage that a number of mobile phase parameters can be manipulated in order to control the retention and separation of the solute ion mixtures. These parameters include the organic modifier content, the concentration and the type of ion-interaction reagent, and the addition of competing ion species as discussed later.

Several mechanisms have been proposed to describe the retention of the solutes in this mode of liquid chromatography. The first view suggests that "ion-pairs" form between analyte ions and the hydrophobic counter ions, and these ion pairs, being neutral and nonpolar, absorb on to the nonpolar stationary phase[3,4]. The second view suggests an ion exchange mechanism[5-8], in which the counterions are first sorbed on to the stationary phase and these charged sites serve as exchange sites for the analyte ions. Since neither the ion pair nor the ion exchange mechanism individually explains all the experimental results, a third model has been proposed[9] which does not require ion pair formation in either phase and is not based on classical ion exchange. This model assumes dynamic

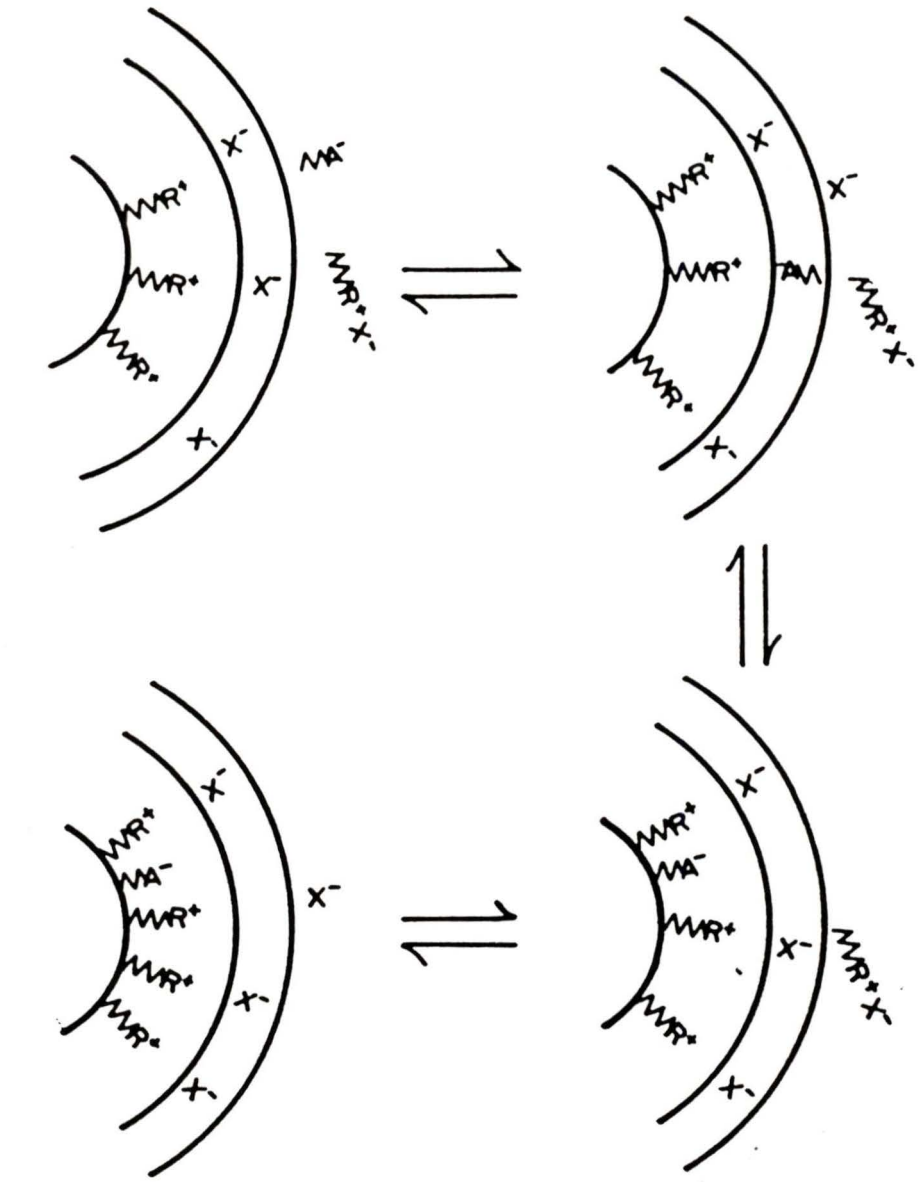


fig. 2. One of the proposed retention mechanisms for Ion-Pair LC.

equilibrium, which is affected by electrostatic, eluphilic or adsorbophobic (i.e. solute attracted more towards the eluent than towards the stationary phase) and eluphobic or adsorbophilic (i.e. solute attracted more towards the stationary phase than towards the eluent) forces. According to this theory the hydrophobic ion of the IIR occupies the primary layer adsorbed onto the non polar stationary phase while the counterion occupies the secondary layer. Thus a dynamic equilibrium is established between this electrical double layer, the hydrophobic salt which is dissolved in the mobile phase and the analyte ion (fig.2). The addition of a negative charge to the positively charged primary ion layer has the net effect of neutralizing a positive charge from this layer. To restore the electrostatic equilibrium, another positive ion can be adsorbed and contribute to the charge of the primary ion layer. However, the net result is that a pair of ions has been adsorbed on to the stationary phase.

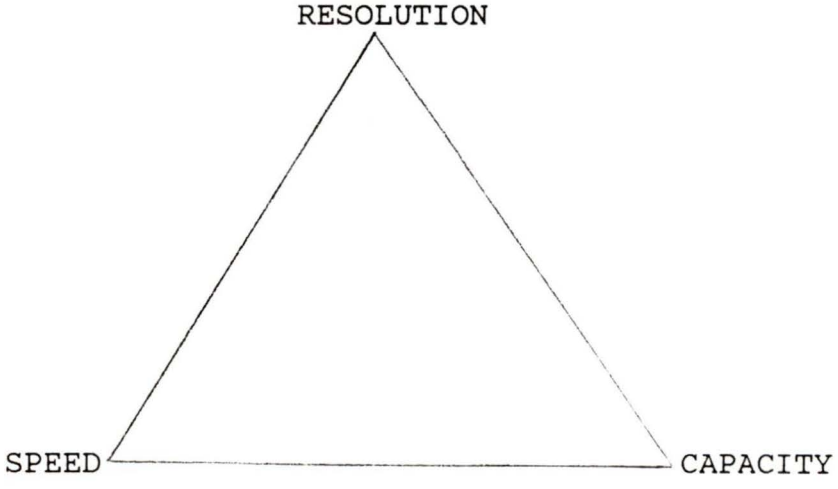
Since this process causes a decrease in the concentration of Ion Interaction Reagent (IIR) in the eluent, it results in a negative peak in the chromatogram if the detector is sensitive to the IIR (for instance, with the IIRs which absorb the light of the detector wavelength when the detector is a UV/Vis spectrophotometer, a negative peak is seen right after the solvent front).

Once absorbed onto the stationary phase, the negatively charged solute ion will tend to be held by electrostatic forces between primary ion layer and solute. For a given concentration of IIR and a given ionic group on the species being eluted, the more adsorbophilic (eluphobic) the hydrophobic counterion (R^+), the greater will be the retention. Thus a greater retention can be achieved with longer carbon chain IIRs.

However the retention mechanism of ion-interaction chromatography is still the subject of some dispute[4].

E. CHROMATOGRAPHIC THEORY[10,11]

A successful chromatographic separation involves making a compromise between chromatographic resolution(R), sample capacity and analysis time as illustrated in the chromatographer's triangle.



Thus it is possible to alter the experimental conditions of a separation to improve one of these qualities at the expense of the other two.

Qualitative understanding of chromatographic theory aids in rapidly optimizing a separation.

All chromatographic separations are based upon differences in the extent to which solutes are partitioned between the mobile and the stationary phase. The equilibrium involved is quantitatively described by means of a thermodynamic partition or distribution coefficient(K), defined here as:

$$K = C_s/C_m \qquad (1)$$ where

C_s = concentration of solute in stationary phase

C_m = concentration of solute in mobile phase

1.Efficiency

According to the "plate theory" of chromatography, the equilibration of the solute between the mobile and the stationary phase is assumed to take place at a series of discrete, narrow, horizontal layers/zones of the packing material within the column, which are called "Theoretical plates". The efficiency of a chromatographic column as a separation device improves as the number of equilibrations increases, that is as the number of theoretical plates(N)

increases. Alternatively, for a given column, the column efficiency is considered to be higher as the thickness of such a layer/zone (i.e. Height Equivalent of a Theoretical Plate or HETP/H) becomes smaller. Thus N and H are related to each other as follows;

$$N = L/H \qquad (2)$$ where

L = length of the packing material

According to the rate theory of chromatography, which considers the rate at which several mass transfer processes occur during migration of a solute along the column, the HETP can be controlled by the adjustment of several experimental variables. These variables include the particle size of the packing, the thickness of the stationary phase (when the stationary phase is a liquid which been coated on solid beads), the viscosity of the mobile phase, the temperature and the linear velocity of the mobile phase. A decrease in the first three variables leads to a decrease in HETP and thus to an increase in column efficiency. The latter two variables affect the column efficiency in a different way (i.e. an optimum column temperature or an optimum velocity must be selected to achieve a minimum H).

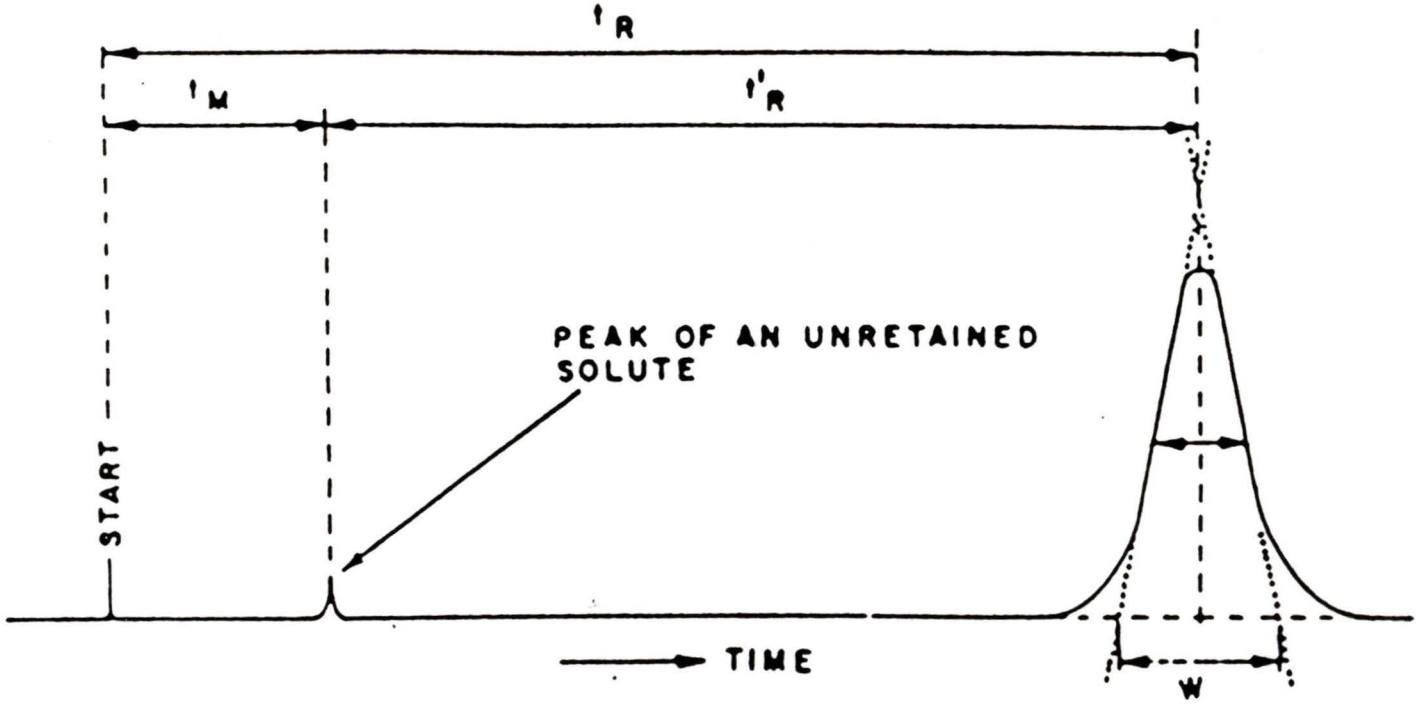


Fig. 3. A typical chromatogram showing the elution of a solute. (t_R = retention time, t'_R= adjusted retention time, t_M = retention time of non-retained solute, W = peak width)

Since the distribution of a solute in a chromatographic column follows random motion, the chromatographic peaks broaden in time. Thus, considering the chromatographic bands/peaks of a chromatogram (refer to fig.3) to be gaussian, the efficiency of a column is defined in terms of variance(σ) per unit length of column.

Thus,

$$H = \sigma^2/L \qquad (3)$$

and

$$N = L^2/\sigma^2 \qquad (4)$$

Therefore, for the experimental evaluation of N and H, the following equation is used, which is obtained by relating the solute retention time to the variance.

$$N = 16(t_R/W)^2 \qquad (5)$$ where

W = peak width

t_R = retention time of the solute (see fig. 3)

The peaks become broader if the solute is retained longer, because a larger volume of eluent is used and the solute sample is therefore diluted.

2.Thermodynamic variables

In addition to the kinetic variables which have been discussed so far there is a set of thermodynamic variables affecting the separation, such as capacity factor(k') and selectivity factor(α).

