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Review

Chromatographic and related electrophoretic methods in the separation of transition metal complexes or their ligands

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Contents

Abbreviations: AA, amino acid (ligand) or atomic absorption (technique); $acac^-$, 2,4-pentanedienato or acetylacetonato ligand; BINOL, 1,1′-bi-2-naphthol; R_2BINOL, 6,6′-(*R*)-1,1′-bi-naphthol; bipym, 2,2′-bipyrimidine ligand; bpy, 2,2′-bipyridine ligand; CE, capillary electrophoresis; Chirasil-Nickel, Ni^{II}(bis(3-heptafluorobutanoyl)-10-methylene-(1*R*))-camphorate; Cp^-, η^5-cyclopentadienide ion ligand; cyclam, 1,4,8,11-tetraazacyclotetradecane ligand; $cydta^{4-}$, 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetate ligand; CZE, capillary zone electrophoresis; dien, diethylenetriamine ligand; DNA, deoxyribose nucleic acid; $dota^{4-}$, 1,4,8,11-tetra-azacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetate ligand; dpaH, 2,2′-dipyridylamine ligand; dppe, 1,2-bis(diphenylphosphino)ethane; dppz, dipyridophenazine; dpq, dipyridylquinoxaline; dpqc, dipyridotetrahydrophenazine; $dtpa^{5-}$, diethylenetriaminepentaacetate ligand; ECL, electrochemically-generated chemiluminescence; $edda^{2-}$, ethylenediamine-*N*,*N*′-diacetate ligand; $eddp^{2-}$, propylenediamine-*N*,*N*′-diacetate ligand; $edta^{4-}$, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate ligand; $ed3a^{3-}$, ethylenediamine-*N*,*N*′,*N*′-triacetate ligand; ESI-MS, electrospray ion mass spectrometry; Et_2dtc^-, diethyldithiocarbamate ion; GC, gas chromatography; $hbed^{4-}$, bis(2-hydroxybenzene)ethylenediamine-*N*,*N*′-diacetate ligand; $hedta^{3-}$, *N*-hydroxyethylethylenediamine-*N*,*N*′,*N*′-triacetate ligand; HPLC, high pressure liquid chromatography; HQS, 5-sulfonato-8-hydroxyquinolato ligand; ICP, inductively coupled plasma spectroscopy; ida^{2-}, iminodiacetate ligand; IMAC, immobilized metal ion affinity chromatography; IMCOS, immobilized metal complexes for organic separations; L, ligand as specified in the text for a complex; MEKC, micellar electrokinetic capillary (chromatography); $mida^{2-}$, *N*-methyliminodiacetate ligand; MMFF94, molecular mechanics force field (1994 version); NAD^+, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); NP, normal phase (chromatography) with hydrophilic stationary phase; nta^{3-}, nitrilotriacetate ligand; *o*-phen, orthophenanthroline ligand; PAR, 4-(2-pyridylazo)resorcinol ligand; phen, orthophenanthroline ligand; py, pyridine ligand; ROH, an alcohol or functional group; RP, reversed phase (chromatography) with hydrophobic stationary phase; R_2dtc^-, bis-substituted dithiocarbamate ligand; SEC, size exclusion chromatography; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; SPARTAN, molecular mechanics program of the Wavetec Corp. that utilizes MMFF94; TAR, 4-(thiazoylazo)resorcinol ligand; TLC, thin layer chromatography, planar chromatography; TPP^{2-}, tetraphenylporhyrinato ligand; $ttha^{6-}$, triethylenetetraaminehexaacetate ligand; tren, tris-(2-aminoethyl)amine ligand; XO, xanthanine orange ligand

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Abstract

It is difficult for most inorganic chemists to be aware of the availability of the many new techniques, described largely in analytical chemistry, environmental chemistry, and biomedical journals, which are increasingly of value in the separation and characterization of complexes of transition metals. Additionally, the design and use of supported transition metal complexes as sites for molecular recognition in the separation of biomolecules and organic materials has the potential to dominate multi-billion dollar industries in proteins and chiral drugs. This review serves as a roadmap for inorganic chemists to the recent chromatographic literature. The review covers advances in separation methods that involve transition metal chemistry which have occurred in the decade of 1992 through early 2003 with 360 references. The review is intended to assist readers in finding key papers that illustrate techniques of chromatography that might be applicable to purposes in the reader's laboratory. Covered topics include the standard separation of inorganic ions and metal complexes, capillary electrophoresis methods (CE, CZE, MEKC), electrochemical detection in flow methods by $[Ru(bpy)_3]^{3+/2+}$ cycling in response to analytes, separations of metal complexes of interest to environmental and biomedical disciplines via size exclusion chromatography (SEC), and detection methods with electrospray mass spectrometry (ESI-MS). Advances in affinity chromatography in the separation of peptide, proteins and biopolymers (IMAC) and of organic substrates (IMCOS) are discussed. Recent advances in understanding the mechanisms of chromatographic separations, and of the technique of polymer imprinting to produce selective recognition sites for metal ions and metal complexes are described.

Keywords: Chromatographic methods; Electrophoretic methods; Transition metal complexes

1. The changing landscape in chromatography

This review describes currently useful methods for the separation and characterization of inorganic complexes. The review covers the advances in inorganic chromatography that have appeared since 1992 through early 2003—a period of substantial change in methodologies—to achieve separations of isomers, chiral complexes, or species which bind to metal complexes via molecular recognition that affects their migration rates.

Separation procedures are often carried out to remove unreacted reagents or side products from a synthesis. Many standard methods are known for these purifications. Advances in techniques for such routine separations by ion chromatography, normal and reversed phase chromatography, and thin layer methods are often limited by the availability of a specialized support material. This review will not emphasize this kind of classic chromatographic information. A reader desiring such information is referred to several recent reviews listed in Table 1 serving that purpose. Rather, the purpose of this review is to highlight inventive methods for species separation that have evolved during the last decade in response to demands from biomedical and industrial applications.

Although the review presents successful examples that illustrate each methodology, the vast nature of chromatography prevents the exhaustive type of referencing that is usually encountered in *Coordination Chemistry Reviews*. It is the author's opinion that having relatively current references to some better examples of these methods as applied to inorganic separations, and having sources of how the techniques have been best performed on representative laboratory samples, far exceeds the utility of an exhaustive reference catalogue on each technique. The review is meant by editorial decisions to be a complementary expansion of a discussion of these techniques that will appear in book chapter of *Comprehensive Coordination Chemistry II* for separations of transition metal complexes and their ligands. The present review contains a more extensive bibliography for most of the methods that are described in both sources. Also, discussions of some techniques including electrochemical detection in chromatography using $[Ru(bpy)_3]^{3+/2+}$ as a mediator with electrode surfaces, electrospray mass spectral detection, size exclusion chromatography (SEC), and environmental bio-inorganic separations—all topics which had to be de-emphasized for text space reasons in the book chapter, but which are of value to specialized segments of the inorganic chemistry community—are presented here.

The review will start with the more classic separations of small molecules using support phases that are useful in the separation of charged complex ions, and separations that occur by differences in molecular recognition between

Table 1
Recent chromatographic reviews on inorganic separations

Reviewed chromatography topic	Reviewer(s)	Number of references	Reference
(1) Classical ion chromatography	Haddad	26	[1]
(2) Simple cation/anion separations	Janos	120	[2]
(3) Acid–base equilibria in ion exchange	Janos	120	[3]
(4) TLC—a 22-year report	Iraqi and co-workers	505	[4]
(5) TLC (planar chromatography)	Sherma	506	[5]
(6) Silica gel attachments to separate inorganics	Biernat et al.	899	[6]
(7) HPLC of glycoaminoglycan Cu^{II} complexes	Wang and Lee	23	[7]
(8) HPLC of metal complexes (1994–1997)	Casoli et al.	190	[8]
(9) HPLC of platinum group metal complexes and anticancer agents	Sommer and Vlasankova	103	[9]
(10) GC of metal complexes	Uden and Henderson		[10]
(11) GC of organometallics with ICP-MS detection	Bouyssiere et al.	117	[11]
(12) GC separations of enantiomers by chiral complexes as the stationary phase	Schurig	102	[12]
	Schurig	96	[13]
(13) GC separation by metal acac complexes as the stationary phase	Slizhov and Gavrilenko		[14]
(14) SEC of bio-metal complexes	Haraguchi	134	[15]
	Haraguchi and co-workers	19	[16]
(15) CZE or CE of metal complex speciation	Timerbaev	175	[17]
	Timerbaev and Buchberger	110	[18]
	Timerbaev and co-workers	104	[19]
	Timerbaev and Shpigun	161	[20]
	Timerbaev	267	[21]
(16) CE of metal complexes	Pacakova et al.	128	[22]
(17) CE with chemiluminescence detection	Huang and Fang	53	[23]
(18) CE enantiomeric separations with chiral complexes as the stationary phase	Kang et al.	77	[24]
(19) CE separation of platinum complexes and anticancer drugs	Timerbaev et al.	70	[25]
(20) Micellar electrokinetic capillary chromatography (MEKC)	Haddad et al.	43	[26]
(21) ECL (electrogenerated chemiluminescence) with $\{[Ru(bpy)_3]^{2+}\}^*$	Fahnrich et al.	280	[27]
(22) Separations of metal chelates in supercritical CO_2 fluid	Wai and Wang	61	[28]
(23) Supercritical fluid extraction of organometallic complexes	Lojkova and Vejrosta	57	[29]
(24) ICP or AA detection of metal-biomolecules	Szpunar and Lobinski	111	[30]
(25) ICP or AA detection of metalloproteins	Sandier et al.		[31]
(26) ESI-MS of inorganic ions; elemental speciation	Stewart	359	[32]
(27) ESI-MS/ICP for metallo-biopolymers	Szpunar	375	[33]
(28) Separation of chiral molecules using cyclodextrin or metal ion additives	Gorog and Gazdag	227	[34]
(29) Separation of chiral Co^{III} chelates	Davies	380	[35]
(30) Chiral phase support columns; supramolecular structures	Wenz	475	[36]
(31) GC enantiomer separations using Chirasil-supported complexes	Schurig	156	[37]
(32) RP chiral column separation of boron clusters	Gruner and Plzak	47	[38]
(33) IMAC separations	Wong et al.		[39]
	Chaga	141	[40]
	Gaberc-Porekar and Menart	118	[41]
	Udea et al.	206	[42]
(34) IMAC-like complexes as gas phase supports	Shi and Fu	39	[43]
(35) IMAC-like GC for organic separations using Cu^{II}- and Cr^{III}-supported complexes	Rykowska and Wasiak	14	[44]
(36) Mesoporous chromatographic hosts with grafted metal complexes for separations	Moller and Bein		[45]
(37) Molecular mechanics/molecular dynamics and Monte Carlo methods in selective substrate-binding site interactions	Lipkowitz	87	[46]
	Lipkowitz	36	[47]