The capacity factor (k') basically describes the extent to which the solute is retained and is defined as follows;

$$k' = (t_s - t_m)/t_m \qquad (6)$$ where

t_s = retention time of the solute in the stationary phase

t_m = retention time of a non-retained solute

Therefore

$$k' = C_s V_s / C_m V_m \qquad (7)$$ where

V_s = volume of solute in stationary phase

V_m = volume of solute in mobile phase

C_s = concentration of solute in stationary phase

C_m = concentration of solute in mobile phase

Thus,

$$k' = K V_s / V_m \qquad (8)$$

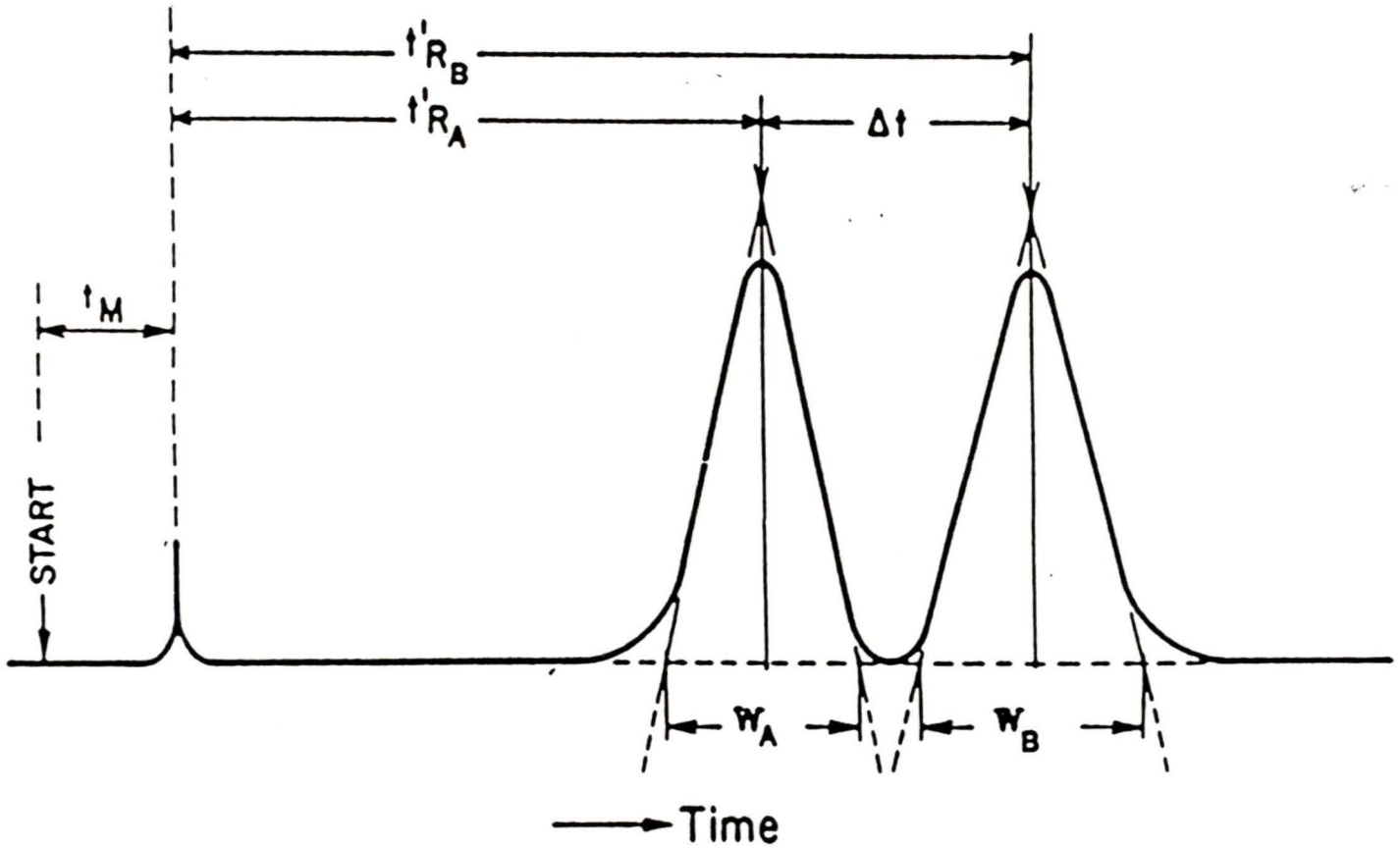


Fig. 4. A typical chromatogram showing the elution of 2 solutes, A and B. (t_M = retention time of non-retained solute, t_R' = adjusted retention time, W = peak width)

Experimentally, k' is defined as follows:

$$k' = (t_R - t_m / t_m) \quad (9)$$

The ability of a column to resolve two solutes is related to the relative magnitude of the partition coefficients of the two species.

The selectivity factor(α) for a separation of two solutes is defined as follows:

$$\alpha = K_B/K_A = k'_A/k'_B \quad (10)$$

Using a chromatogram as shown in fig.4, α is experimentally defined as follows:

$$\alpha = \{(t_R)_B - t_m\}/\{(t_R)_A - t_m)\} \quad (11)$$

The resolution(R) of the two solutes is quantitatively defined, using the same chromatogram, as follows:

$$R = \Delta Z/(W_A/2 + W_B/2) \quad (12)$$

Assuming we are dealing with 2 solutes A and B having retention times that are close enough to one another,

$$W_A \approx W_B = W$$

Therefore,

$$R = \{(t_R)_B - (t_R)_A)\}/W \qquad (13)$$

It can be shown that,

$$R = \sqrt{N}/4 \ \{(\alpha-1)/\alpha)\}\{k'_B/(1+k'_B)\} \qquad (14)$$

(i.e. The product of an efficiency term, a selectivity term and a capacity term respectively.)

This is a significant equation because it serves as a guide to the choice of conditions that allows the chromatographer to achieve the desired separation in a minimum time.

3.Optimization

In optimization of the column performance, in order to achieve a desired separation, k'and N can be adjusted more or less independently.

As discussed earlier, N could be altered by changing several parameters, among which the most convenient in general for a given column is changing the flow rate to minimize H. In LC however, the effect of flow rate on H is not very significant.

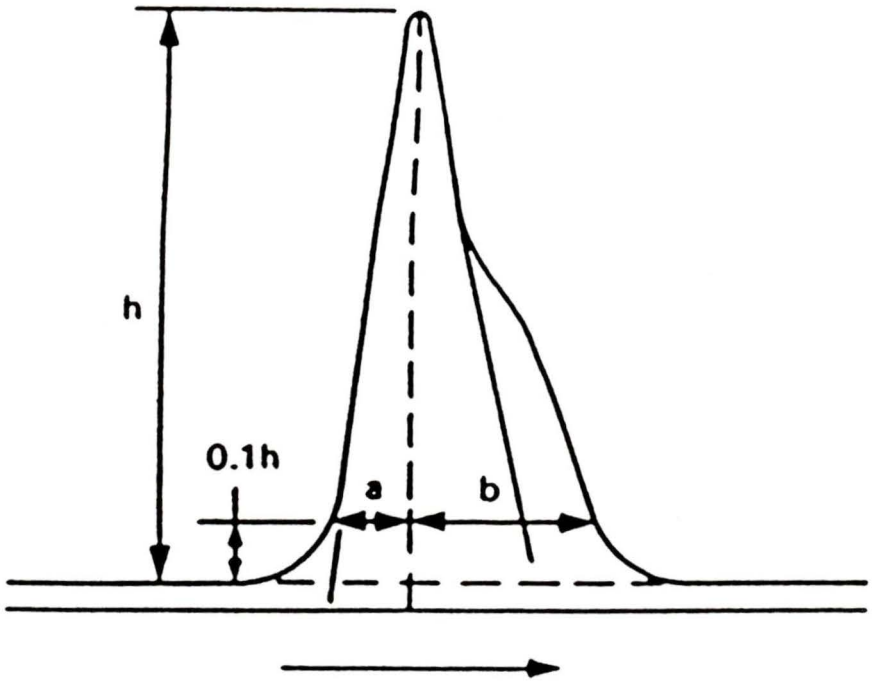


Fig. 5. Definition of symmetry measurement.

%S = a/b x 100 where %S = percent symmetry.

k' can be varied easily by varying the mobile phase composition. Less conveniently, a different type of column packing can be employed.

In seeking the parameters which affect the resolution, another important parameter which must be considered is the symmetry factor of a peak. Although, previous theory assumed gaussian peaks, if the distribution of the solute in the stationary phase is not random, the peaks show asymmetric tails. Therefore, the parameter N, which has been introduced assuming gaussian peaks, becomes no longer useful. Since the resolution (R) is dependent upon the peak width, if the peaks emerge with long tails the resolution decreases. A peak symmetry factor can be quantitatively defined as shown in fig.5. (a and b are measured at 10% peak height.)

The experimentally controllable parameters which can be used to govern the retention characteristics in Ion-interaction chromatography include the type and concentration of the IIR, the type and concentration of the organic modifier used in the eluent, and the type and concentration of added salts as discussed later.

These parameters are normally used to change the capacity factor (k') which in turn could change the resolution (R) according to the equation 14. The longer the carbon chain length and the higher the concentration of the ion-interaction reagent, the greater will be the

capacity factor. The more polar and the higher the concentration of the organic modifier, the lower will be the capacity factor in reversed phase mode. Salts may be added to the eluent, which normally decreases the capacity factor. The effect is more pronounced with organic salts compared to inorganic salts[12]. Furthermore, this effect is enhanced with increasing the concentration of the added salt.

F.CONSIDERATIONS AND OBJECTIVE OF THIS WORK

The use of chromatographic techniques for the separation and identification of charged metal coordination complexes is still in its infancy. Although ion exchange chromatography (IEC) has proved useful in separating isomeric coordination metal complexes of increasing complexity and charge, and has allowed the resolution of certain enantiomeric complexes when used with optically active eluents[13], such separations are generally tedious. Especially if the solute ions are highly charged, long columns and many hours are required to effect the complete or partial separation of related species bearing a similar overall charge. Attempts by several workers[14,15], to use high performance ion exchange chromatography (HPIEC) have in general been disappointing. Resolutions could be marginally improved over IEC but long elution times were still required. Also, the additional care and time

required to set up the experiments have limited its usefulness for analytical and repetitive investigations.

On the other hand studies using inert supports such as silica and non polar solvents (i.e. adsorption chromatography) have been largely reserved for uncharged coordination metal complexes, although some charged complexes have also been investigated. This method was in fact superior to IEC but again was time consuming[16], and gave very broad, tailed chromatographic peaks[17] with charged metal coordination complexes. However, underivatized silica lacks the discriminatory power for most purposes.

Valenty and Behnken were the first[18] to use the reversed phase high performance ion pair chromatography (RP-HPIPC) technique when they separated some monoester-monocarboxylate and dicarboxylate derivatives of $Ru(bipy)_3^{2+}$ using $CH_3SO_3^-$ or $CH_3(CH_2)_6OSO_3^-$ as ion pair reagents in aqueous tetrahydrofuran. Since then several workers[13,14,19-22] have been using this method for similar purposes, but most of this work has dealt with cationic metal complexes. What little work has been done with anionic metal complexes[23] has not been very satisfactory.

Thus the major concern in the present study is with the separation of highly charged anionic coordination metal complexes and incresing the resolution of highly charged cationic coordination metal complexes. Since it was not

possible to achieve a complete separation of some of the mixtures under consideration, possible ways of improving resolution have also been considered. This includes attempts to improve the selectivity factor (α), column efficiency (N) and peak shape (%S). A number of mobile phase parameters were manipulated including the organic modifier content, the concentration and type of the ion interaction reagent, and the concentration and type of the added salt.

Peak splitting was observed when higher concentrations of added salt were used. This phenomenon has been investigated further.

The possibility of separating geometrical isomers of Cr complexes, using the "added salt" method was also investigated.

EXPERIMENTAL

1.GENERAL INSTRUMENTATION

Separations were carried out on a reversed phase column. The stationary phase was ODS, with a particle size of 10μm. The columns used were either of 4 mm internal diameter and 30 cm length (VARIAN), or of 4.6 mm internal diameter and 25 cm length (Scientific Products & Equipment Ltd). The guard column used was from Upchurch Scientifics. The packing used was Perisorb RP-18 30-40 μm pellicular particles.

A VARIAN 5000 chromatograph, with a micro processer-controlled pump to mix and deliver the two eluents, was used for analysis. This chromatograph comprises of three solvent reservoirs, a gradient forming component, a solvent delivery system, a sample injector, a column enclosure and an electronic cabinet housing a control keyboard and a CRT.

A pair of proportioning valves admit solvent in an accurately metered ratio to the pump. The processor controlled, single piston, reciprocating pump then delivers the solvent mixture to the column, via a flow controller, mixing chamber, pulse damper and pressure transducer.

The keyboard allows the user to control the operating conditions of the instrument and includes keys to select solvent reservoir, flow rate and solvent composition. The CRT also displays the status of all instrument parameters and shows a complete listing of the current program.

Samples were injected via a high pressure loop injector, using a 10µl loop. A 10µl Hamilton syringe was used to inject the samples into the loop.

Detection was via a Varian VARI-CHROM Variable wavelength detector (0.005 - 2 absorbance units full scale/AUFS) which uses a 1.0 mm diameter, 10 mm long flow cell (Varian # 951440). The sample cell was horizontally fixed, and the band width was set at 2 nm. The capacity of the flow cell was 7.9 µl. The absorbance scale used for the detection was 0-0.2A. The chromatograms were recorded on an Omniscribe strip chart recorder.

A Metrohm model E-388 potentiometer, with Ingold low temperature glass combination electrode was used to adjust the pH of the eluents.

2.PREPARATION OF COMPOUNDS AND ELUENTS

1.Compounds

NOTE:- The compounds, e to o, prepared by several workers, were available in the laboratory. The preparation methods involved the corresponding literature methods as indicated.

a. $K_3Co(CN)_6$ was prepared by reacting $CoCl_2.6H_2O$ and KCN, as described in the literature[24].

b. $K_3Co(CN)_5Br$ was prepared both by the literature method[25] and by the displacement of NH_3 from $[Co(NH_3)_5Br]Br_2$ by KCN.

c. $K_3Co(CN)_5I$ was prepared both by the previous method[25] and photochemically in which case an aqueous solution of $K_3Co(CN)_6$ is irradiated in the presence of excess KI.

d. $K_2Co(CN)_5H_2O$, $K_3Co(CN)_5NCS$ and $K_3Co(CN)_5N_3$ were prepared photochemically[26] by the irradiation of an aqueous solution of $K_3Co(CN)_6$ alone, and in the presence of KCNS and NaN_3 respectively.

The UV/Vis absorption spectra and the molar absorptivity values of the complexes prepared were in agreement with literature[24,25,26].

e. $K_3Co(CN)_5Cl$ was prepared according to Adamson et al[25].

f. $[Cr(tn)_2FNCS](ClO_4)$ and $[Cr(en)_2FNCS](ClO_4)$ were prepared according to Vaughn et al[27]. The method employed was to remove the coordinated water molecule of the starting materials $[Cr(tn)_2FH_2O](NCS)_2$ or $[Cr(en)_2FH_2O](NCS)_2$ by heating.

g. $[Cr(NH_3)_4FCNS]ClO_4$ was prepared by the replacement of H_2O in $[Cr(NH_3)_4FH_2O]$, by NCS. This was done by stirring the complex and NH_4NCS in dry methanol for 24 hours at 28 - 30°C[28].

h. $[Cr(NH_3)_4Cl_2]Cl_2$ was prepared by passing dry HCl through a suspension of $[Cr(NH_3)_4(C_2O_4)_2]Cl_2$ in dry methanol. The complex $[CrCl(H_2O)(NH_3)_4)]Cl_2$ was prepared according to literature[29]. This complex was treated with potassium oxalate and oxalic acid to get $[Cr(NH_3)_4(C_2O_4)_2]Cl_2$.

i. $[Cr(en)_2Cl_2)ClO_4$ was prepared from $[Cr(en)_2F_2]ClO_4$. Fluoride in $[Cr(en)_2F_2]ClO_4$ was replaced with Cl, by stirring a mixture of the complex and conc. HCl for 24 hours at room temperature[30].

j. $[Cr(tn)_2Cl_2]ClO_4$ was prepared according to Pederson[31].

k. $[Cr(pn)_2Cl_2]ClO_4$ was prepared according to McLean[32], using chlorodimethylsulphoxidechromium(III) as the starting material.

l. $[Cr(en)_2F_2]Br$ and $[Cr(tn)_2F_2]Br$ were prepared according to Vaughn et al[33].

m. $[Cr(tn)_3](ClO_4)_3$ was prepared according to Pederson[31].

n. $[Cr(tn)_2(NH_3)_2](ClO_4)_3$ and $[Cr(tn)(NH_3)_4](ClO_4)_3$ were prepared during the course of other recent research[34] in this lab, as follows:

1.$[Cr(tn)_2(NH_3)_2](ClO_4)_3$:-

Trans difluoro(1,3-propanediamine)chromium(III) chloride was prepared by a literature method[27]. It was converted to dibromo complex, by stirring with 48% HBr for 24 hrs[30]. The dibromo compound, on treatment with liquid ammonia in a Carius tube, gave the crude complex $[Cr(tn)_2(NH_3)_2]Br_3$. This complex was dissolved in water and 60% $HClO_4$ was added

to give the tri perchlorate salt. The complex was further purified by recrystallization from 0.001M $HClO_4$.

2.$[Cr(tn)(NH_3)_4](ClO_4)_3$:-

Aquopentamminechromium(III) nitrate was prepared by a literature method[29]. This was converted to oxalato tetrammine complex by treatment with potassium oxalate and oxalic acid. The dichlorotetrammine was prepared by passing dry HCl for six hours through a suspension of the oxalato complex in dry methanol[35]. This product was recrystallised from 6M HCl and was suspended in a mixture of DMSO and tn. This suspension was stirred for 2 hours, excess ethanol was added and the resultant precipitate was filtered. The mono tn complex was extracted by adding small volumes of 0.1M $HClO_4$ followed by the addition of 60% $HClO_4$ and cooling in ice. It was washed with ethanol, ether dried, and recrystallized with 0.001M $HClO_4$.
(warning:- These organic perchlorates are potentially explosive and should not be subjected to sudden shock or heat.)

UV absorption spectra, IR spectra and elemental analysis were used to confirm the structures and the purity of the above complexes[34].

o. Cis[27] and trans[30] isomers of $[Cr(en)_2H_2OCl]Br_2$ were prepared according to the literature.

p. propylamine, butylamine, octylamine, tetradecylamine, cetyltrimethylammonium bromide, aniline,

N,N-diethylaniline, tetraethylammonium bromide, triethylamine, trimethylamine, 1,6-hexanediamine, 1,3-butanediamine and dibutylamine were from Aldrich Chemicals and were specified to be better than 95% pure in all instances. Hexylamine was from Eastman Chemicals. N,N-dimethylhexylamine was prepared according to literature[35], and recrystallized from ethanol.

q. Hexanesulphonic acid, toluenesulphonic acid and butanesulphonic acid were from Aldrich Chemicals and were specified to be better than 98% pure.

r. Sodium citrate, tartaric acid, sodium oxalate and ammonium hexafluorophosphate were from Matheson Coleman and Bell manufacturing Chemists.

s. HPLC grade methanol, from Fisher Scientifics, was used to prepare the eluents.

t. Organopure water was used for the preparation of all the eluents and was prepared using a SYBRON/Barnstead Nanopure-A system.

u. The HCl and NH_4OH used to adjust pH were from Fisher Scientific and AMACHEM respectively.

v. Benzene and toluene were used to check the column performance were from Fisher Scientific and ANACHEMIA Chemicals respectively.

2.Eluents

a. The eluents prepared with hexanesulphonic acid and with butanesulphonic acid followed a modification of the procedure given in the literature by Buckingham et al[14].

The two eluents, 25mM sulphonic acid in 95% methanol and 25mM sulphonic acid in organo pure water were prepared as follows;

Sulphonic acid (50 mmol) was dissolved in 50 ml of water, and filtered through a 0.45µm cellulose acetate filter (Millipore, Bedford, Mass., U. S. A.). The volume was made up to 100 ml with water. Water was added to 50 ml of this mixture, up to a final volume of 1l (25mM sulphonic acid in water). HPLC grade methanol was added to the other 50 ml of the mixture, up to a final volume of 1l (25mM sulphonic acid in 95% methanol). The solutions were adjusted to pH 3.5.

When the UV/Visible detector was used at 220 nm, the absorbance of the eluents had to be adjusted, since methanol absorbs at 220nm and water does not. Very small amounts of toluenesulphonic acid were added to the eluents until both had the same absorbance at 220 nm. In this way a steady base line was obtained even when gradient elutions were carried out.

b. Eluents were made up with amines following more or less the same procedure. If the amine was a liquid, conc. HCl was added dropwise to make the amine hydrochloride which was then dissolved in water. The desired eluent pH values were obtained using HCl and NH_3. The differential absorbance of water was not a problem in anion analysis, since the detection was always done above 230 nm and an isocratic elution mode was used in most instances.

3.Procedure

Immediately before each run,the column was prepared by washing with methanol, followed by equilibration with appropriate eluent compositions. A sample of benzene or toluene was usually injected initially to check the column, the detector (270 nm) and the retention time. On completion of each run, the column was brought to the next required composition using a linear gradient, and equilibrated with that composition. When not in use, the column was thoroughly flushed and left filled with methanol.

The eluent pHs were adjusted to ensure that the ion interaction reagents and the competing ions were in the required ionic form, based on tabulated pK values.

In order to determine whether the compounds of interest could be detected under the conditions used, whether they are retained by the column and elute from the

column, and to determine the approximate composition of the two eluents, the following experiment was done:

Using the two eluents made with water and with methanol, a short blank linear gradient from 0% to 100% was completed. This was repeated until a steady baseline was obtained. Subsequently, the same gradient was repeated, with an injection of the sample solution. If the peaks were closely grouped together, isocratic conditions had to be used to separate the sample mixture, whereas if they were widely spaced, a gradient had to be used for the separation process. In order to calculate the approximate composition to be used, the composition of the mobile phase when the peak emerges from the column was calculated, or directly read on the CRT screen.

Once the approximate composition to be used was determined, a few runs were done by varying the composition, based on the evaluation of the previous run, in order to optimise the separation.

UV/Vis spectra of the eluents and of the complexes were taken and molar absorptivity values were calculated. These data allowed the determination of a wavelength at which the solutes have a reasonable absorbance and the eluents have the least possible absorbance. Also, the molar absorptivity values helped in the determination of the sample size required to obtain reasonably large peaks with the absorbance scale used.

Retention time was measured from the point of injection to the peak maximum. The peak width was found by drawing tangents at the points of inflection of the peaks and measurement of the distance between the intersections of the tangents with the peak base line. The t_m was determined by measuring the retention time of nonretained solutes such as alcohols which absorb at the detection wave length. This time was usually the same as the time between the point of injection and the first observable change on the base line of those chromatograms obtained using ion interaction reagents in the eluent.

A. Analysis of cationic Cr(III) complexes

The ion interaction reagent used for the separation of cationic Cr complexes was hexanesulphonic acid. 25 mM solutions of hexane sulphonic acid in water and in methanol were used as the two eluents. The sample size was about 0.1 mmol. The samples were dissolved in organo pure water and filtered through a 0.45 µm millipore filter. The peaks were detected by their absorption at 220 nm. The gradient used for the elution was 25% $\underline{15\ \text{min}}_{>}$ 35% (where the percentage of methanol is indicated) for the singly charged cationic Cr complexes. In order to elute the triply charged cationic Cr complexes, the percentage methanol required was about 90.

In the analysis of triply charged cationic Cr complexes, butanesulphonic acid was used instead of hexanesulphonic acid, since the retention of a solute is shorter for a shorter carbon chain length IIR. Solutions of butanesulphonic acid (25mM) in methanol and in water were prepared and detections were performed at 236 nm. The elution was isocratic with 14% methanol.

B. IEC for anionic Co(III) complexes

Anion exchange chromatography was attempted for the triply charged Cr complex $K_3Cr(SCN)_6$, using the resins Amberlite IRA-400 (F^- form), SP-Sephadex A-25 (Cl^- form) and Hamilton HAX8 (Cl^- form). The eluents used were $(NH_4)_2SO_4$ (1M and 2M solutions) and $Al_2(SO_4)_3.18\ H_2O$ (0.25M, 0.3M and 0.4M), with a high concentration of positive ions and highly charged positive ions respectively.

C. Analysis of anionic Co(III) complexes

The IIR which was initially used for the separation of anionic Co complexes was octylamine hydrochloride. Solutions of octylamine hydrochloride (25mM) in methanol and in water were used as eluents. The detection was performed at 240 nm. The sample size was around 0.5 mmol. Samples were dissolved in distilled water and filtered through a 0.45 μm millipore filter. The gradient used for

the elution was 50% 25 min> 55%. The compounds were singly run initially and then separations were carried out according to the results obtained for the single runs. Several runs were needed to determine the optimum composition for a particular separation.

D. Use of longer chain IIR

Cetyltrimethylammonium bromide was the ion interaction reagent used in the study of retention time dependence on the concentration of IIR in the eluent. Solutions of cetyltrimethylammonium bromide (25mM) in water and in methanol were prepared and fractions of that solution were diluted, to make the concentrations required. The solute used was $K_3Cr(CN)_6$. For each concentration of the IIR, the column was thoroughly flushed with methanol and a blank run was done immediately before the run using the same conditions. This was necessary because a gradient (80% 10 min> 95% 10 min> 100%) was run and the detection wavelength was 240 nm in which region methanol and water have different absorbances. Thus the base line was not steady requiring a comparison to recognize the actual peak. In order to determine the eluting conditions to be used, initial runs were done with the lowest and the highest concentrations of the IIR in the eluents. Separation of a mixture of the compounds $K_3Co(CN)_5X$ (X = CN, Cl, Br, I) was also attempted with 25 mM solutions of this IIR. The

initial single runs were done with the elution condition 92% isocratic.

E. Changing the nature of IIR

In the study of the effect on resolution of the number of carbon atoms of the alkyl chain, as well as the number of alkyl groups attached to the N atom of the amine hydrochloride which was used as the IIR, the two solutes used were $K_3Co(CN)_6$ and $K_3Co(CN)_5I$. The effect of the charge of the IIR on the resolution was also tested by including diamine hydrochlorides as IIRs. The amines used were 1,3-diaminopropane and 1,6-diaminohexane. In these instances, the pK values of the amines were checked and the pH of the eluents was adjusted, in order to achieve doubly charged IIR molecules in the eluents. The eluents were prepared in a similar manner as in the previous cases. The detection wavelength was 270 nm. When different IIRs were used, the retention time of the second peak was kept constant by varying the percentage of methanol in the eluent. This made it possible to be able to compare the resolution achieved with different IIRs, within a particular time.

F. Changing the eluting conditions

Since the variables which affect N, and thus R, also include column temperature and the flow rate, experiments

were carried out to determine the extent to which these variables could affect the parameters concerned. For these experiments, octylamine hydrochloride was used as the IIR. The solutes used were $K_3Co(CN)_5I$ and $K_3Co(CN)_5NCS$.

In order to determine the variables which could significantly affect the peak shape, some other experiments were done. These include changing the solvent composition and using solutes with different chemical properties. The IIRs used were octylamine hydrochloride and hexanesulphonic acid.

G. Use of "added salts" in the eluent

In the study of the effect of added ion on peak shape, the eluent used consisted of 25 mM octylamine hydrochloride and 25 mM trimethylamine hydrochloride at pH 3. In addition, 50 mM and 100 mM concentrations of trimethylamine with 25mM octylamine hydrochloride were attempted.

In addition, another set of eluents was prepared consisting of 25 mM octylamine hydrochloride and of 1.5 mM $K_3Cr(C_2O_4)_3$. (It was not possible to get a base line within the detection range when higher concentrations of this complex were used in the eluent.) The solutes used were $K_3Cr(C_2O_4)_3$ and $K_3Co(CN)_6$, with the eluting conditions 70% and 65% respectively.

Also, eluents of 25 mM octylamine hydrochloride plus 20 mM sodium citrate were prepared. The pH was adjusted to

6.5 at which value a substantial amount of triply charged anionic citrate was available, since the pK_3 of citrate is given as 6.39.

A study of competing ion dependence on the peak shape and resolution used tartaric acid, ammonium hexaflurophosphate, sodium oxalate and sodium citrate in the eluents. The eluents used were initially prepared with octylamine hydrochloride. The pH's of the eluents were appropiately adjusted, according to the pK values of the ions, in order to achieve the required charge of the ions in the eluents.

In the study of the effect on the capacity factor of the concentration of the competing ion, the eluents used were of 25mM octylamine hydrochloride plus the required concentrations of sodium citrate, in a 50% mixture of water and methanol.

Separation of a mixture of geometrical isomers was attempted with the "added salt" method, using cis and trans $[Cr(en)_2H_2OCl]Br_2$ as the solute mixture. The eluents were of 25 mM hexanesulphonic acid plus 25mM triethylamine hydrochloride at pH 3. The elution condition was 14% isocratic. The effect of added ion on the retention behaviour was also studied with the above solute and $[Cr(tn)_2(NH_3)_2](ClO_4)_3$.

H. Study of peak splitting

In order to investigate the reason for and the factors affecting the peak splitting, a few more experiments were done. These included changing the flow rate, the sample size, the temparature and dissolving the samples in the eluent used which contained both IIR and added salt, instead of dissolving them in distilled water. The sample used for all these experiments was $K_3Co(CN)_6$.

In the study of the effect of sample size, the amounts injected were 263 and 669 nmols.

In the investigation of the effect of temperature on the peak splitting, the column temparature was changed using the thermostat attached to the instrument.

RESULTS

In the absence of IIR, none of the complexes showed retention on the column. On addition of a small concentration (<= 25mM) of IIR, the solutes were retained, making it possible to resolve certain mixtures.

A. Separation of cationic Cr(III) complexes

The results of the separation of singly charged Cr complexes, using hexanesulphonic acid as the IIR can be presented as follows (the number of plates follows the retention time for each peak):

1. $[Cr(AA)_2FNCS]^+$ AA = tn (9.2 min, 936)
   en (4.3 min, 413)
   $(NH_3)_2$ (2.9 min, 389)

   (see fig. 6)

2. $[Cr(AA)_2F_2]^+$ AA = tn (5.9 min, 556)
   en (4.0 min, 441)

   (see fig. 7)

3. $[Cr(AA)_2Cl_2]^+$ AA = tn (6.3 min, 784)
   pn (4.0 min, 316)
   en (3.5 min, 296)
   $(NH_3)_2$ (2.7 min, 238)

   (These results are only from single runs.)