complexes in a moving phase upon exposure to a stationary phase. These general methods have evolved for the separation of isomers and chiral complexes upon supports designed to differentiate species of different geometry or pairs differing by chirality.

Two newly evolved techniques are discussed that are making impact on what the inorganic synthetic community can achieve in speciation and separation. These apply to small amounts of ionic complexes or metal ions chelated by complicated ligands of biochemical origin: (1) electrochemically-assisted separations that may be applied to relatively small samples (capillary zone electrophoresis and derivative methods), and (2) identification of the number and nature of ligands in complexes that may be synthesized only in a solution phase by the mass-to-charge ratio (electrospray mass spectrometry; ESI-MS). Methods of separation that have been motivated by needs of the biomedical community include the separation of ions attached to biopolymers (e.g. metallo-enzymes, regulatory peptides, DNA oligomers and cleavage fragments, transport

proteins, etc.). These methods include size exclusion chromatography and immobilized metal ion affinity chromatography (IMAC). IMAC developments rest upon providing molecular recognition sites on a polymer support by means of the attachment of inorganic complexes that can coordinate and retard the migration of biopolymers. Additionally, the separation of organic feedstock mixtures is taking a developmental cue from IMAC bio-separations. A newly evolving technique that uses metal complexes, supported on polymeric materials, to retard the chromatographic migration of species (IMCOS) is described. Also included in this review is the new field of molecular mechanics modeling of chromatographic binding sites for the purpose of optimizing separations. Interestingly, it is inorganic chemists who have contributed the most to design in this frontier area. The newly evolving field of polymer imprinted molecular recognition sites for metal ions and ternary metal complexation is discussed.

If a researcher intends to use this review as a guide to selecting a type of chromatography that will assist progress in his or her own laboratory, he or she will need to take note of the charge and structural arrangement of their complexes which are to be separated. Additionally, the sample size is another aspect that controls the choice of method. How these parameters parallel the example cases that are provided in this review will dictate the applicability of a given method as a service to his or her research.

2. Other review sources

Various aspects of chromatography as applied to inorganic systems have been reviewed recently by others in the field. The reader is referred to the following previous reviews as detailed sources on the following topics (Table 1). Some 15 new reviews appeared in 2002–2003, particularly in electrochemically-connected chromatography methods and in chiral separations.

3. Recent advances in inorganic separations

3.1. Reminders about the origin of chemical separations

The inorganic chemist is reminded that reversed-phase (RP) chromatography occurs with a non-polar stationary phase and relatively polar mobile phase. Conversely, normal phase (NP) methods utilize a polar stationary phase and a non-polar mobile phase.

In any chromatographic separation procedure, various species for separation (S_1, S_2, S_3, ...) distribute between a mobile phase and a stationary phase. The migration rate depends on the relative time spent by the species in the mobile phase. For convenience, one can consider the mobile phase environment as "site A" and the differing environment of the stationary phase as "site B". The "on–off" equilibrium of the substrates (S_1, S_2, S_3, ...) at the binding site B produces the retardation to migration of S_1, S_2, S_3, ..., and hence, results in the separation of S_1 from S_2 and S_3 ... based on the differing affinities of these substrates for binding sites B in the stationary phase.

It is useful for further discussions throughout this review to recall that this solution (mobile) phase to stationary phase equilibrium is influenced by the following factors:

- charge attractions between (S_1, S_2, S_3, ...) and the stationary site B; this is the fundamental origin of separation in cation or anion chromatography;
- hydrophobic contacts of substrates $S_1, S_2, S_3, \ldots$ for structural features of the binding site B; this adds to the affinity of individual substrates for binding site B and adds to the binding constant, and hence, can be a function of ligand structure for metal complexes;
- hydrogen-bonding contacts of S_1, S_2, S_3, ... for H-bondable structural units at the binding site, such as amines, ROH, carboxylates, etc. so that ligand structures of metal complexes can be highly influencing of separation behavior for differing complexes;
- the multi-contact effect—this extra affinity of a particular substrate is a manifestation of the hydrophobic effect (2) and the H-bonding effect (3), but it exists only in highly organized orientations, such as when a three-oxygen interaction of a phosphate group makes simultaneous H-bonding interactions with a receptor site; the result produces a free energy of binding that is greater than the sum of the individual enthalpic contributors.

The presence of an additive to the mobile phase can also influence the "on–off" equilibrium, and hence, the rate of transport within the mobile phase. (It is assumed for discussion that the additive does not bind at site B as a competitive inhibitor.) Two effects that are important for the current applications of inorganic chromatography are produced by the addition of a counter-ion (X^n) that can form an ion-pair with charged analytes (S_1, S_2, S_3, ...), and by chiral recognition additives (CR) that form association complexes with the analytes. In the case of electrophoretic methods where migration in the mobile phase is induced for ions by means of an electric field gradient, the formation of ion-pairs normally reduces the migration rate because the net charge on the ion-pairs (S_1X^n, S_2X^n, S_3X^n, ...) are lower than on the original analytes (S_1, S_2, S_3, ...), and the additional mass and size produces more diffusional drag, resulting in a lower mobility. The same mass and cross-sectional area influence on the mobility of substrates occurs for the association complexes (S_1CR, S_2CR, S_3CR, ...). Thus, these also tend to move slower in the presence of a chiral recognition additive.

However, the reader is cautioned that the addition of ion-pairing reagents X^n or chiral recognition reagents CR can, in fact, accelerate the migration rate of analytes (S_1, S_2, S_3, ...) if by virtue of their strong binding in competition with the binding site B, the additives greatly favor that the substrates reside in the mobile phase. Therefore, additives

can either enhance or retard the mobility of analytes. The influence of additives may be used by the experimentalist who performs the chemical separation to his or her advantage.

The general features of chromatography and the influence of additives during chromatographic procedures, as discussed above, will appear in applications throughout the following sections of the review.

3.2. Standard chromatographic separations of metal complexes

A number of reviews related to inorganic separations have been listed in Table 1. Haddad has given a retrospective report on classical ion chromatographic separations [1]. Simple cation/anion chromatographic separations and the competitive influence of acid–base equilibria have been discussed by Janos [2,3]. Thin layer chromatographic methods for simpler aqua ions have been reviewed by Iraqi and co-workers [4] and by Sherma [5].

The progress in modifying silica gel supports with surface R group attachments that vary the surface polarity and terminal functionalities has been extensively discussed by Biernat et al. [6]. The use of supercritical fluid CO_2 as a mobile phase for organometallic complexes including transition metal and lanthanide complexes of organophosphates, acetylacetonates, dithiocarbamates, calixarenes, and triazolato crown ethers has received two reviews [28,29].

The use of chiral transition metal complexes as the stationary phase in the separation of enantiomers of organic molecules and other transition metal complexes is an active, recent addition to the fields of gas chromatography and capillary electrochromatography [11–14,37,44,45]. This work is discussed in later sections of this report.

Computer methods applied to the energetics of complementary host–guest attractions, and hence substrate-chromatographic binding site interactions, are in their infancy as an approach to improving separations; initial attempts to use computer methods have been reviewed by Lipkowitz [46,47].

Other aspects of general reviews of chromatographic methods from Table 1 will be mentioned in the more detailed sections devoted to these topics throughout this report. Recent results from simple ion chromatography, LC and TLC will now be described.

Lucy has discussed how instrumental advances in detectors have kept simple ion chromatography as a useful tool in spite of the rapid rise of capillary electrophoresis (CE) to replace it [48].

The use of chloro-8-hydroxyquinolines to separate V^V, Ni^{II} and Co^{III} using RP methods showed that binding to non-polar support phase was enhanced compared to the mobile phase residence time as the number of 5-chloro and 7-chloro substituents increase, making the phenolate donor less basic (ionic) [49].

Conversely, coordinating anions enhance the mobile phase residence time in RP methods as shown by the assisted movement of 20 different d-block metal ions by the addition of oxalic acid or succinic acid to the mobile phase of ions adsorbed on talc or starch/talc support for RP-TLC [50].