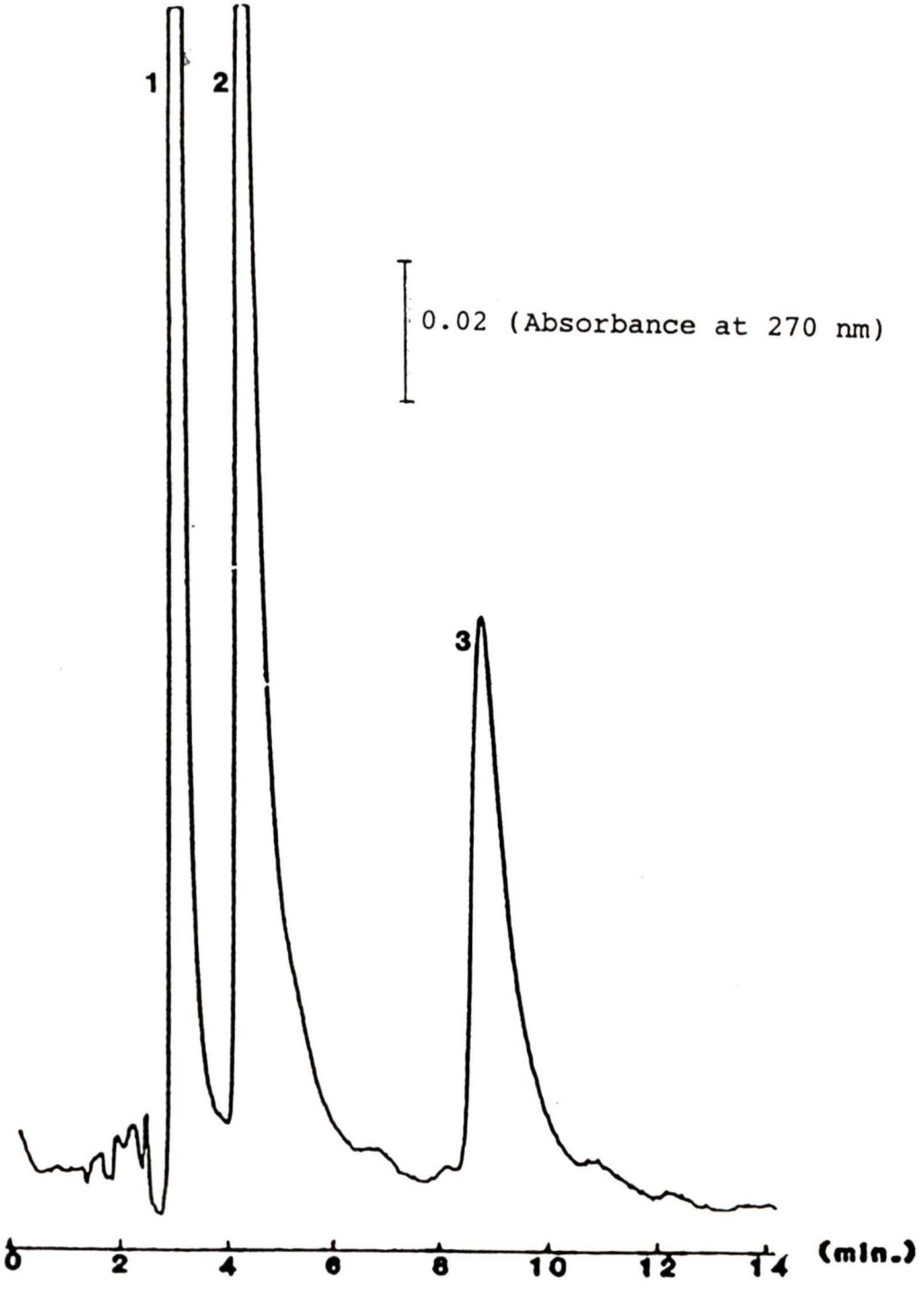


Fig. 6. Chromatogram of $[Cr(AA)_2FNCS]^+$ complexes where AA = $(NH_3)_2$, en, tn in order of elution.
(methanol/water, IIR = hexane sulphonic acid, gradient = 25% 15 min.> 35%)

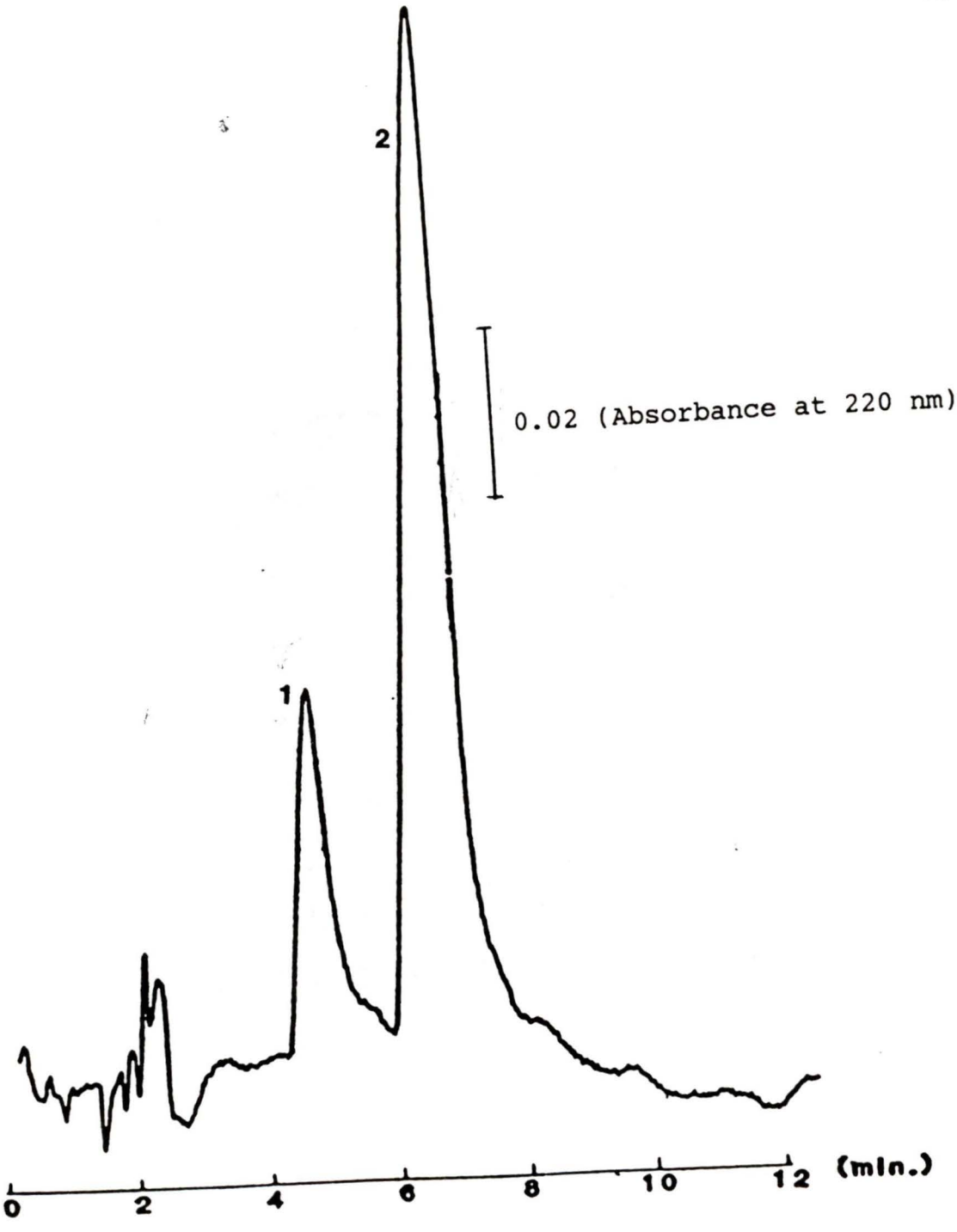


Fig. 7. Chromatogram of $[Cr(AA)_2F_2]^+$ complexes where AA = en, tn in order of elution.

(methanol/water, IIR = hexane sulphonic acid, gradient = 25% 15 min.> 35%)

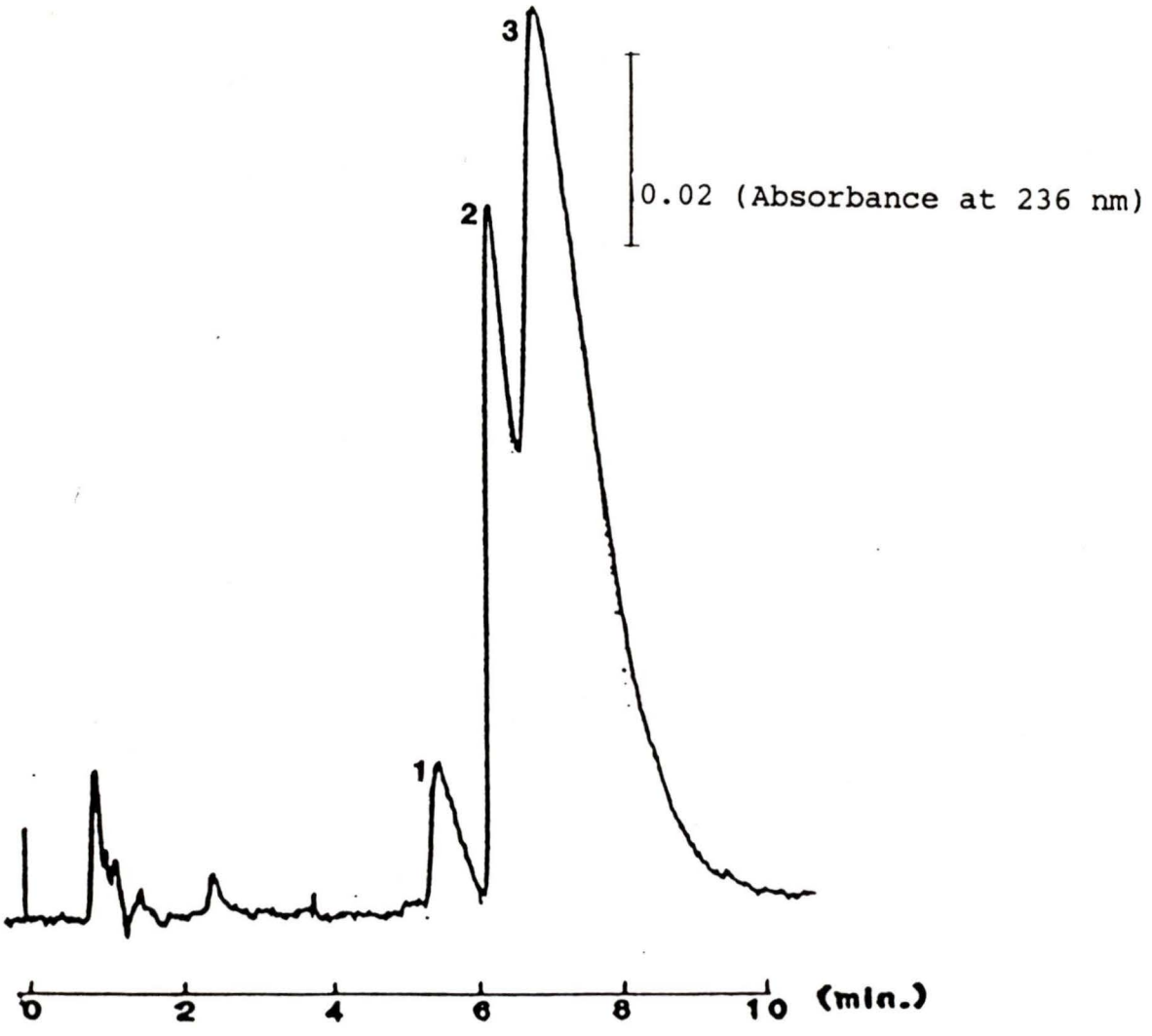


Fig. 8. Chromatogram of $[Cr(tn)_x(NH_3)_{6-2x}]^{3+}$ complexes where x = 1, 2, 3 in order of elution.

(methanol/water, IIR = butanesulphonic acid, 14% isocratic)

The results for the separation of triply charged Cr complexes, using butanesulphonic acid as IIR is as follows:

$[Cr(tn)(NH_3)_4]^{3+}$ (5.5 min, 988)

$[Cr(tn)_2(NH_3)_2]^{3+}$ (6.3 min, 703)

$[Cr(tn)_3]^{3+}$ (7.0 min, 100)

(see figure 8 for the chromatogram)

B. Ineffectiveness of IEC

The elution of triply charged anionic Cr complexes using Ion exchange chromatography was a total failure. With all three resins used and with all the eluents used (as described in the experimental section), the elution was carried out for three days. The reddish purple band of the sample $K_3Cr(NCS)_6$ on the top of the resin bed never moved at all.

C. Separation of anionic Co(III) complexes

The results of the single runs for different anionic Cobalt complexes, using octylamine hydrochloride as IIR were as follows:

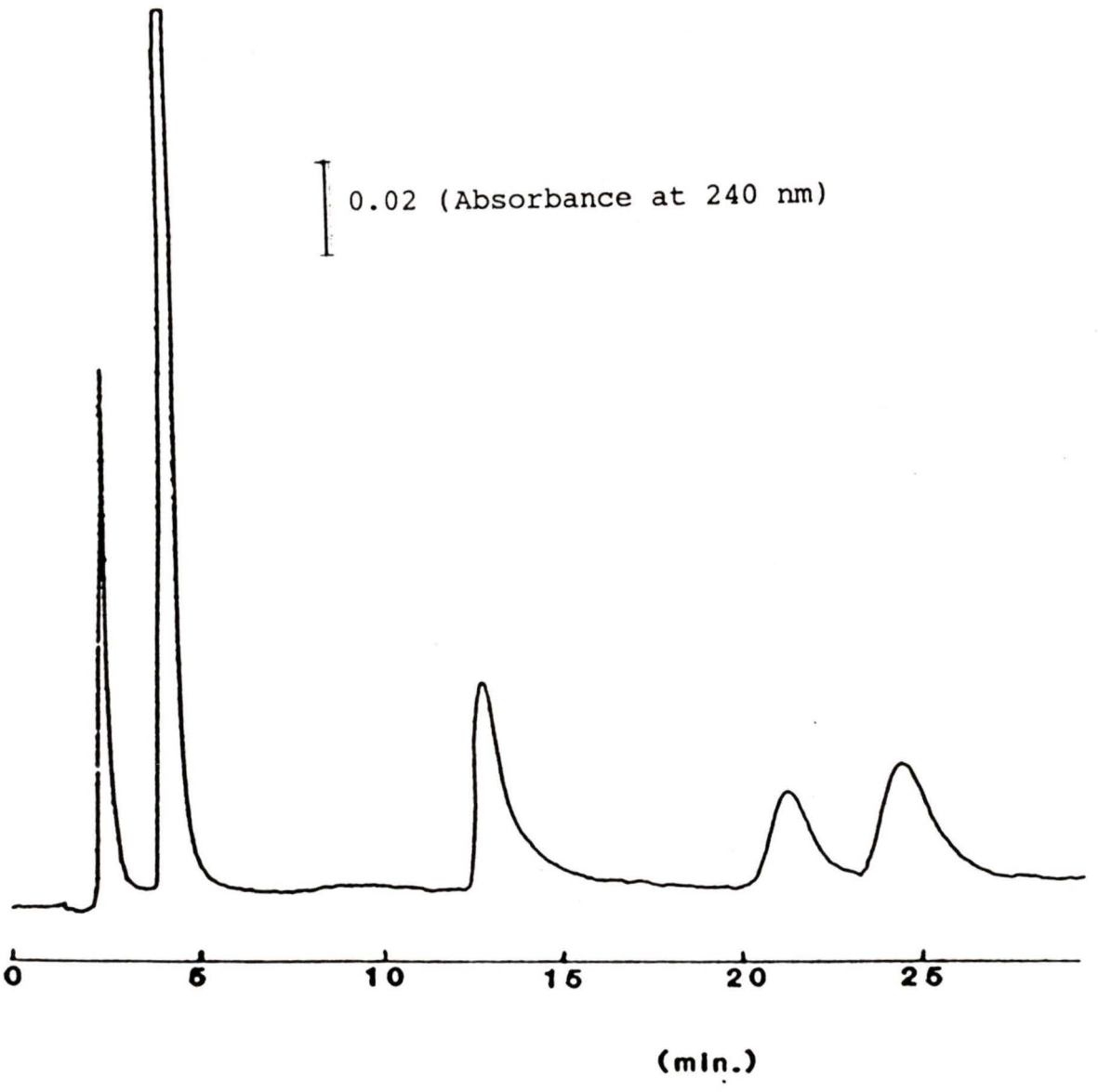


Fig. 9. Chromatogram of $K_3Co(CN)_5X$ complexes where X = CN, Cl/Br, I in order of elution. The first two peaks are due to decomposition products. (methanol/water, IIR = octylamine hydrochloride, 50% isocratic)

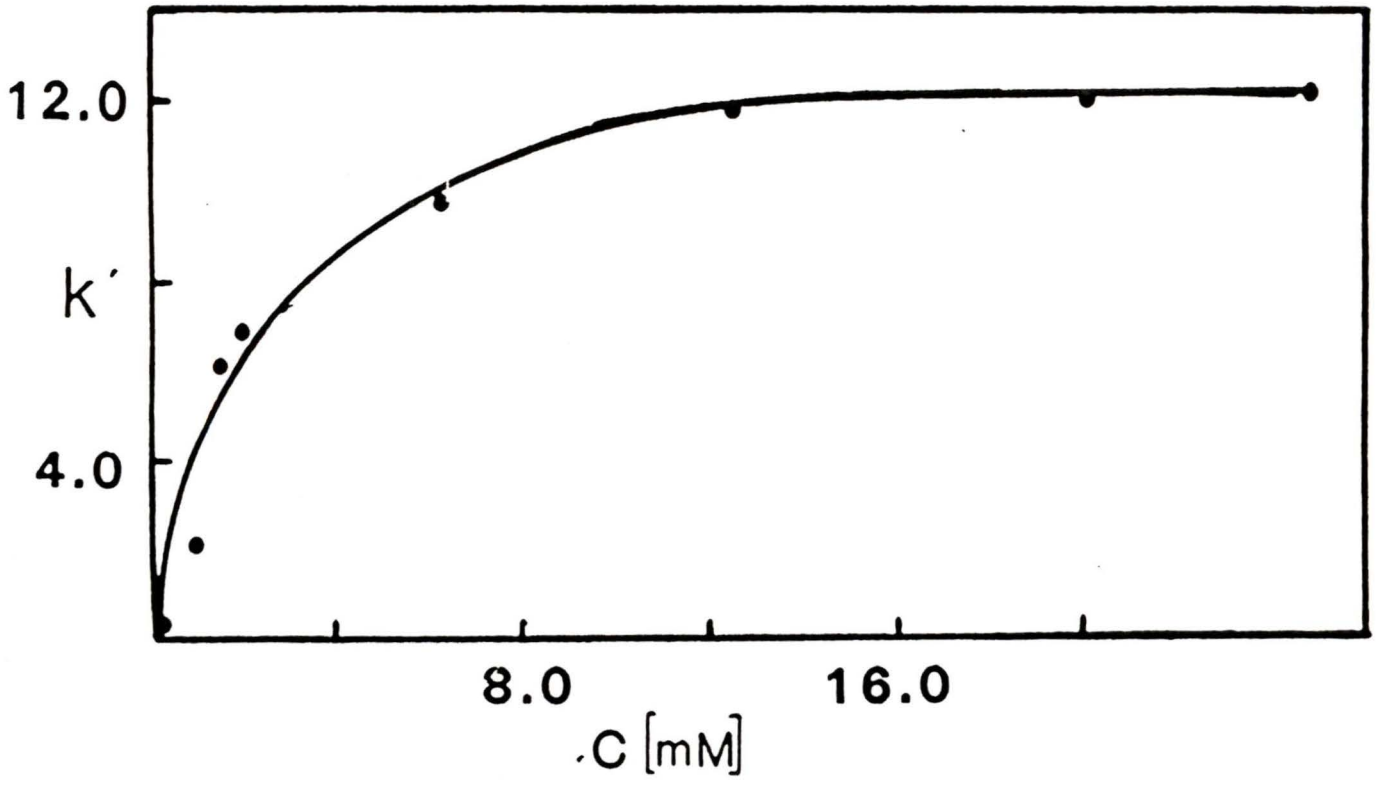


Fig. 10. Effect of cetyltrimethylammonium bromide concentration on the capacity factor of $K_3Cr(CN)_6$. (methanol/water, 80% <u>10 min.</u> 95% <u>10 min</u> 100%)

$K_3Co(CN)_5X$ X = CN (12.2 min, 1409)

N_3 (15.1 min, 1262)

NCS (16.0 min, 1417)

Cl (17.6 min, 1372)

Br (18.0 min, 1600)

I (19.7 min, 1408)

Since NCS, Cl and Br complexes showed similar retention times and since the solute peaks were quite broad when a mixture of all three was run, they could not be resolved. However, each of them could be separated from the CN, I and N_3 complexes completely. Several elution conditions were tried and the one which gave the best α values was 50% isocratic. Separation of one of the mixtures, with the 50% isocratic condition is shown in fig. 9.

D. Effect of longer chain IIR on the retention behaviour

The relationship between the capacity factor of $K_3Co(CN)_6$ versus the concentration of the IIR cetyltrimethylammonium bromide in the eluent is shown in fig. 10.

The single runs for different Co complexes, carried out using cetyltrimethylammonium bromide as IIR, gave following retention times:

IIR	% CH_3OH	t_R(CN) (min.)	N_{CN}	% S_{CN}	t_R(I) (min.)	N_I	% S_I	α	R
$CH_3(CH_2)_{13}NH_2.HCl$	91	6.6	310	27	11.4	1440	12	1.9	3.6
$CH_3(CH_2)_7NH_2.HCl$	65	6.0	290	11	11.9	890	50	2.2	3.9
$CH_3(CH_2)_5NH_2.HCl$	45	5.3	120	12	12.0	440	26	2.6	3.1
$CH_3(CH_2)_3NH_2.HCl$	2.5	4.8	220	4	11.4	540	28	3.2	4.2
$C_6H_5NH_2.HCl$	3	5.3	180	3	11.9	570	8	2.4	3.7
$C_6H_5N(C_2H_5)_2.HCl$	15	6.0	710	98	11.6	1270	46	2.7	5.1
$(C_2H_5)_4N^+Br^-$	3	4.8	260	7	11.2	1660	18	2.8	5.5
$(C_2H_5)_4N^+Br^-$ (recrys.)	2	5.0	280	7					
$(C_2H_5)_3N.HCl$	2	5.0	400	8					
$(C_4H_9)_2NH.HCl$	25	7.6	170	7					
$CH_3(CH_2)_5N(CH_3)_2.HCl$	40	6.7	180	15					
$CH_3(CH_2)_7NH_2.HCl$ + sodium citrate	44	4.2	1760	95	11.6	2660	90		11.4

TABLE 1

Effect of the nature of the IIR on the separation of $K_3Co(CN)_6$ and $K_3Co(CN)_5I$.

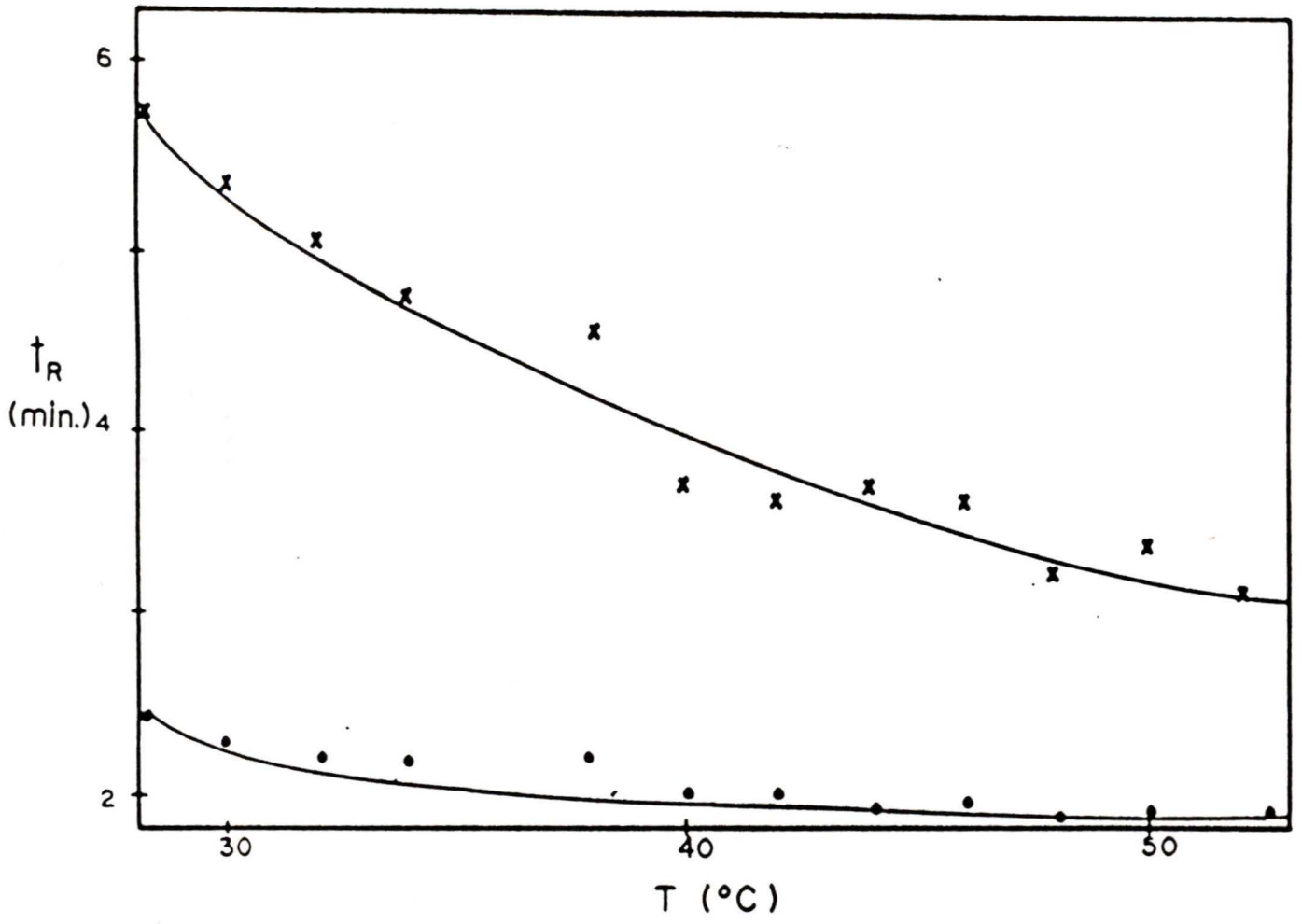


Fig. 11. Effect of temperature on the retention time of $K_3Co(CN)_5I$ (above) and $K_3Co(CN)_5NCS$ (below). (methanol/water, IIR = octylamine hydrochloride, 50% isocratic)

$K_3Co(CN)_5X$ X = CN (7.8 min)

Cl (10.5 min)

Br (11.1 min)

I (11.7 min)

When a mixture of these complexes was run, only two peaks resulted. One corresponded to CN complex and the other was a combination of the other three complexes.

E. Dependence of resolution on the nature of IIR

In table 1 are tabulated the results obtained for the effect on peak shape and resolution, of the length and number of alkyl chains attached to the N atom of the amine hydrochloride used as IIR.

With propylamine hydrochloride as IIR, the compounds were not retained. The concentrations of propylamine used were 25mM, 50mM and 100mM. None of these concentrations changed the retention behaviour observed. But with four or more carbon atoms the retention by the IIR was dramatically increased. In order to compare the resolution, $(t_R)_B$ was kept constant. To achieve this it was necessary to adjust the methanol concentration for different amine hydrochlorides, as shown in table 1.

When diamines were used as IIRs, there was no retention observed. The peaks were eluted just after the solvent front, even when the concentration of the IIR was increased to 50mM.

F. Dependence of resolution on eluting conditions

The effect of temperature on N was not significant. That on the retention time is shown in fig. 11 as a plot of retention time versus temperature.

The effect of flow rate on N is shown below (table 2):

flow rate(ml/min)	N
1.0	50
1.8	79
2.0	126
2.2	146
2.5	64
3.0	63

The effect of the concentration of IIR on N was also studied. The results are shown below (table 3):

[IIR]mM	%methanol	t_R(min)	N
25	62	2.4	342
20	62	2.0	346
15	62	1.5	394
10	62	1.1	423

The effect of solvent composition on N is shown below (table 4):

[IIR]mM	%methanol	t_R(min)	N
25	70	7.0	1225
15	62	7.2	394
25	65	5.3	555
10	62	5.4	423

The effect of chemical properties of the solute, on N can be shown as follows (table 5):

compound	% methanol	t_R(min.)	N
$KCr(C_2O_4)_2(H_2O)_2$	7%	8.8	4096
$K_3Cr(C_2O_4)_3$	65%	8.2	1328
$(Bu_4N)_3H_2OCo(CN)_6$	65%	6.2	961
$(Bu_4N)_3Co(CN)_6$	65%	6.5	1056
$K_3Cr(C_2O_4)_3$	75%	3.2	1820
$(Bu_4N)_3Co(CN)_6$	75%	2.8	1600
$K_3Co(CN)_6$	75%	2.7	1296
$[Cr(tn)]_3^+$	70%	4.8	510
$[Cr(tn)_2F_2]^+$	7%	9.0	2645

G. Effect of "added ions" on the resolution

The use of short chain tertiary amine hydrochloride to mask the active sites on the stationary phase[37] did not improve the peak shape. The concentration of trimethylamine hydrochloride was increased from 25 mM to 50mM and then to 100 mM. However, no improvement in peak shape was observed.

Preparing the eluents with $K_3Co(C_2O_4)_3$ as the added salt, failed to change both the retention time and the peak shape.

With the eluents prepared using sodium citrate as the added salt, a dramatic improvement in the peak shape and the selectivity factor was observed. Thus, a significant difference in resolution was observed in the separation of $K_3Co(CN)_6$ and $K_3Co(CN)_5I$ as shown in table 1* and in fig. 12. Separation of the mixture $K_3Co(CN)_5X$ (X = CN, Cl, Br) was attempted this way and a complete separation of the components was achieved, as shown in fig. 13. Also, a mixture of $K_3Co(CN)_5X$ (X = CN, Cl, Br, I) could be separated in less than 8 minutes, as shown in fig. 14.

The results obtained in the study of the effect of added salt on the peak shape, selectivity factor and resolution are summarised in table 2.

The variation of the capacity factor (k') with the concentration of the added salt sodium citrate is shown as a plot of k' versus concentration of sodium citrate in fig. 15.

The effect of the concentration of added salt on the peak shape could not be studied because of the peak splitting observed when higher concentrations of added salt were used.