Dye molecules that can chelate metal ions have been used to aid in visualization of the location of metal complexes as these are transported through a chromatography column. Bruzzoniti et al. have used alizarin violet N (LH_2^-) which forms a 1:1 $[Cu^{II}(L^{3-})]^-$, but a $[Co^{II}(L^{3-})(LH_2^-)]^{2-}$ complex to show that ion-pairing to cationic column sites obeys an electrostatic model [51].

Many metal–edta complexes of the type $[M^{II}(edta)]^{2-}$ and $[M^{III}(edta)]^-$ are popular in chromatographic separations, particularly from environmental and geological samples. The M(edta)-type anionic complexes are separated by amine-based cationic RP columns. Shpigun and co-workers have shown that the more hydrophobic "$hbed^{4-}$" analogue of $edta^{4-}$, which has two benzyl-*o*-phenylate arms symmetrically replacing two glycinato arms of $edta^{4-}$, achieves a separation of ion mixtures including Fe^{III}, Co^{III}, Mn^{III}, Al^{III}, Cu^{II}, Ni^{II}, Zn^{II} as $[M(hbed)]^{n-}$ complexes with a 10-fold lower detection limit than for the M(edta's) separated on RP columns. This is indicative of column affinity as the key aspect of separation of ion mixtures and that $[M(hbed's)]^{n-}$ exhibit greater differentiation in affinities for the non-polar support phase [52]. The separations are ligand-dependent and -independent of the nature of the stationary phase.

Because of their substitution inertness, substituted acetylacetonates (R-acac$^-$) and thio-acetylacetonates (R-(S)acac$^-$) have been studied for uses in separations of M^{III} complexes by GC (M^{III} = Cr^{III}, Co^{III}, lanthanide III, etc.). This has been extended to the separation of M^{III}(R-acac)$_3$ and M^{III}(R-(S)acac)$_3$ complexes on polyacrylonitrile sorbents [53]. Substitution of exterior R groups into the acac$^-$ structure generally increases retention time, as does replacement of the acac$^-$ O donors by S in the thio derivatives; e.g. less polarity or more hydrophobic contacts via R increase the residence time in the non-polar phase of RP columns. Replacement of the CH_3 groups of the acac$^-$ ligand progressively by R = $[(\phi O(CO))_2CHO]$ with M^{III} = Co^{III}, Cr^{III} or Ru^{III} in M^{III}(acac's)$_3$ increased the residence time in the non-polar support phase with non-polar eluents, and decreased the residence time with polar aqueous eluent solutions [54].

Separations of Co^{III} complexes of polyaminocarboxylate or amino acid chelates have received much attention [55–57]. SP-Sephadex is a popular chromatographic support medium for the separation of Co^{III} β-diketonate and polyamine complexes. On silica gel, cellulose, polyacrylonitrile, or nitrile-modified silica gels, adding $(NH_4)_2SO_4$ to the carrier phase decreased the R_f value (poorer transport and greater support affinity implied) and this effect amplifies as the size of the backbone chelate ring is increased [55–57]. Polyacrylonitrile gave the highest separation factors among similar Co^{III}(aminocarboxylate) complexes of the $edta^{4-}$ and amino acid families [58], being superior to silica gel and cellulose supports.

Dithiocarbamate (R_2dtc^-) complexes are robust, and form many $M^{II}L_2$ and $M^{III}L_3$ complexes. In a chromatographic study on C-18 RP columns, complexes of M = Zn^{II}, As^{III}, Fe^{III}, Cd^{II}, Pb^{II}, Ni^{II}, Cu^{II}, Hg^{II}, Co^{III}, and Cr^{III} were separated using methanol/water or acetnitrile/H_2O or acetonitrile/CH_3OH/H_2O mixtures [59]. Those $M(R_2dtc)_n$ complexes with R of C-3 to C-5 size were separated by the ternary solvent system (except for R = isopropyl), showing that a crucial polarity balance is required to differentiate various metal complex polarities. The related dialkyldithiophosphates of Zn^{II}, [$Zn(R_2PS_2)$] have been separated from lubricating petroleum oils by hexane-based normal HPLC [60].

Cu^{II} amino acid complexes are important in biochemistry; these as Cu^{II}(AA) complexes are separable by RP methods. The column affinities follow the hydrophobicity of the side chain R functionality of the amino acid (AA) [61]. Cu^{II}(AA) complexes appear in environmental samples produced by exposed sediments. It was shown that transport of Cu^{II} into the environment by amino acid chelation occur with rates depending upon the electrostatic charges of the complexes (accelerating transport with higher charge) and their hydrophobicities (suppressing transport for more hydrophobic AAs) [62].

4-(2-Pyridylazo)resorcinol = PAR and 4-(thiazolylazo)-resorcinol = TAR (Fig. 1) are popular "N_2O chelate" ligands for chromatographic separations of higher oxidation state metal complexes. RP columns eluted with citrate buffers have been used to separate Nb^V and Ta^V using PAR in the mobile phase [63], to separate Pd^{II}, Rh^{III}, Pt^{II}, and Ru^{III} as PAR complexes by RP-HPLC [64], and for Nb^V and Ta^V from granites and basalts as PAR complexes with citrate buffers [65]. TAR complexes of Ru^{III}, Rh^{III}, Os^{IV}, and Co^{II} have been separated by capillary electrophoresis [66].

3.3. Silica surface modifications in chromatography of metal complexes

Treatment of silica gel with $SiCl_3$(vinyl), followed by acrylamide, and digestion with ethylenediamine provides silica-anchored amide/amine functionalities as indicated:

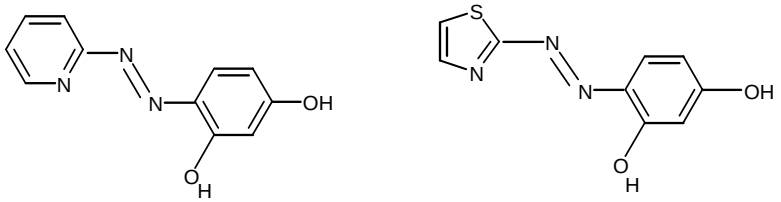
silica-O-(CH_2CH_2)(C=O)N*H(CH_2CH_2)N*H_2

The terminal amide/amine chelation (starred chelation points) has been used to separate Co^{II}, Cu^{II}, Fe^{III}, and Ni^{II} with better column capacity than for attached 8-hydroxyquinoline or monoamine-supported columns [67]. Mercaptopropyl and cyanopropyl attachments to silica were derivatized with $CuCl_2$ to make supported complexes; these supported Cu^{II} complexes were used to separate a 10 component mixture of C-2 through C-4 olefins, but also CH_4, C_2H_6, C_3H_8, and C_4H_{10} [68]. This new separation technique, immobilized metal complexes for organic separations (IMCOS), is discussed in a later section of this review.

Pyridinyl groups in the H^+ form or quarternary ammonium tails bonded to silica have been used to separate $M(edta)^{n-}$ and $M(cydta)^{n-}$ complexes on Silasorb-C-18 support materials [69], while *N*-ethylpolyvinylypyridine attachments were used to detect 1–5 ng of Mn^{II}, Co^{II}, Cu^{II}, Ni^{II}, Zn^{II}, Cd^{II} or Pb^{II} as $M(edta)^{2-}$ complexes with UV detection from columns of 6000 theoretical plates [70]. Silica, itself, was shown to be anionic and able to separate Co^{III} complexes from 3+, 2+, 1+, and 0 charges (and does not bind anionic complexes) using KCl, KI or K_2SO_4 in the eluent [71]. 5-Sulfonato-8-hydroxyquinoline (HQS) complexes of various metal ions have been separated on octadecyl-modified silica with determination by fluorescence [72].

Normal phase (polar supported stationary phase with non-polar eluting phase) separations of metal tetraphenylporphyrins (TPP^{2-}) with M^{II} = Co^{II}, Ni^{II}, Cu^{II}, Zn^{II} were performed on silica derivatized by (4-pyridyl)ethyl, 4-hydroxy-5-ethylimidazole, and propylamine functionalities; the best separations were obtained for M(TTP) complexes that desire axial coordination that could be afforded by the pyridyl or imidazole moieties [73].

Robards and Patasalides have compared the utility of GC versus LC chromatographies on complexes prepared from salicylaldimines, Schiff bases, fluorinated β-diketonates, β-dithiones, and a hexadentate macrocycle with lanthanides, transition metals, VO^{2+}, Pt^{II}, Pd^{II}, and Zn^{II} [74]. Ligand instabilities under the thermal conditions of GC is the main limitation for GC separation methods. The instability problem may be overcome by adding volatilizing R groups (C_2F_5, C_6F_5, etc.) to the ligand structure, allowing for mobility of the complexes at a lower temperature. In general, however, it was concluded that for ultratrace determinations only the LC approach has the versatility, sensitivity and ability to separate differing species [74].



PAR TAR

Fig. 1. Structures of resorcinol-based dye chelates. PAR = 4-(2-pyridylazo)resorcinol and TAR = 4-(thiazolylazo)resorcinol.

The attachment of metal acac chelates to silica as the stationary phase for GC separations has been reviewed by Slizhov and Gavrilenko [14].

3.4. Organometallic separations

Separation of methyl and ethyl complexes of Hg, Cd, Zn, Sn, Pb, Te, and As by GC methods using ICP-MS detection has been reviewed by Lobinski and co-workers [11].

Supercritical CO_2 separations have economic advantages for industrial separations. Developments in the use of this solvent phase for inorganic systems are relatively few, in part because of the specialized apparatus that is required for high pressures. Separations of metal chelates under supercritical CO_2 conditions have been reviewed [28].

Supercritical CO_2 fluid has been used to separate bis-cyclopentadienide complexes with $FeCp_2$ and $NiCp_2$ as trial examples; the transport rate follows the solubility of the MCp_2 complex in the mobile phase CO_2 [75]. Ferrocene-labeled materials are of increasing interest for markers in synthesis, biochemistry, and commercial uses. Tags of $FeCp_2$ bonded to estrogens were used in separation of estrogens by HPLC methods, using UV and electrochemical detection [76]. Separation of Pt from sources has been achieved by designing chelating ligands that afford the transport of $PtCl_6^{2-}$ into the supercritical CO_2 phase [77]. Bechman and Powell project the use of supercritical CO_2 for other separations of precious metal compounds [77].

NP methods using hexane/diethylether eluents were used to separate tetra(pentafluorophenyl)propholactone complexes in the elution order of PdL > AgL > ZnL > NiL. The elution order should be the order of increasing polarity of the complex, as those bound more strongly to the polar stationary phase transport more slowly [78]. Stilbenediamine Schiff base complexes are readily modified as to their hydrophobicity by varying the R group. Their M^{II} metal complexes (M^{II} = Ni^{II}, Pd^{II}, VO^{2+}, Cu^{II}) are readily separated on Nova Pac C-18 RP columns [79].

Few species are as representative of "organometallic" complexes as the Fischer carbenes. $[Cr(CO)_5(=C(OR)R')]$ and $[Cr(CO)_5(=C(NHR)R')]$ complexes have been separated by HPLC, followed by mass spectral detection [80]. Presumably, these separations serve as models for the separation of other metal carbonyls.

Zn^{II} and Ni^{II} complexes of C-10 ether-*para*-substituted dithiobenzoic acid, ML_2, form metalmesogens, liquid crystal phases. $Ni^{II}L_2$ metalmesogens have been used as self-contained non-polar phases linked to metal differentiation regions to separate polyaromatic hydrocarbons ("PAH") samples using NiL_2 at 140–230 °C on Chromosorb; the $Zn^{II}L_2$ species separate R_2S substrates [81]. Phenols were separated with either the $C_{10}H_{21}OC_5H_4CS_2^-$ complexes adsorbed to 5% on Chromosorb, or by siloxane attachment of the *p*-$C_5H_4CS_2^-$ binding site to glass capillary surfaces as a wall coating [82].

3.5. Bio-sample separations

This topic will be covered in a separate section of this review. However, a few studies are mentioned here as example techniques. Chelation of a desired metal from a biological sample often precedes chromatographic separation. The residual cisplatin (*cis*-$PtCl_2(NH_3)_2$) concentration in a cancer patient's blood may be reacted with bis(isovaleroacetone)ethylenediimine to make a chloroform-extractable complex that separates readily on BP1 or BP5 columns. Using an FID detector, the range of 246–253 ng/ml is detectable [83]. Cu^{II}, Ni^{II} or Pd^{II} in the blood separate as complexes of the diimine wholly separate from the cisplatin derivative, allowing quantification.

Multi-dimensional chromatography, sometimes called double or two-dimensional chromatography, can be used to first separate multiple metal complexes. A first type of chromatography is followed by a second variety of chromatography in order to separate components or isomers. For example, pheophytin a and b make Cu^{II}, Zn^{II}, Pb^{II}, Hg^{II}, and Ce^{III} complexes. The metal ion mixture was separated as (a plus b) components using RP-HPLC with hexane/butanol eluent, followed by separation of the pheophytin a from pheophytin b of each metal using NP chromatography with acetonitrile/ethanol/acetic acid eluent [84].

4. Separations of isomers and chiral metal complexes

One of the most useful applications of chromatography is in the area of separation of isomers and of chiral complexes for preparing catalysts and metallodrugs. Many potentially chiral transition metal undergo rapid inversion of configuration or fluxional processes that racemize the complexes, particularly, the tetrahedral ones, or those that can undergo trigonal bipyramidal to square pyramidal Berry twist rearrangements. In these cases, the ligand field rearrangement barriers are low, affording pathways for racemization. Thus, it is no surprise that most work on chiral transition metal complexes has been carried out with low-spin d^6 octahedral (usually Ru^{II} and Co^{III}) and d^3 octahedral (Cr^{III}) transition metal complexes which have high ligand field barriers to isomerism.

Three recent reviews discuss aspects of chromatography for separation of chiral molecules [34,85,86]. The separation of enantiomers for the pharmaceutical industry by GC and HPLC using π contacts with supported metal complexes has been presented [85]. The technique of adding chiral molecules to the mobile phase to separate enantiomers has been discussed [34]. Often organic chiral-binding molecules such as cyclodextrins are used to alter the mobility of enantiomer pairs, but chiral metal complexes which have in-solution affinities for second-sphere molecules in the "solvation" shell are also used [34]. The separation of *cis* and *trans* isomers has been reviewed by Queiroz and Batista, but the utility is limited to those who read Portuguese [86].

4.1. Separation of isomers of Co^{III} complexes

Co^{III} complexes often offer the best chance for isomer separation via chromatography. fac- and mer-$Co^{III}L_3$ (L = 5-methoxy-1,2-benzoquinone-2-oxime) complexes have been separated on silica gel with 1:1 toluene:CH_2Cl_2 as the mobile phase, allowing the structure of the fac isomer to be obtained on a "quinone-oxime" chelate [87]. [Co^{III}(tren)(acac)] atopimers were separated on Celluofine-200 (a modified polysaccharide ion exchanger) without the need to resort to adding chiral agent to the mobile phase, as is required for such separations on sephadex [88]. [Co^{III}(eddp)(AA)] complexes (eddp^{2-} = *N*,*N*′-propylenediaminediacetate and AA = an amino acid), and [$Co^{III}(AA)_3$] complexes were separated into fac and mer isomers (fac isomers have three N donors on one face of an octahedral complex) using TLC on polyacrylamide support [6,86]. This is an example of the multi-contact type of differentiating association that occurs due to a structural feature in the molecule wherein the presence of the structural arrangement provides a higher affinity than an equal number of functionalities that are not properly arranged for maximum binding.

Separation of Co^{III} acacs of the various permutable formulas [$Co^{III}(acac)_{3-n}(\phi acac)_n$] was also accomplished [89]. The mer isomers exhibited higher R_f values for all three categories of Co^{III} complexes in the study [89], indicative of less binding to column support sites. A three-point association with the chromatographic surface, available with the fac isomers, gives the highest binding constants and slower migration rate.

Sargeson and co-workers used chromatographic methods to separate numerous Co^{III} complexes that are obtained from commercial tetraethylenepentamine, resolving linear, branched trenen, and other isomeric complexes [90]. Isomers of Co^{III} complexes have been separated by LC on clays containing a Ru^{II} complex adduct as a resolving feature [91]. Co^{III} and Pt^{II} isonitrosothiocamphor, isonitrosoacetylacetone complexes, and imine complexes of Ni^{II} were separated by TLC and column methods [92].

4.2. Separation of isomers of Ru^{II} complexes

The separation of $Ru^{II}L_3$ complexes into Δ and Λ isomers has been stimulated by the interest in the higher affinity of Δ-[Ru^{II}(*o*-phen)$_3$]$^{2+}$ over the Λ-isomer for the major groove of calf-thymus B-DNA [93] or in the minor groove for short oligonucleotides of 10–20-mer size [94], and by the search for useful DNA-nicking (antitumor) agents or gene-specific cleavage agents. "Chiragen" ligands are two 2,2′-bipyridine ligands linked together by a bridging unit such as *m*-xylyl, $(CH_2)_5$, and $(CH_2)_6$. The linker enforces chiral coordination as with [Ru^{II}(chiragen)Cl_2] by Murner et al. [95]. Binuclear complexes were then prepared using 2,2′-bipyrimidine as a bridge between Ru^{II} sites as [(chiragen)Ru^{II}(bipym)Ru^{II}(chiragen)]$^{4+}$ complexes.

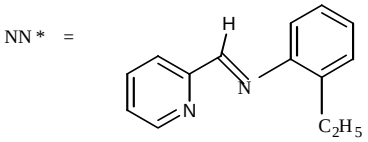


Fig. 2. Pyridineimine ligand structure in Nuber's chiral bis-(2,2′-bipyridyl)(2-*N*-[(*S*)-1-phenylethyl]pyridineimine)ruthenium(II) complex [97].

Pure Δ, Δ; Λ, Λ; and mixed Δ, Λ isomers were separated [95]. A related bipym-bridged complex with four 4,4′-dimethyl-2,2′-bipyridines replacing the chiragen ligands of the Murner complex has been studied by Fletcher and Keene [96]. This complex has two diasteromers, rac and meso, which were observed by ^{1}H NMR to have differing affinities for various organic counter-ions. The authors point out the importance of such chiral discriminations for isomer separations in chromatography.

Starting from *cis*-[Ru^{II}(bpy)$_2Cl_2$], Nuber et al. added NN* ligand (Fig. 2) to make the Λ, Δ isomer pair, which were then chromatographed to obtain pure Δ-[Ru^{II}(bpy)$_2$(NN*)]-$(PF_6)_2$ [97].