Separation of the geometrical isomers cis and trans $[Cr(en)_2H_2OCl]^+$ was not possible by the "added salt"

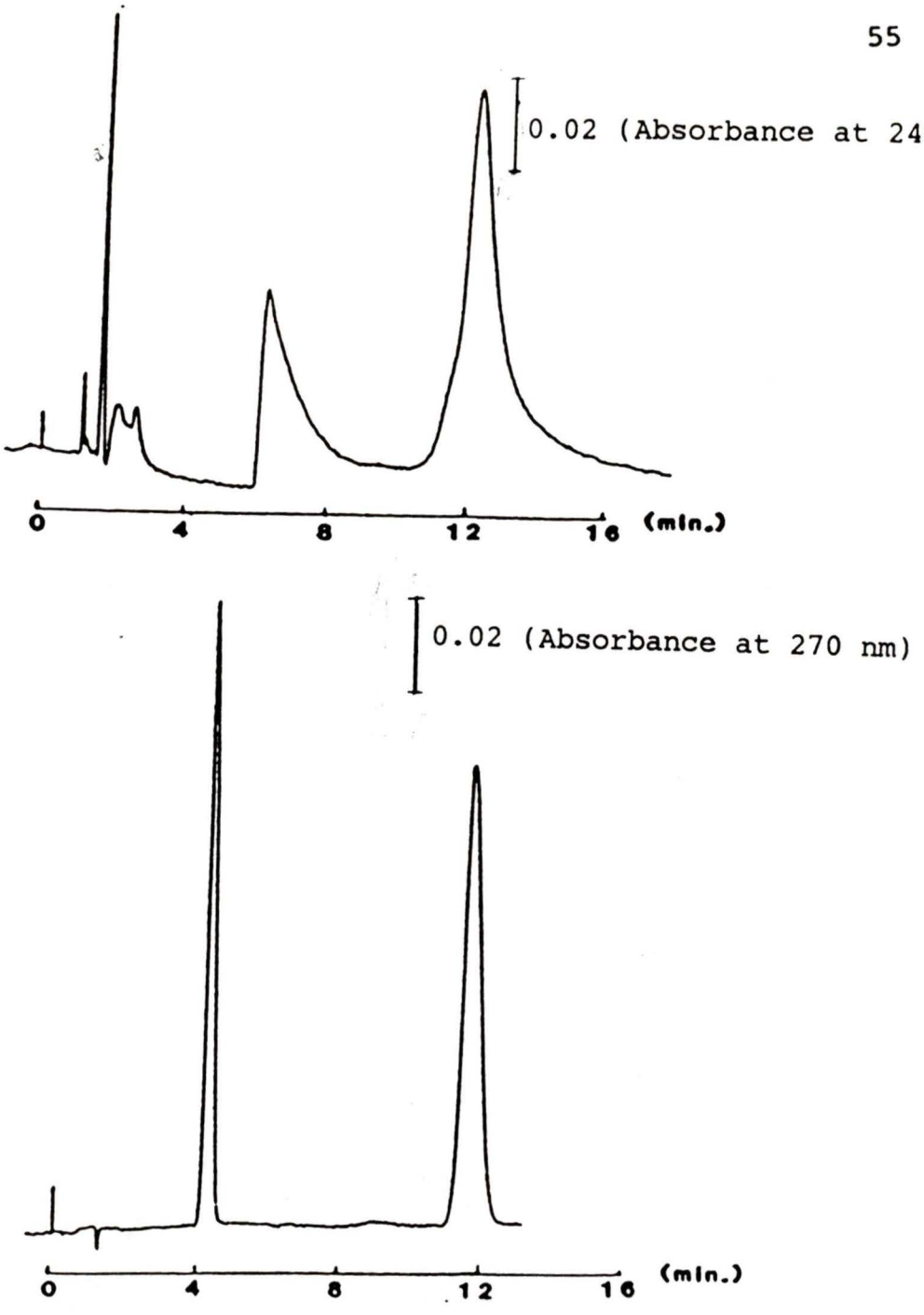


Fig. 12. Chromatograms corresponding to line 2 and line 12 of table 1.

(above: eluent contained only octylamine hydrochloride, below: eluent contained octylamine hydrochloride and citrate)

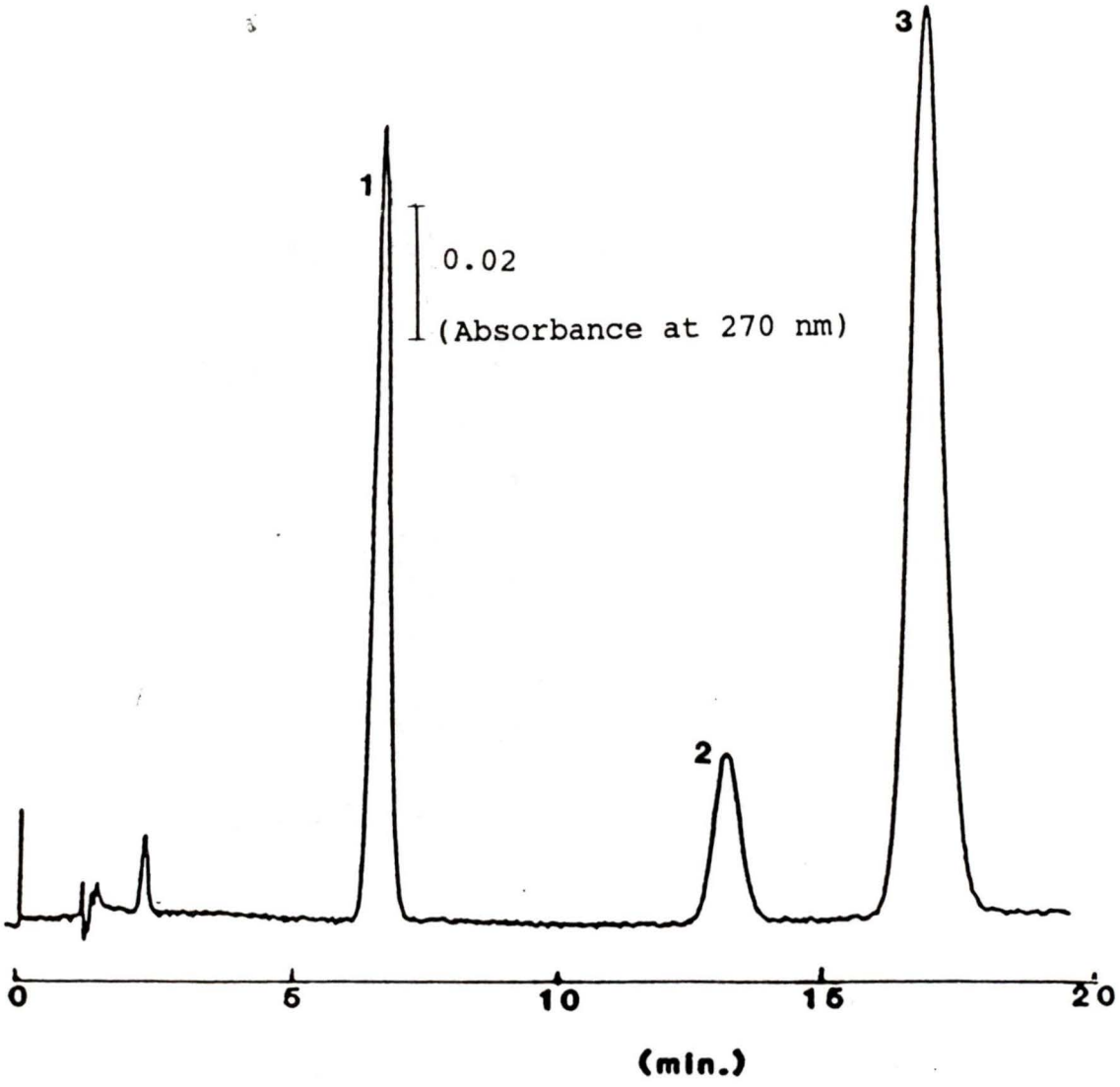


Fig. 13. Chromatogram of $K_3Co(CN)_5X$ complexes where X = CN, Cl, Br in order of elution.
(methanol/water, IIR = octylamine hydrochloride, 30% isocratic, added salt = sodium citrate)

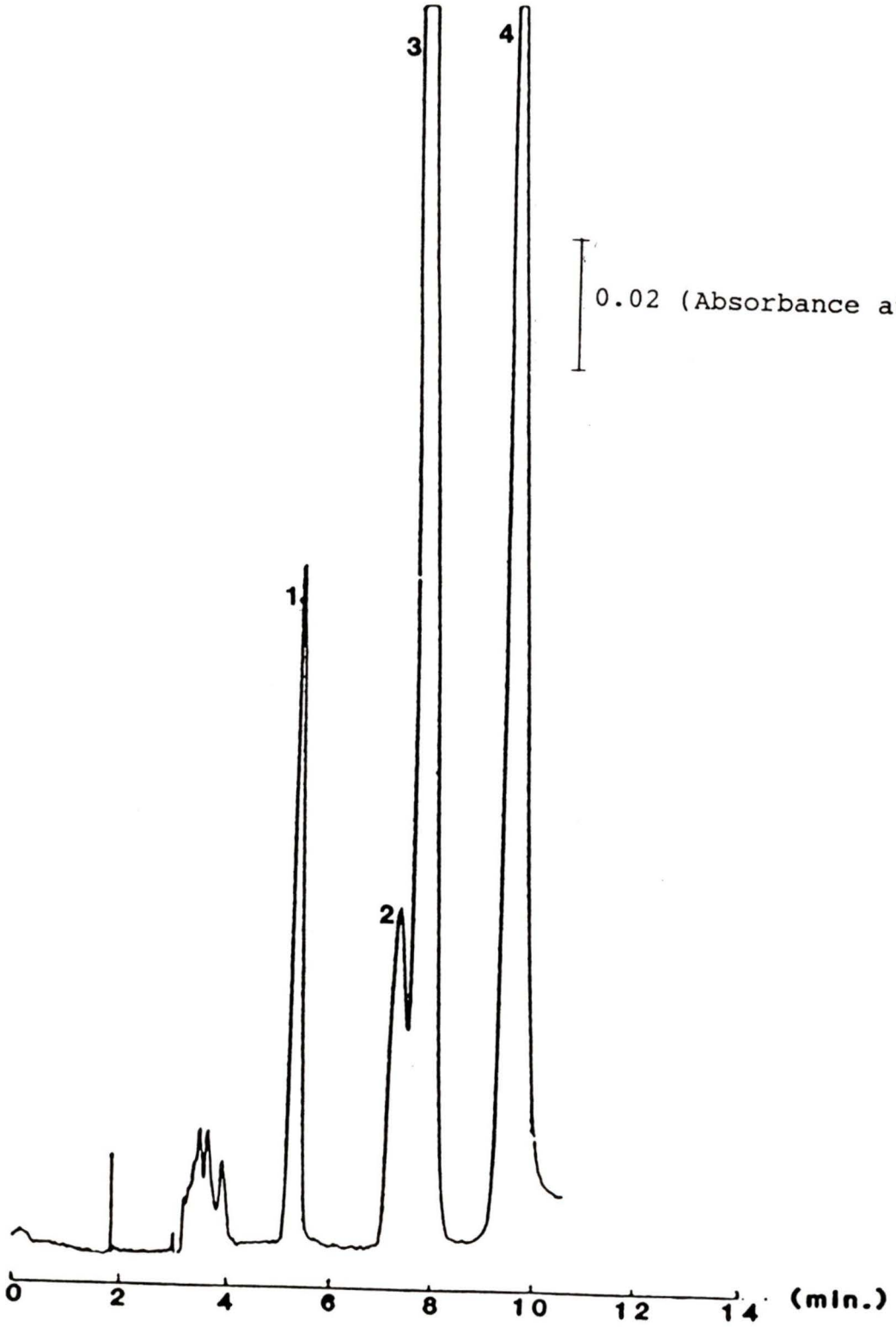


Fig. 14. Chromatogram of $K_3Co(CN)_5X$ complexes where X = CN, Cl, Br, I in order of elution. (methanol/water, IIR = octylamine hydrochloride, 50% isocratic, added salt = sodium citrate)

Added salt	pH	% CH_3OH	t_R(Cl)	α	N_{avg}	$R_{CN/Cl}$
sodium citrate	6.5	30	13.4	2.2	2464	8.3
tartaric acid	5.8	53	13.0	2.0	928	4.4
NH_4PF_6	5.0	45	13.5	1.8	1724	5.1
sodium oxalate	6.5	50	13.4	1.9	1509	5.4
-	3.0	60	14.0	1.8	766	3.6

TABLE 6

Effect of the nature of the added salt on the separation of $K_3Co(CN)_6$ and $K_3Co(CN)_5Cl$.

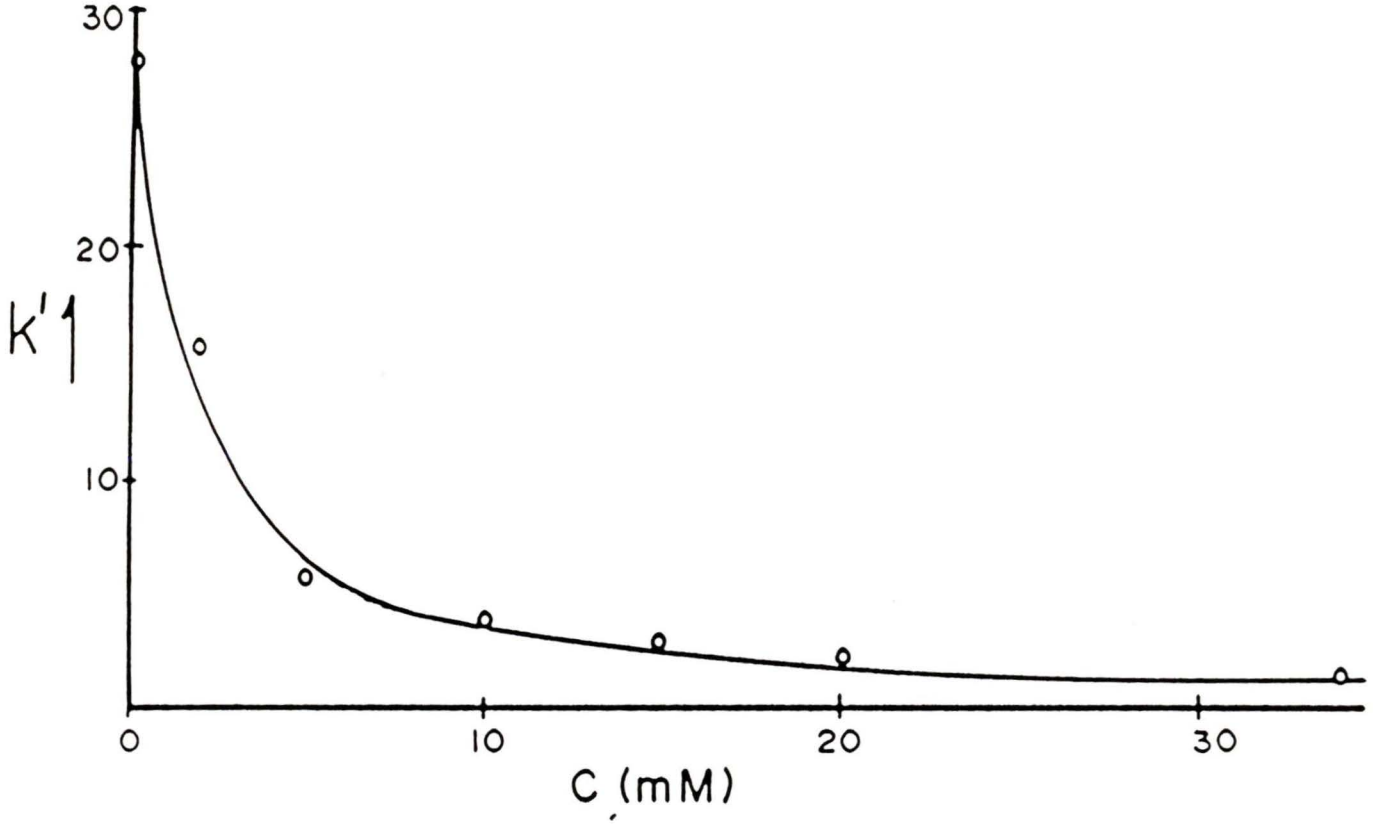


Fig. 15. Effect of citrate concentration on the capacity factor k' of $K_3Co(CN)_6$.
(methanol/water, IIR = octylamine hydrochloride, 30% isocratic, added salt = sodium citrate)

method. The single runs, with 14% isocratic eluting condition, resulted in the same retention time for both cis and trans isomers. But there was a pronounced decrease in the retention time of both isomers, due to the addition of the salt. Therefore, lower concentrations of methanol could be used, as shown below (table 7):

compound	% Methanol	eluent contents	t_R (min)
$[Cr(en)_2H_2OCl]^+$	30	$CH_3(CH_2)_5SO_3H$	15
	14	$CH_3(CH_2)_5SO_3H$ + $(C_2H_5)_3N.HCl$	15
$[Cr(tn)_2(NH_3)_2]^{3+}$	70	$CH_3(CH_2)_5SO_3H$	3.3
	50	$CH_3(CH_2)_5SO_3H$ + $(C_2H_5)_3N.HCl$	5.5