As a twist on the DNA-binding scheme by chiral metal complexes, Williams and co-workers prepared DNA-supported HPLC columns which were then used to separate the Λ and Δ isomers of $Ru^{II}L_3^{2+}$ complexes (L = *o*-phen, quinoxaline), based upon the chiral discrimination of the major groove of DNA for the $Ru^{II}L_3^{2+}$ isomer [98]. Such separations are limited by the size of the $Ru^{II}L_3^{2+}$ species that can fit into the major groove of supported DNA.

Using an SP-Sephadex C-25 column, Fletcher et al. have separated fac and mer isomers of [Ru^{II}(bpy-R)$_3$]$^{2+}$ complexes, even when recrystallizations failed to separate the various isomers [99]. Kane-Maguire and co-workers have separated [Ru^{II}(diimine)$_3$]$^{2+}$ complexes by the addition of chiral anionic agents (antimonyl-D-tartrate or dibenzoyl-L-tartrate) to the mobile phase of a capillary electrophoresis separation method in the presence of micelles, acting as the stationary phase, suspended throughout the mobile phase [100]. This is termed MEKC, which is discussed in Section 5.1 of this report. The migration rate of isomers of [Ru^{II}(diimine)$_3$]$^{2+}$ are influenced by the presence of chiral anions by formation of ion pairs which distribute selectively into micelles in the mobile phase. Thus, separations of racemic mixtures may be achieved due to the differences in partitioning between the mobile phase and the micellular phase, along with differences of mobility of the isomers due to the ion-pairing equilibria and altered net charges of the ion pairs in the mobile phase.

Pellegrini and Aldrich-Wright have used the addition of Na_2[(−)*O*,*O*-dibenzolyl tartrate] to achieve the separation of Λ and Δ isomers of [Ru(4,4'-Me_2bpy)$_2$L]$^{2+}$ complexes on sephadex for L = phen, dipyridylquinoxaline (dpq), dipyridotetrahydrophenazine (dpqc), and dipyridophenazine (dppz) which showed the affinity order of complexes with L = phen < dpq < dpqc < dppz in studies used to show

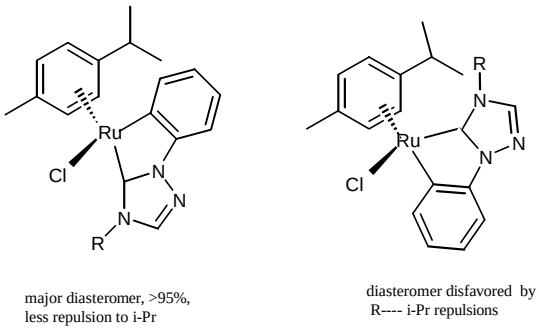


Fig. 3. Diasteromers of [(η^6-cymene)(triazolinylidene)ruthenium(II)] ({Ru^{II}(cymene)Cl[=CN(R)$CHN_2(C_6H_4^-)$]}) complexes described in [104].

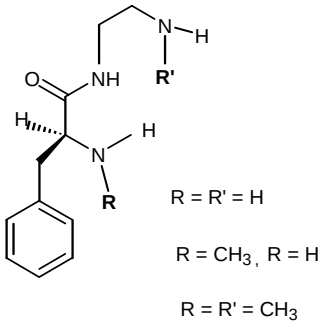


Fig. 4. Chiral amino–amide ligands of Marchelli and co-workers described in [107].

that the addition of the 4,4′-dimethyl substituents strongly influence (and reduces) the DNA groove binding compared to their $[Ru(bpy)_2(L)]^{2+}$ analogues [101].

The same technique of adding chiral anions has been used by Kane-Maguire and co-workers to separate complexes of Ru^{II}, Ni^{II}, Cr^{III}, and Co^{III} by capillary zone electrophoresis using chiral anion additives of threo-D-*S*[+]-isocitrate, antimonyl-D-tartrate, or dibenzoyl-L-tartrate [102]. Curiously, the elution order of isomers is reversed for complexes of 3+ charge compared to all the other ion combinations [102].

More standard separations were also achieved, such as the separation {*cis*-[Ru(cyclam)Cl_2]}Cl from the *trans* isomer, by means of RP-HPLC on octadecylsiloxy-derivatized preparative columns [103].

Enders et al. reacted the η^6-(cymene)$RuCl_2$ and Cp^*RhCl_2 dimers with *N*-phenyl triazolium perchlorate to make the pseudo-tetrahedral triazolinylidenes shown here with a 95% ee purity for the Ru^{II} derivative with cymene [104]; the synthesis itself is highly enantioselective as addition in the opposite sense produces a steric confrontation between the isopropyl group of cymene and the N–R group of the triazole. However, the diasteromers were further purified by simple chromatography on silica with CH_2Cl_2 as the mobile phase prior to obtaining an X-ray diffraction which established the *R* chirality for the Ru center (Fig. 3).

4.3. Copper(II) and nickel(II) chiral applications in chromatography

The lability of most Cu^{II} and Ni^{II} complexes often prevents structurally rigid species for study. However, their rapid ligand on–off rates allow for equilibria in solution which establish interactions with chiral ligands, usually in a preferential (not statistical) distribution. This has been used in two ways to assist chromatographic separations: (1) by adding Cu^{II} or Ni^{II} to the mobile phase, complexes that are not attached to column support sites, but which are chiral and have differing affinities for other chiral ligands, alter the ligand mobility through the column and separate of the chiral pairs of the ligands or (2), by attachment of Cu^{II} or Ni^{II} as supported complexes which have coordination discrimination for the chiral ligands can be used to separate isomers of the ligands.

Examples of the additive effect on mobile phase transport have been given by Bazylak for the separation of chiral amino-alcohols using optically active Ni^{II} Schiff base complexes added to the acetonitrile/water mobile phase [105]. This work was applied to the separation of ephedrine isomers in cough syrups and medicinal tablets, and follows the scheme introduced using Cu^{II}(L-proline)$_2$ as chiral mobile phase additives [106].

Engle and Purdie used achiral meso-tartrate Cu^{II} complexes plus chiral pseudo-ephedrine to show that chiral complexes migrate at different rates in LC [106].

A most interesting study by Marchelli and co-workers showed that RP-HPLC may be used to separate D,L-amino acids and dansylated-amino acids by adding Cu^{II} terdentate complexes of the ligands as shown to the mobile phase [107] (Fig. 4).

At pH 6, the deprotonated central amide and the two terminal amino groups of Marchelli's Cu^{II} complex are all coordinated in three "in-plane" sites of the Cu^{II} center. This affords only one in-plane site for coordination of an N-terminal donor of an amino acid. However, the axial interaction of the carboxylate functionality affords two locations for the placement of R groups of the amino acid side chain as shown here for phenylalanine. The isomer with preferential π to π ring stacking, shown at the left, makes a stronger complex that keeps the D-amino acid more complexed, and more adsorbed by the column sites, eluting later of the D,L pairs (Fig. 5).

For other amino acids with R groups other than phenyl, the separation is poor unless the amino acids are N-terminally dansylated, affording multiple π-stacking contacts (see structure) and higher column affinity for the dansylated D-amino acids [107]. The Marchelli group has also made covalently bonded *S*-phenylalaniamide and *R*-phenylalaninamide phases which bind added Cu^{II} and then coordinate to dansylated amino acids. Separations in this mode are correlated to the bulkiness of the amino acid side chain R group [108].

A similar approach using a RP C-8 HPLC column coated with N-T-*n*-decyl-L-spinacine has been described by

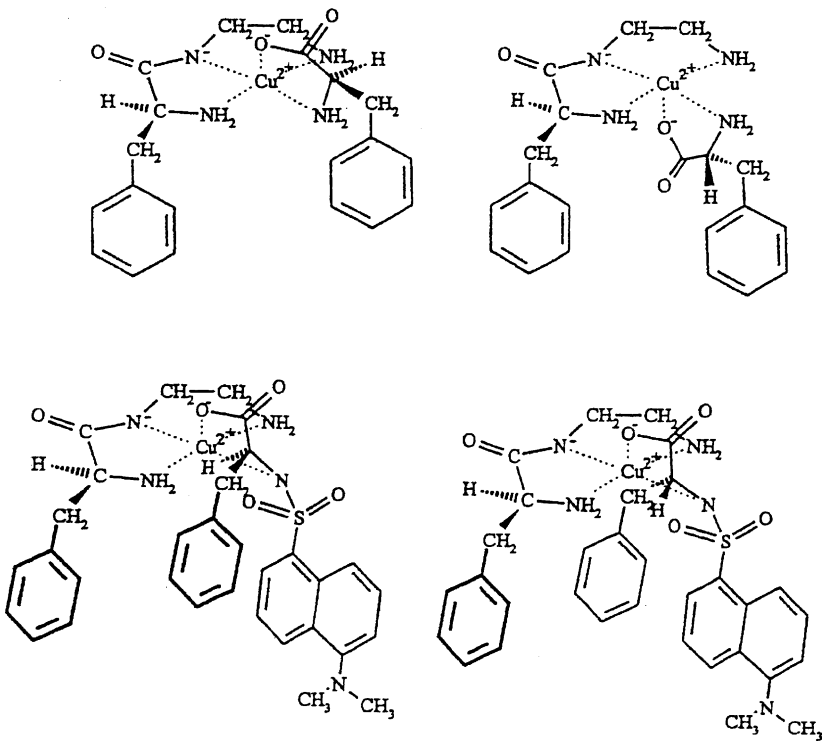


Fig. 5. D- and L-phenylalanine and *N*-dansylated D- and L-amino acid complexes of Marchelli's Cu^{II}(amine–amide ligands) for chromatographic resolution of D,L pairs (reproduced from [107] with permission of the copyright holders).

Remelli et al. [109]. After adsorbing the *n*-decyl-L-spinacine, Cu^{II} was introduced to load the column's recognition sites. Racemic mixtures of amino acids or oligopeptides were then separated by RP chromatography to achieve chiral ligand separations based upon differing affinities of the D or L forms with the Cu^{II}(spinacine) binding sites [109].

Marchelli and co-workers have recently prepared a Cu^{II}(diamino–diamido) complex (CuL) derived from the tetradentate amide-linked ligand formed from ethylenediamine and two L-phenylalanines. The four-coordinate Cu^{II} complex forms adducts with amino acids by means of the dissociation of one-half of the tetradentate diamido-diamine chelate, forming a ternary Cu^{II}(L)(AA) complex [110]. The pendant phenylalanine portion of the displaced chelate is available for hydrophobic contacts with the stationary phase and the bound amino acid. The ternary complex, Cu^{II}(L)(AA), exhibits differing lipophilic affinities for a C_{18} RP column depending upon whether the amino acid ligand has the D or L configuration, thus allowing separation of the D and L isomers of the amino acids [110].

Chen and Hobo have carried electrophoresis of Cu^{II} complexes of dansylated amino acids on silica gel prepared with L-prolinamide attachments as a chiral selector phase to separate D from L amino acids; the complexes of the D form move ahead of the L complexes except for serine [111]. Similarly, Schlauch et al. have used penicillamine-supported Cu^{II} complexes for the HPLC separation of amino acids [112]. These researchers concluded that coordination of amino acids is necessary for separations, but it is the hydrophobic contacts between the substrates and the support surface that is crucial for chiral discrimination in binding between different amino acids and enantiomers [112a].

Schurig and co-workers have attached Ni^{II}(bis(3-heptafluorobutanoyl)-10-methylene-(1*R*))-camphorate, termed "Chirasil-Nickel", to capillary surfaces [113], and on polydimethylsilane [114], to carry out GC separations of ligand enantiomers at 170–180 °C. The derivatized column is also useful with supercritical CO_2 fluid as the mobile phase. The structure of the Chirasil-Nickel monomer complex as determined from an X-ray diffraction study of its pyridine adduct is shown in Fig. 6. Upon incorporation with a polymer support, the repetitive chromatographic phase, suited to enantiomer separations, is produced (Fig. 6). This is depicted in the drawing beside the Chirasil-Nickel monomer complex. The 3-heptafluorolbutanoyl substituent has also been replaced by the 3-perfloroacyl group in producing M^{II}(bis-[3-perfluoroacyl]-(1*R*)-camphorate) analogues of Chirasil-Nickel as chiral chromatographic binding sites. Schurig et al. have reviewed in detail the application of the chiral M^{II}(bis-*R*-(1*R*)-camphorates) for gas phase enantiomer separations [12,13,37] and for capillary electrochromatographic separations [24].

R,*R*-, *S*,*S*-, and meso-isomers of bpp = 2, 6-bis(pyrrolidin-2-yl)pyridine have been separated as their Cu^{II} complexes on SP-Sephadex with a C-25 column; the $[Cu^{II}L(H_2O)]$ complexes were eluted with buffers containing CO_3^{2-} and OH^- which eluted the species with differing mobilities [115]. The resolved ligands were obtained by acid dissociation

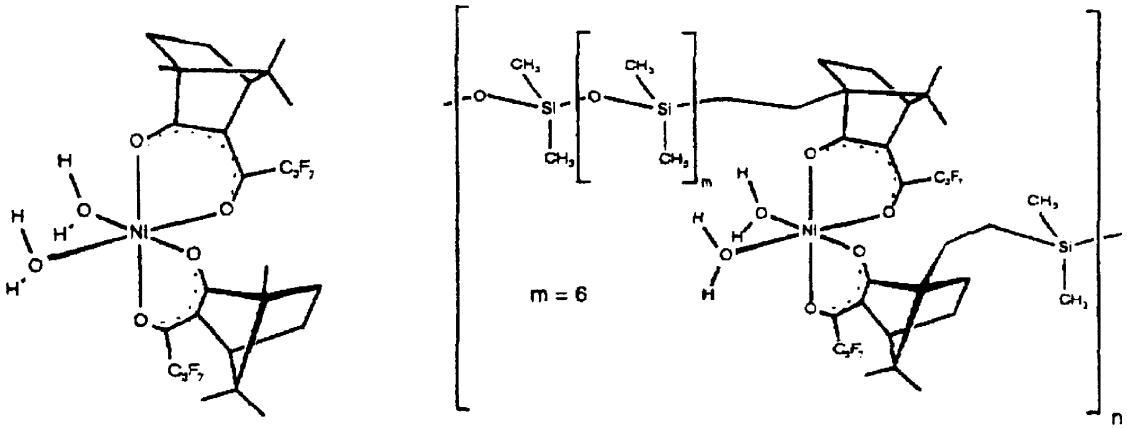


Fig. 6. X-ray structure of Chirasil-Nickel and polymer-supported Chirasil-Nickel according to Schurig (reproduced from [37] with permission of the copyright holders).

of the isolated $[CuL(OH)]^+$ or $[CuL(CO_3)]$ complexes [115].

4.4. Organometallic chiral complexes

cis and *trans* $Cu(CO)_2$[Fe(CO)(indole)] complexes as pseudo-square planar Cu complexes were separated [116]. Other pseudo-planar chiral complexes of $Cp^*Rh(COD)$ was obtained with a reported 100% ee, and $Cp^*Fe(CO)_2R$ complexes in 50% ee by adding cyclodextrin to the mobile phase to alter the isomer migration rates on polyamide columns [117].

Disubstituted and tetrasubstituted cyclopentadienides form Co^I(1,5-cyclo-octadiene) complexes, e.g. [(CpR_n)-Co(1,5-cod)] which are readily separated in enantiomerically pure form on silica support under Ar by common chromatography procedures [118].

Metallopthalocyanines are prepared by condensation reactions with a templated metal center. A mixture of isomers is, therefore, obtained for any condensing component that bears a peripheral R group. Garlach et al. have separated for isomers of In^{III}, Ni^{II}, and Zn^{II} using C-30 HPLC columns [119]. The Ru^{II} phthalocyanines have uses in photodynamic therapy [119b].

Two diasteromers of tribenzylidenemethane in $Ru(C_6H_6)$-(TBM) were separated on alumina with hexane [120]. In one diasteromer, the three benzyl groups are symmetrically placed as a propeller about the Ru–C axis (as shown), whereas in the other diasteromer the rotational direction of the set is disrupted. X-ray diffraction ORTEP diagrams of the two isomers are presented in Fig. 7.

Paisner and Bergman found that Chiracel OD HPLC columns, which have 3,5-dimethylphenyl carbamate derivatized cellulose (Fig. 8), could be used for separation of sterically hindered and chelating cyclopentadienyl dihaloiridium(III) (Fig. 9) and tricarbonyl(R)molybdenum(II) complexes ($R = Cl^-$, CH_3^-, $C(O)CH_3^-$), but application of the same chelating cyclopentadienide dichloroTi(IV) and

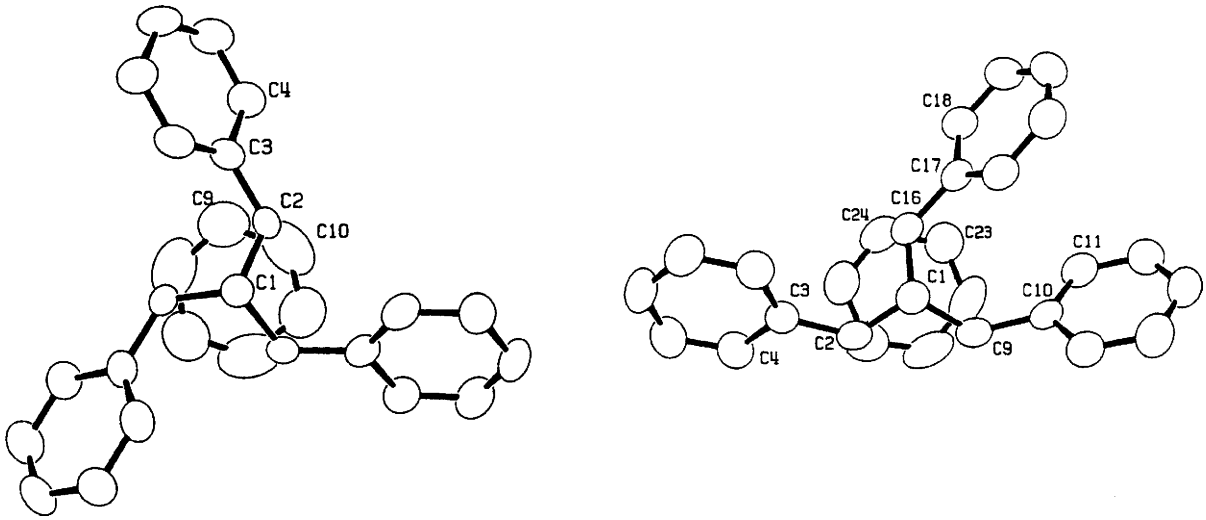
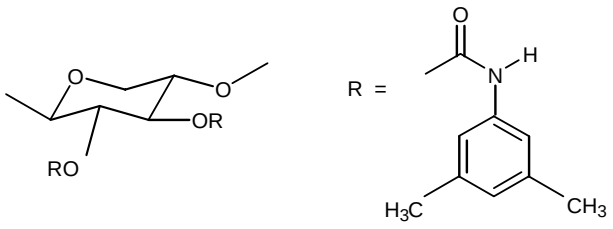


Fig. 7. ORTEP diagrams of the two possible isomers of [(η^6-benzene)(tribenzylidenemethane)ruthenium] (reproduced from [120] with permission of the copyright holders).