H. Study of peak splitting

The peak splitting, which was observed at higher concentrations of added salt, could not be avoided by dissolving the sample in the eluents instead of dissolving it in water. Changing the flow rate also failed to prevent

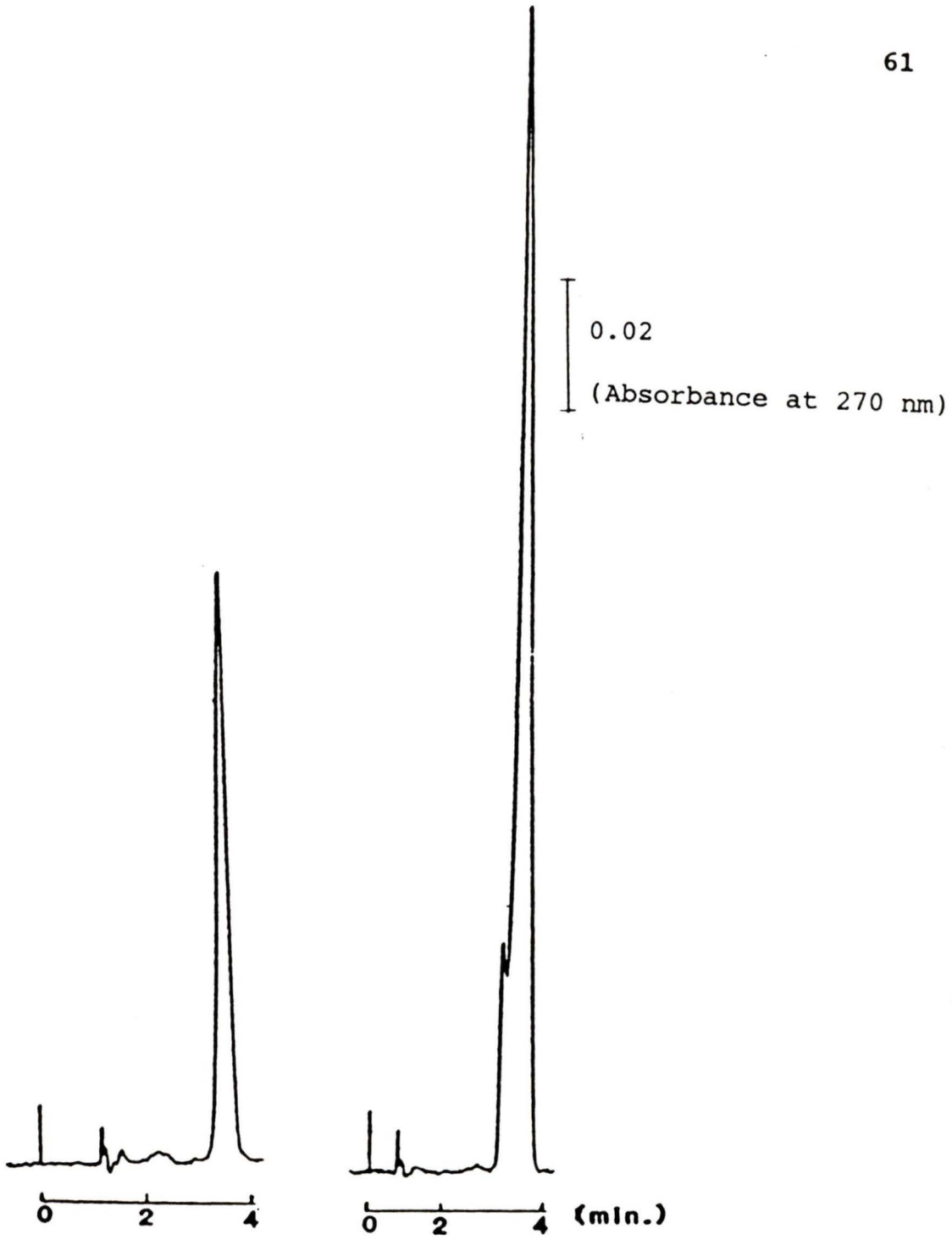


Fig. 16. Effect of sample size on the peak splitting.
sample sizes = 0.263 m mol, 0.669 m mol
(sample = $K_3Co(CN)_6$, methanol/water, IIR = octylamine hydrochloride, 50% isocratic, added salt = sodium citrate)

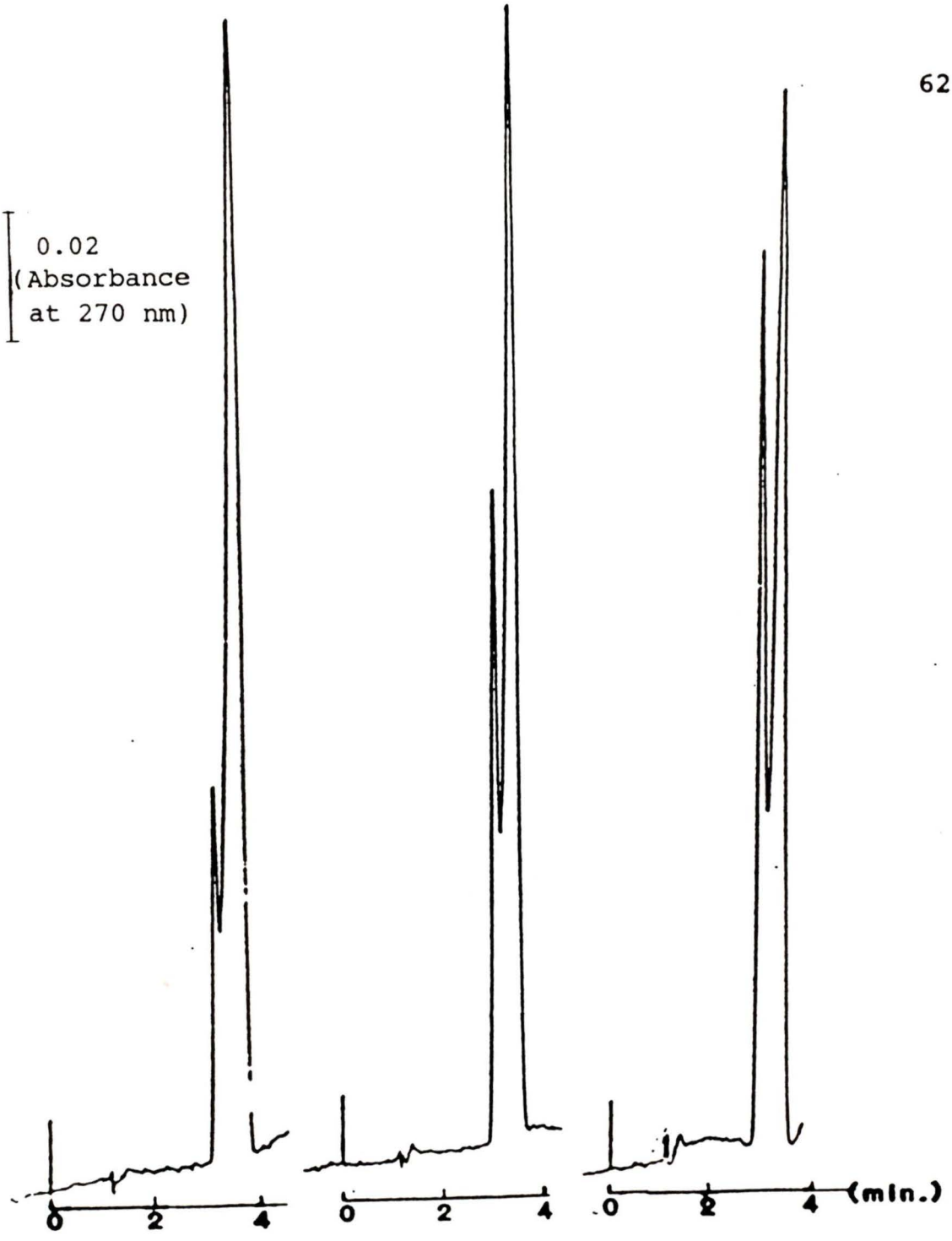


Fig. 17. Effect of temperature on peak splitting. temperatures = 30°C, 40°C, 50°C respectively. (sample = $K_3Co(CN)_6$, methanol/water, IIR = octylamine hydrochloride, 50% isocratic, added salt = sodium citrate)

the peak splitting. It was observed, that when the sample size was reduced, with the concentration of added salt kept constant, the peaks did not split (fig. 16). As the temperature was increased, the first peak grew, relative to the second as shown in fig. 17.

DISCUSSION

A. Preliminary separations of Cr and Co complexes with IEC and Ion-pair RP HPLC

In Ion exchange chromatography the charge/size ratio of the solute ion determines the extent of retention of the solute ion onto the exchange site. The solute ions associate with water before retention. For cations this association (hydration) is easier than for anions, because cations can electrostatically attract the oxygen atoms of the water molecules to form a hydration sphere around the ion. For the anions however, this association has to occur between the hydrogen atoms of the water molecules and the ion, which is geometrically and energetically less favourable. Therefore the cations are heavily hydrated. This leads to a decrease in the charge/size ratio of the cation because the size of the ion increases. But the decrease in the charge/size ratio of the anion in this way is less significant because of weak hydration. Thus, the anions are usually retained more strongly on the ion exchange column compared to cations despite their larger crystallographic radii.

The size of hydration sphere in cations does not significantly differ with the charge of the ion. Therefore, the size of the hydration sphere for a lower charged cation is similar to that of a higher charged

cation. Thus among cations the factor which determines the charge/size ratio, and hence the extent of retention, is mainly the charge.

In Ion exchange chromatography, when used in the present work for the highly charged complexes (especially anionic), elution becomes almost impossible because the solute molecules bind more or less permanently on to the "exchange sites".

Usually, in separating singly and doubly charged cationic metal complexes using IEC, the time required for the separations were long and often resulted in rather broad bands. In separating cationic Co(III) complexes[14,15], even the transfer to high pressure ion exchange chromatography (HPIEC) has given only marginal improvement with recycling often being required to obtain a complete separation.

Therefore, reversed phase HPLC was used in the present study to separate metal complexes. When a C_{18} stationary phase was used for the analysis, in the absence of IIR, the charged metal complexes were not retained on the column. This was because the non polar stationary phase has no attraction to the polar substance. But when the IIR is incorporated into the elution process, if the retention is by "ion exchange" mechanism, there is an electrostatic attraction for the charged IIR molecules which are dynamically adsorbed on to the non polar C_{18} stationary

phase and the charged solute molecules. If the retention is by the "ion pair" mechanism, there is a non polar-non polar attraction between the non polar C_{18} stationary phase and the neutral "ion pairs" which are formed between the charged solute molecules and the oppositely charged IIR molecules.

These electrostatic or non polar-non polar attractions are responsible for the retention of the solutes, and therefore lead to the resolution of the components in a solute mixture, as the extent to which the solutes are retained differs for each of the components being resolved.

In the preliminary separations, only the conveniently changeable parameters were manipulated. These include changing the percentage methanol, the hydrophobicity of the IIR and the mode of elution.

Since the triply charged complex ions are retained more strongly, a higher concentration of the strong solvent methanol is required to elute them as shown in results section A. When no IIR is used, Bidlingmeyer[9] found that at a given methanol concentration, log k' is a first order function of the number of carbon atoms in the solute molecules, and for a given number of carbon atoms, log k' is a first order function of the methanol concentration. (The solutes were long chain organic amines and organic sulphonic acids which have sufficient hydrophobicity to be retained by the C_{18} stationary phase despite their ionic

nature. Thus there was no need of an IIR to assist the solute retention.) In the present work, when an IIR is used in the eluent, a similar trend was observed for the charged metal complexes (i.e. higher percentage of methanol was required to elute more strongly retained complexes.).

Also, the retention of oppositely charged ions usually increases with increasing carbon chain length (i.e. increasing hydrophobicity) of the pairing ion of the IIR, when the mobile phase composition is kept constant. Therefore a lower concentration of methanol was used to elute triply charged Cr(III) complexes, with butanesulphonic acid as the IIR instead of hexanesulphonic acid (which was used as the IIR for the separation of lower charged complexes). Similar results were obtained in the literature[38] in the analysis of adrenalin in the presence of different organic sulphates in the eluent.

The elution orders, $(NH_3)_2$ > en > pn > tn and tn > $(tn)_2$ > $(tn)_3$ (see results section A p.40) show the importance of the hydrophobic property in the retention process. This behaviour has been observed also by Buckingham[14] with Co(III) complexes of different d and l amino acids.

Since the attempt to separate the mixture of Co(III) complexes using octylamine hydrochloride as IIR was not successful with the gradients used, isocratic conditions were used for the elution. This was done in order to

increase the k', expecting a better resolution with a higher k' according to equation 14. But with all the isocratic conditions used, the selectivity factors (α) of the mixture of complexes were not significantly increased, with the increase in capacity factor. Thus significant improvement in resolution was not observed. Indeed, a broadening of the peaks occurred with the increase in k' which actually resulted in a decrease of the resolution.

B. Methods of improving Resolution

In improving the resolution, the major concern was either to increase N or to improve the peak shape (increasing %S). The mobile phase parameters were manipulated in order to achieve an improvement in N, S or α within a given elution time.

1. Changing the parameters related to IIR

The first attempt at improving resolution was to investigate the effect of changing the nature and the concentration of the IIR. The plot of capacity factor versus the concentration of IIR (fig. 10) was in accordance with Bidlingmeyer's[9] and Tomlinson's[39] results for this kind of plot. The capacity factor increases with the increase in IIR concentration and reaches a plateau at a certain value. Also, there was a dramatic increase in the

capacity factor with cetyltrimethylammonium bromide compared to octylamine hydrochloride as the IIR.

Although the capacity factor (k') was increased by the longer carbon chain length and higher concentrations of IIR, there was no improvement in selectivity factor(α), according to the results (section D p.47) obtained with cetyltrimethylammonium bromide. Furthermore, very high concentrations of methanol were required to achieve sufficiently low k' values, making the method uneconomical.

The attempt to vary the number of carbon atoms of the alkyl chain of the IIR to achieve a better resolution had to be started with butylamine hydrochloride, since unlike all other amine hydrochlorides, propylamine hydrochloride failed to make the solute retain on the C_{18} stationary phase. Based on the ion-exchange mechanism, this may be due to the insufficient hydrophobicity of the IIR, preventing it from forming a charged layer on the non polar C_{18} stationary phase. In terms of the ion-pair mechanism there is a better possibility for the ion pair formation since the size of the IIR is small. But it may be because of the insufficient hydrophobicity of these ion pairs that the retention onto the C_{18} stationary phase becomes impossible. This is an important observation which shows that the minimum number of carbon atoms in an amine hydrochloride, used as an IIR with methanol and water as

solvents should be four, for the retention of negatively charged metal complexes.

When elutions were attempted using diamine hydrochlorides as IIR, the pH of the eluents was appropriately adjusted, in order to have doubly charged IIR molecules in the eluent. The purpose of this experiment was to determine the effect of the charge of the IIR on the retention behaviour. But these IIRs failed to make the solute retain on the column. In this experiment, the IIR molecule was expected to be bent and trap the solute ion with its two ammonium ends to form the ion pair in the mobile phase or in the stationary phase. Probably the insufficient hydrophobicity of this bent alkyl chain of 1,3- propanediamine and 1,6- hexanediamine is responsible for the inability to make the solute retain on the C_{18} stationary phase.