Chiracel OD

Fig. 8. The structure of Chiracel OD: 3,5-dimethylcarbamate-derivatized cellulose chromatographic support phase.

dichloro Zr(IV) analogues failed, resulting in decomposition [121].

Chiracel OD columns were also used to separate $Cr(CO)_3$(R-naphalenes) (R = Cl, CH_3, $Si(CH_3)_3$, $Sn(CH_3)_3$) wherein the attachment of the aromatic naphthalene to $Cr(CO)_3$ may be by either the nonsubstituted or by the R-substituted ring [122] into pure isomers. Studies then showed that racemization occurs in noncomplexing solvents (hexane and C_6F_6) by an intermolecular path with 1% conversion at 85 °C in 40 h, in coordinating aromatic solvents ($CH_3C_6H_5$) by an intramolecular walking migration (20% conversion; same conditions), and in solvating solvents (thf or dibutyl ether) with >90% conversion at 85 °C, 40 h. The $Cr(CO)_3$(R-naphathalenes) are retained more strongly when $Cr(CO)_3$ is on the R-substituted ring with electron releasing Rs and more weakly retained by the column when two electron withdrawing groups (R = $Cr(CO)_3$ and Cl) share the ring.

4.5. β-Cyclodextrin–ligand–metal ion chiral recognition particles

β-Cyclodextrins have been used as additives to mobile phases to alter the mobilities of chiral species in solution. An alternative approach has arisen recently in which the β-cyclodextrin component is adsorbed on silica particles. The "mouth" of the β-cyclodextrin is derivatized by a ligating moiety that binds a metal ion to provide a charged, polar entity. The ligand region can be further embellished with a side arm functionality which imparts steric and polarity effects. Molecules of differing chirality which move past the {(silica particle)-β-cyclodextrin–ligand–metal ion-side-chain} assembly exhibit differing degrees of association to the surface of this assembly. Recent examples include β-cyclodextrins attached to crown ether—Na^+ or K^+ chromophores [123], cyclam-R ligands for Ni^{II} [124], and imidazole-attached Cu^{II} side-arms [125]. These have been used as stationary phases in the chiral recognition/enantioselective separation of ionic and polar chiral species [123,124]. In the β-cyclodextrin–imidazole system, the ternary complex of the Cu^{II}(L-tryptophan) unit has provided separation of D,L-racemates [125].

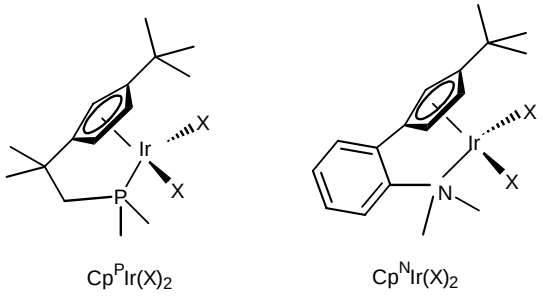


Fig. 9. Chiral cyclopentadienyl–phosphine and cyclopentadienyl–amine Ir^{III} dihalo complexes as described by Paisner and Bergman in [121].

This methodology in which the (silica particle)-β-cyclodextrin–ligand–metal ion components yield a species adapted to chiral recognition of substrates in the mobile phase bears a significant similarity to the immobilized metal ion affinity chromatography (IMAC method) described later in this report. In the IMAC method there is a support–ligand–metal ion assembly that provides a chromatographic binding site for peptides and proteins. The attraction in IMAC rests upon bond formation to the chromatographic metal binding site. The β-cyclodextrin–ligand–metal ion systems act by structural and shape recognition, although in the Ni^{II} and Cu^{II}-supported systems there can be the additional discrimination afford by bonding via heteroatom donors on the chiral substrate.

5. Capillary electrophoresis and capillary zone electrophoresis of metal complexes

The first separation of inorganic ions by capillary electrophoresis (CE) involved the separation of Bi^{3+} and Cu^{2+} in the presence of chelating lactic acid [126]. In CE, the ions in solution move toward an electric field plate of opposite charge. The technique termed CE refers to all electroseparations performed in capillaries (see below when a sub-technique, CZE, is described). The result is similar to gel electrophoresis which uses macromolecular ion movement in a field of ~200 V to separate biomolecules. The electrophoresis technique is not used on large solution volumes because the ion current at high voltage produces too much heat for sample stability. However, with very small samples in capillary tubing where the ratio of solution volume in contact with a wall cooling surface to the solution volume in the interior is high, heat dissipation is rapid enough to allow the study of ion migration under controlled temperature conditions.

The advantages of CE include the ability to analyze small samples, with speed, by the movement of ions within a small bore capillary under high voltage between the cathode and anode of the cell. The voltage can be raised to 25 kV to separate the sample ions efficiently in time.

Ions of greater net charge move farther than ions of lesser charge; ions of equal charge, but with differing mobilities due to radial size and molecular weight are sorted by

their respective mobilities. A differentiated zone for ions of like charge (hence, capillary zone electrophoresis) appears. CE/CZE is rapidly becoming the major technique for the determination of simple inorganic ions. As such, it has been heavily reviewed in recent times by Timerbaev and co-workers [17–21,127], by Pacakova [22], and by Cheng and co-workers [128,129].

An expert reviewer in the field has noted that CZE is a type of CE method in which the analyte moves through a liquid electrolyte solution. Since most people equate CZE with CE, he has advised the author that it is preferable to use one term (methodology), CZE, to describe all of these methods inclusively as the correct terminology. The author gladly defers to experts in the field. However, readers may encounter papers in which their authors continue to distinguish these techniques.

For conservation of journal space, these excellent presentations and discussion of the method's limitations [127] will not be reproduced here. However, the reader may consult the extensive tables of ion applications in the separation of all common Group I and NH_4^+, Group II, Group III, ca. 40 different transition metal ions (M^{2+}, M^{3+}, M^{4+}, and M^{5+}), and lanthanide ions. In those tables, the buffer, electrolyte, and complexing carrier ligands that are required for selective separations are specified [22]. Cheng and co-workers' extensive review has tables that emphasize the use of organic complexing ligands to modify the migration of about 50 transition metal, lanthanide, and actinide ions [128]. In Cheng's report, many uses of the azo dyes, quinoline dyes, porphyrins, dithiocarbamate, and edta-family of ligands ($edta^{4-}$, $cydta^{4-}$ < nta^{3-}, $dtpa^{5-}$, $ttha^{6-}$), as well as CN^- and Cl^-, for the separation of both hard and soft transition metal ions are discussed.

Haddad and co-workers mention the several organic dye molecules (notably PAR, BrPADAP) as metallochromic ligands (Fig. 10) that are used widely in CZE methods [130] for many separations. The same dye ligands are also used in the related micellar electrokinetic capillary (MEKC) separations in which the complexed metal ions are allowed to partition between micellar stationary phases while undergoing migration in the CE cell in the "mobile" phase. Haddad has shown that it is necessary to use co-ligands such as $edta^{4-}$ to tie up "background" ions in the determinations of ions of interest by CE. The ions of interest are bound by the metallochromic ligands, and the background ions by $edta^{4-}$, because the entire population of cations in a sample affect the electrostatic balance of the capillary wall at siloxy groups. The charges on the capillary wall in turn influence the migration rates of the metallochromic metal complexes, and the observed ion currents. The advantage for the metallochromic ligands is that the desired ion may then be detected as they migrate past a selected point in the CE cell