The change in the number of carbon atoms of the alkyl chain of the IIR hardly affected the resolution of the two solutes concerned as seen in table 1. Although the number of alkyl groups attached to the N atom of the amine hydrochloride did not affect the selectivity (α) significantly, it was observed that the number of theoretical plates (N), and therefore the resolution was relatively good with tertiary amine hydrochlorides and quaternary ammonium salts as IIR, as shown in table 1. This observation is in agreement with the literature[37]

since the ability of tertiary amine hydrochlorides to mask active sites is better than that of the other amine hydrochlorides as discussed later in section 3.

In the literature[14,15], commercially available chemicals are habitually recrystallised prior to using them as IIR. In order to investigate whether there is a significant effect of the purity of the IIR on the peak shape, the eluents were made using both commercially available and recrystallized triethylamine hydrochloride as IIRs. But as seen in table 1, the value of N was hardly affected by the purity. This may be because the concentration of the IIR used was in the plateau region of the plot of k' versus concentration of IIR (which means a little distraction by any other material or a small change in concentration of eluent would not affect the retention mechanism significantly.)

2.Changing other parameters

The effect of the temperature on N was so insignificant (results section F p. 51) that changing the temperature obviously would not help in improving peak shape or resolution. According to usual standards in chromatography the van-Deemter curve (H versus flow rate) for LC is flat, i.e. the effect of flow rate on N is very small. The results obtained in the study of the dependence of N on the flow rate (section F p. 51) do indeed show only

a small effect with the optimum flow rate at around 2 - 2.2 ml/min.

According to the results (section F p. 52) there was no significant influence of the concentration of IIR on the peak shape either. Since the concentration of IIR did not affect N significantly, the study of the effect of the solvent composition on N could be done with different IIR concentrations in order to keep the retention time constant. According to the results shown in section F, it is obvious that the increase in percentage methanol in the eluent increases N. This effect has been observed in the literature[4, 40]. This increase can probably be attributed to the lower viscosity of methanol compared to that of water, since lower viscosity of the solvent promotes the mass transfer of the solute between the stationary phase and the mobile phase.

As seen in the results obtained for the variation of N with the change in chemical properties of the solute, the compounds bearing similar charge gave similar N values within a given retention time. As the charge decreases, there was a dramatic increase in N in spite of the very high percentage of water used (although a decrease in N was anticipated according to the previous paragraph), for the same time of elution, $KCr(C_2O_4)_2(H_2O)_2$ gave a very high N, compared to $K_3Cr(C_2O_4)_3$, which required a very low percentage of water for elution. As far as cations are

concerned, the same behaviour is observed, i. e., even when a higher percentage of water is used, there is a large increase in N with lower charged compounds compared to higher charged compounds. Highly charged solutes make the mass transfer from the stationary phase to the mobile phase more difficult. This means broad peaks are an inherent property of highly charged compounds when using this technique.

3.Consideration of peak tailing and the introduction of the "added salt" method

Since the parameters manipulated so far were not able to give a significantly small peak width (in terms of N), the methods by which the peak shape can be improved (increase in %S) were considered.

According to the results obtained in section F, the peak broadening observed in the separation of certain Co(III) complexes can be partially attributed to the high charge. But the peak tailing was considered to be due to the most frequently cited cause: active sites were present on the stationary phase.

During the process of chemically attaching linear organic molecules or organo silanes to the surface hydroxyl groups of a normal (silica) stationary phase, some free hydroxyl groups remain. The smaller the number of residual hydroxyl groups, the better will be the quality of the

column. However, no commercially available column can be completely free of these active sites. Furthermore, continuous usage of the column, mechanical shocks due to changes in pressure, or the use of eluents of pH > 7.5 could cause degradation of the stationary phase. The effect of these active sites on the peak shape, i.e. peak tailing, is more pronounced when the charge of the solute is higher.

An attempt to separate cationic Co(III) complexes using a normal phase column resulted in extensively tailed peaks[17]. This in fact, supports the idea that the poor peak shape observed with reversed phase may be due to the residual hydroxyl groups on C_{18} column.

The use of an amine hydrochloride[41] as IIR in separating main group inorganic anions and anions of weak organic acids, and the use of cetrimide[42] as IIR in separating main group inorganic anions, using a C_{18} stationary phase in both cases, also resulted in peak tailing.

Recently, Kiel and Morgan[37] used a silica based column to study the retention of positively charged solutes. The three solutes concerned were a primary, a secondary and a tertiary amine. When no IIR was used in the eluents, it was found that, as the eluent pH was increased from 5.5 onwards, the retention of tertiary amine was increased significantly more than at lower pH values. (All the

amines were protonated at eluent pH levels below 8.) This increase in retention at higher eluent pH values has been attributed to ion exchange effects between the positively charged protonated amine and the negatively charged unprotonated silanol sites on the stationary phase (since the pK of SiOH is 9.5).

The retention of the solutes at pH levels below 4 was due to the decrease in Na^+ concentration which causes cationic competition with the solutes, for the ion exchange sites on the silica surface. (The Na^+ comes from the NaOH used to increase the pH.)

Also, it was found that, over the pH range 2 - 8, the tertiary amine exhibited a greater peak asymmetry than the primary and secondary amines. They found that the peak shape of all three amines can be improved by the addition to the mobile phase of an organic amine modifier in low concentrations. Also, due to the cationic competition these amine modifiers decrease the k'. The effect of reducing k' and the improvement in peak shape were greater with the tertiary amine modifiers compared to primary and secondary amine modifiers. This behaviour was attributed to the greater ability of tertiary amines to hydrogen bond to the silanol sites and mask them from the solutes.

The amine modifier molecules with shorter hydrophobic side chains will be less strongly attached to the stationary phase carbon chain and will tend to adsorb

better on to the silanol support. Further, the short chain amines can efficiently penetrate to the support silanol groups and block the potential solute interactions with them.

Considering the results of this work, and since the resolution obtained with tertiary and quaternary amine hydrochlorides as shown in table 1 were better than that of other amine hydrochlorides, the elution of the Co complexes under consideration in the present study was attempted with both octylamine hydrochloride and trimethylamine hydrochloride in the eluents. However, no improvement in the peak shape was observed in the elution of negatively charged Co(III) complexes.

Thus, an anionic masking agent was used in the eluent. The masking agents used were $Cr(C_2O_4)_3^{3-}$ and citrate. As described in the results section G, the oxalato chromium complex as a masking agent, failed to improve the peak shape even though citrate did. This failure can be attributed to the very low concentration (1.5 mM) of the complex compared to the citrate concentration (25 mM).

In addition to the improvement in peak shape, a dramatic reduction in the retention time was also observed. As shown in table 1 not only the peak shape (in terms of N and S) but also the selectivity factor (α) was increased, resulting in a tremendously high resolution. With this

method it was possible to achieve the target of the present work which was a complete separation of certain Co(III) complexes. These results, shown in fig. 12, 13 and 14 were quite satisfactory owing to the following reasons:

1. The separation required less than 8 minutes, although all the components possessed very high negative charge.
2. A very good peak shape was achieved despite the very high percentages of water in the eluent (a high content of water in the eluent generally causes peak broadening as discussed in section E).
3. This was an unusual improvement of resolution, because there was no sacrifice in the speed of separation. (Indeed, the addition of a competing ion decreased the retention time, or the capacity factor; according to equation 14, this should result in a decrease of the resolution.)

The reduction in retention time by the addition of ions to the eluent was observed in the literature as described below. It was observed[43] that when the counterion of the IIR used is multivalent, the solute retention time was shorter than when the counterion is univalent. Also, Skelly[43] showed that the addition of inorganic anions like chloride, sulphate or phosphate, to the eluents prepared with an amine hydrochloride as IIR, decreases the retention time of the anionic solutes.

Pletrzyk and Iskandarani[44, 45] observed the same phenomenon and they ordered the added ions according to the effect they had in reducing the analyte retention. These anions included both inorganic and organic ions.

Billiet and Gy Vigh[38] showed that when salt control is used, IIRs with different chain lengths result in identical solute retention times.

Hill[46] used salts in the eluent in separating methoxy anilines using a C_{18} column and he did not use an IIR in the eluents (since the solute itself had sufficient hydrophobicity). He called this method "competing ion reverse phase HPLC". According to Hill, when added to the mobile phase, an ionic compound of similar charge to the solute molecule, it is adsorbed on to the stationary phase to form a charged layer. Hence, solute molecules are repelled from the stationary phase and show reduced retention and improved peak shape. He recommended this technique, especially when silica based columns are used, which are not completely end capped (i. e. not all the active hydroxyl groups are silanized) or partially hydrolyzed by harsh mobile phases.

As discussed earlier, in the present work, which used an IIR in the eluents, similar results were obtained. The decrease in retention time in this case may be due to the competition of the solute ion and the added ion for the exchange sites, which consists of the IIR molecules. (i.e.

assuming the formation of the "charged layer" of IIR on the C_{18} stationary phase according to ion exchange mechanism.) The improvement in the resolution may be due to two factors

1. The increase in N because of the relatively easy mass transfer of the solute from the stationary phase to the mobile phase when the competing ion is present in the eluents.
2. The increase in peak symmetry because the active sites are masked by the added ion and because of the competition of the solute and the added ions for the active sites.

According to the plot of k' versus concentration of added salt shown in figure 15, the retention of the solute decreases with increasing concentration of added competing ion. The curve very slowly approaches a constant value. According to Horvath et al[4], added salts tend to reduce the capacity factor, because they can compete with IIR molecules in forming ion pairs with the solute. Theoretically, they expect the capacity factor to fall precipitously with the addition of salt until it reaches a value which is unaffected by the addition of more salt. However, the capacity factor at high salt concentrations does not reach a constant value, but decreases somewhat.

Although the decrease in retention time and the improvement in peak shape due to the addition of a competing ion has been observed in the literature as discussed earlier, the increase in the selectivity factor,

shown here in table 1 has not. However, the effect of added salt on α was not as great as that on N. Thus, the improvement in resolution is attributed mainly to the enhancement in N, or column efficiency.

In the study of the effect of the nature of the added salt on the resolution (see table 2), the order of efficiency observed was:

citrate > oxalate > tartrate > hexafluorophosphate

Highly charged small molecules effectively can mask the active sites and compete for the exchange sites. Thus, as far as organic salts are concerned one could expect the above order for improving resolution and decreasing retention time. This order is in agreement with Gy Vigh's results[12], according to which the effect is greater with organic ions and is dependent on the structure of the added ion (when amine hydrochlorides and inorganic cations were used as the added salts in suppressing the retention of amine hydrochlorides). According to his results there is no apparent effect of the counterion of the added salt on the capacity factor.

The separation of geometrical isomers attempted hexanesulphonic acid as the IIR and trimethylamine hydrochloride as the added salt, was not successful. Both isomers gave very similar retention times. This may be because the increase in α caused by the added ion used was

not high enough to allow the separation of geometrical isomers.

C.Peak splitting

As discussed in the results section H, peak splitting was observed when higher concentrations (>20mM) of added salt are used. The normal peak was preceded by an abnormal peak. Presumably this must occur while the solute travels through the column, since dissolving the sample in the eluent could not avoid peak splitting. Also, with constant eluent parameters, a larger sample size resulted in peak splitting whereas a smaller sample size did not (fig. 16). Furthermore when the temperature was increased, as shown in fig. 17, the relative importance of the first or abnormal peak grew.

In a separation of a mixture of cationic Co(III) complexes using tosylate as IIR, Buckingham et al.[19] observed that the peaks split when the column is overloaded by the sample. The normal peak always was preceded by an abnormal peak which was also observed in the present work. They found that the retention time difference between the normal and the abnormal peaks was related to the total amount of complex loaded rather than to an excess of any one species. This again agrees with the results obtained in the present work because in this case the retention time

difference between the two peaks was constant when a constant amount of sample was used with different added ion concentrations and with different temperatures. They also were able to demonstrate[19] that the splitting is related to the events occurring on the column and is not related to injection problems or to any other loading abnormalities at the top of the column. In this work, they always used more than one complex in the sample and they believed the splitting occurs while one complex overtakes the other complex on the column, according to their results (i.e., the first peak split when the sample corresponding to the first peak is injected 10 seconds after the injection of the sample corresponding to the second peak. But it did not when the delay was increased to 30 seconds. When the loading is done in the reverse order, no splitting was observed since the complex ions never cross. As the concentration of the second peak was increased, the delay between the loadings could be correspondingly increased to maintain the peak splitting.) They concluded that the concentration of solute species in the cross over zone is a factor in determining splitting.

Since, according to their work, the splitting was related to the total amount of the complex, overloading should be a major factor in determining the peak splitting. However, they also observed that the peaks split when the concentration of the IIR in the eluent is low. This in

fact is in agreement with the results obtained in part H of the results section. According to the results, when the competing ion concentration is increased, the number of molecules of IIR available for the solute becomes lower due to the competition (i.e. IIR depletion effect), and the peaks split.

By dissolving the samples in the eluents used, the conditions available for the sample to undergo elution were made uniform. Since the peaks split even after doing so, the phenomenon must be related also to the stationary phase rather than only to the mobile phase. The results shown in fig. 16 concerning the sample loading, are again in agreement with Buckingham's[19] results.

For the peak to be split, the sample should split into two fractions within the column. The splitting of the sample should occur at the beginning of the column. These fractions stay as two fractions (do not mix), since the flow rate remains constant.

The splitting may occur due to either or both of the following factors:

1. IIR depletion:- This could be due to a decrease in concentration of IIR or an increase in the concentration of competing ion.

2. Overloading:- An increase in the concentration of solute with a constant number of exchange sites could result in peak splitting.

(These exchange sites consist of IIR ions adsorbed onto the stationary phase.)

The column can be considered as consisting of several hypothetical regions. In the above mentioned circumstances, when the solute is introduced on to the column, only a fraction of the solute molecules can occupy the sites available in region one. The rest of the molecules (fraction two) flushes through without retaining to the second (or successive) region, where there are vacant exchange sites. This way, fraction one starts travelling through the column from region one, whereas fraction two starts from a later region. Thus fraction two lies ahead of fraction one, and gives an abnormal peak which precedes the normal peak.

As the temperature increases, due to faster motion, retention becomes more difficult. Hence fewer solute molecules are retained, fraction two becomes larger, resulting in a larger abnormal or first peak.

Thus peak splitting in the present work occurs due to the "crowding" of the solute molecules at the beginning of the column. In Buckingham's[19] work, this "crowding" is created somewhere within the column. This was done by injecting a slower moving solute prior to the injection of the solute under consideration. Thus the solute molecules experience "crowding" when they cross the area which was already occupied by the slower moving solute. Therefore, a

fraction of the solute occupies the remaining exchange sites in the cross over area and the others (fraction two) moves faster and so on. When the delay between the two injections is increased, due to dilution of the slow moving solute, the cross over zone becomes less "crowded". Thus fraction one predominates resulting in a small or no first peak. With the incresed delay of the injections, the first peak can be made to grow by increasing the concentration of the slow moving solute (i.e. increase "crowding" of the cross over zone.)

D.Summary

In separating charged metal complexes, HPLC is found to be superior to IEC. A C_{18} stationary phase with a mobile phase containing an IIR allows a relatively complete and very fast separation of charged metal complexes.

Changing the mobile phase parameters is found to be the most effective way to improve resolution. Among the mobile phase parameters, changing the organic solvent composition and using added salt in particular allow a dramatic improvement in resolution to be achieved. However, the increase in concentration of added salt in the eluent is limited due to the peak splitting phenomenon which is encountered as the concentration of added salt exceeds a certain limit.

This technique can be very useful for kinetic studies and photochemical studies of charged metal complexes for which a fast and an efficient method of separation is required.

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