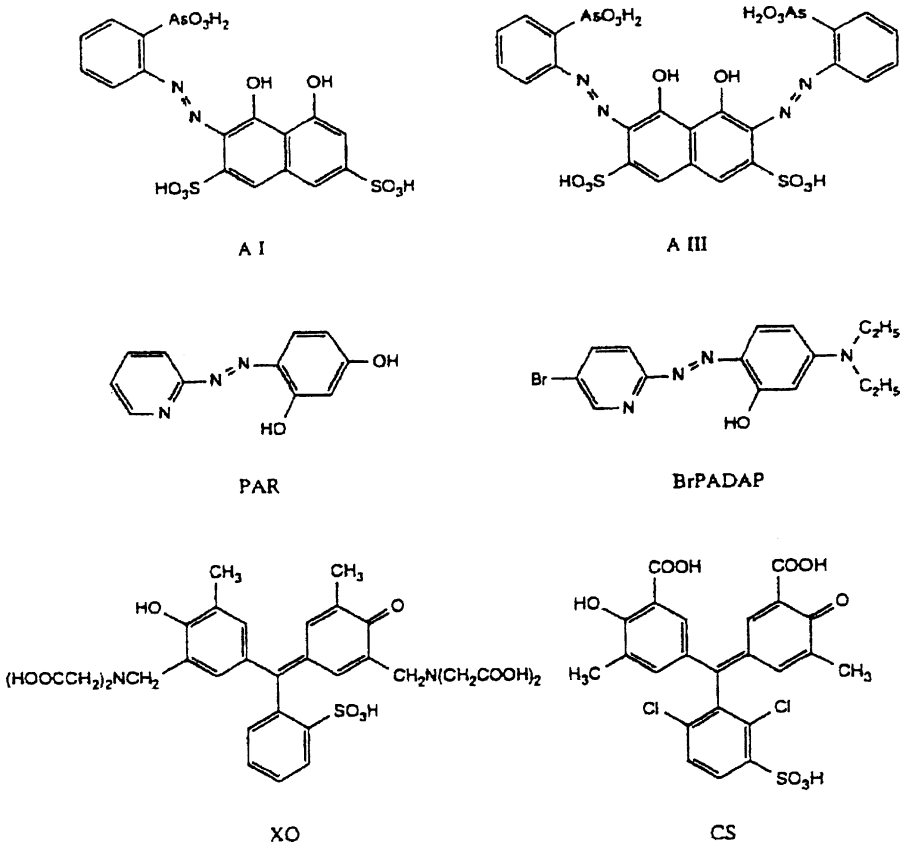


Fig. 10. Structures of six metallochromic ligands discussed by Haddad and co-workers in [130].

by means of UV-Vis spectroscopy, or at low detection limits by laser fluorescence.

Timerbaev and Buchberger have noted that the most interesting applications of CE separations are in applications of "hyphenated techniques" such as CE-ICP-MS for the identification of the formulations of chemical species as well as the quantity of the central metal center, particularly for metallated biomolecules and macromolecules [18]. In such schemes, the CE aspects separate the desired species from a multi-species matrix. The separation step by CE is followed by the sampling of the separated ions for analysis as to element kind (ICP), and as to the nature and number of ligands in the mobile unit (by MS techniques). Timerbaev and Buchberger give a succinct table for the applications of metallochromic ligands in separations of Cr^{III}, Ni^{II}, $Co^{II/III}$, Cu^{II}, $Fe^{II/III}$, Al^{III}, Pb^{II}, V^{IV}, Zn^{II}, Ba^{II}, Sr^{II}, U^{VI}, and La^{3+}(lanthanides) by various workers in the discipline [18].

A few representative applications of CE or CZE separations to metal complexation chemistry include the following recent uses. Metal leaching from automobile catalytic converters has been studied by making cyano $M^a(CN)_n{}^{m-}$ complexes ($m = n - a$) for Fe^{II}, Cu^{I}, Ni^{II}, Pd^{II} and Pt^{II} [131]. Pd^{II} and Pt^{II} cyano complexes were differentiated by CE in 30 mM phosphate buffer with 15% added CH_3CN [132].

Chiral separations have been achieved by the CE method using [Cu^{II}(L-amino acid amides)] in the mobile phase to retard the migration of D- and L-amino acids [133]. The L-enantiomers generally have longer retention times as these associate more favorably with the [Cu^{II}(L-amino acid amide)] chiral selective additives. However, with dansylated amino acids, the Dns-D,L-serine racemic mixture showed that the L-isomer migrated more rapidly [133]. This is the same result as Marchelli and co-workers' [107] in which the D-enantiomer of dansylated amino acids are selectively bound by chiral Cu^{II} amino acids for separation by reversed phase chromatography as discussed earlier in this report.

Separations of enantiomers by the capillary electrochromatography procedures are also possible using chiral stationary phases formed by coating the capillary walls with a supported chiral metal complex such as Chirasil-Nickel [24].

CE has been applied in the separation of Λ and Δ enantiomers of [$Ru^{II}(phen)_2$(5-(4-hydroxybutyl)-5′-methyl-2,2′-bipyridine)]$^{2+}$ by the addition of carboxymethyl-β-cyclodextrin as a chiral discrimination reagent that associates with the bipyridine substituents; as anticipated the same procedure fails to separate enantiomers of $[Ru(bpy)_3]^{2+}$ or $[Ru(phen)_3]^{2+}$ [134].

The MEKC method with micelles present as a secondary equilibrating region to enhance the separation of ions of like charge has been applied by Haddad and co-workers to the separation of Co^{II}, Pt^{II}, Pb^{II}, Cd^{II}, Ni^{II}, Bi^{III}, Cr^{II}, Cu^{II} and Hg^{II} as bis(2-hydroxyethyl)dithiocarbamate complexes, achieving separation and detection in the 22–133 parts-per-billion (ppb) range [133].

Timerbaev and Semenova showed that lanthanide (Ln^{3+}) complexes of edta-family ligands migrate in CE conditions based on the ligand charge-to-size ratio [135]. Using cyclohexylethylenediaminetetraacetate ($cydta^{4-}$) as the chelating ligand, these workers separated Al^{III}, As^{III}, Bi^{III}, Cr^{III}, Hg^{II}, Pd^{II}, Ag^{I}, Tl^{I}, Ti^{IV}, U^{VI}, V^{IV}, V^{V}, and Zr^{IV} by CE [136], and concluded that $cydta^{4-}$ was the best ligand for CE separations when the detection was <1 μg/ml [137]. In an interesting but predictable study, the addition of 8-hydroxyquinoline-5-sulfonate (HQS) reversed the movement of M^{n+} toward the positive electrode in forming $M(QS)_2{}^{m-}$ complexes, whereas their aqua M^{n+} ions migrate towards the negative pole [18,138,139]. Most methods now use preformed complexes, prepared in pre-column operations that are followed by CE separation [139].

Although the CE method was conceived for ion separations, the separation of ligands bound to those ions has also been exploited. Harvey used Cu^{II} added to mixtures of $edta^{4-}$, $hedta^{3-}$, nta^{3-}, $edda^{2-}$, $dtpa^{5-}$ and $ed3a^{3-}$ to show that ligand separation may be achieved by CE and MEKC (see below for a discussion of MEKC). The edta-family ligands were separated, riding the Cu^{II} cation with differing mobilities based on the CuL^{n-} charge-to-size ratio [140]. Milk proteins were separated by CE after addition of Cu^{II} citrate to cow's milk, or by adding M^{II} (Cu^{II}, Ni^{II}, and Zn^{II}) to the milk [141]. The complexation of the metal ions by the proteins via histidyl and cysteine side chains allowed the proteins to change charge, and to be moved in the CE field.

Rodriguez and co-workers have separated numerous metal ion binding proteins including albumin, carbonic anhydrase, conalbumin, cytochrome *c*, hemoglobin, myoglobin, superoxide dismutase, and transferrin by CZE using capillaries coated with polyvinyl alcohol as the stationary phase [142].

Environmental samples containing Co^{III}, Bi^{III}, Fe^{III}, Cr^{III}, V^{IV}, Pb^{II}, Hg^{II}, Co^{II}, Cu^{II} and Ni^{II} were separated by forming $edta^{4-}$ or $cydta^{4-}$ complexes and placing the solution, adjusted to pH 8.5, into a CE small bore capillary with a plate voltage of 25 kV [143]. This "real sample" separation basically duplicates the "in-lab" separations of Timerbaev and co-workers.

Pt^{II} analysis has become important for the biomedical field. Pt^{II}, Pd^{II}, and Pt^{IV} may be determined by CE separation as their NCS^- complexes; the detection of the migrated species at 305 nm for the NCS^- complexes is easier than for Cl^- at 214 nm [144]. The CE method is being applied to the analysis of Pt drugs and their metabolites for chemotherapy. A recent review by Timerbaev et al. has been presented [25].

The exact formulation of metal ion-chloro-aqua species and the amounts present under solution phase equilibria are often questions that ask for experimental evidence. For Ir^{IV} as the $IrCl_6{}^{2-}$ salts, it was shown that a solution of K_2IrCl_6 contained Ir^{IV} as $IrCl_6{}^{2-}$, $IrCl_5(H_2O)^-$, and $IrCl_5(OH)^{2-}$, but that reduction in solution via oxidation of Cl^- or water produces Ir^{III} as $IrCl_5(H_2O)^{2-}$ and $IrCl_5(OH)^{3-}$, as well as dimers [145]. Thus, assignment of charge followed by

electrochemical study or spectroscopy can give the required in formation on non-labile species by CE [138,145].

5.1. Micellar electrokinetic capillary chromatography (MEKC)

A significant sub-area of separations using electro-migration procedures of CZE is the modification in which surfactant micelles have been added to the mobile phase in concentrations above the critical micelle concentration. The complexes of interest which migrate in the electric field are also able to establish "on–off" equilibria with the micelle phase, much in the manner of the stationary phase-to-mobile phase equilibria of liquid chromatography. This adds a dimension to the separation procedure that further differentiates species that may have comparable ion mobilities in the absence of the presence of micelle binding sites. If a metal ion is attached to a chiral ligand, the separations also become enantioselective [26].

The separation of cisplatin and its hydrolysis products was carried out using SDS micelles under MEKC conditions [146]. The anionic surface of SDS micelles affords a competitive binding site for $[Pt^{II}(NH_3)_2Cl_2]$ and its hydrolysis products, $[Pt^{II}(NH_3)_2Cl(H_2O)]^+$ and $[Pt^{II}(NH_3)_2(H_2O)_2]^{2+}$. Electrochemical detection of the eluting Pt^{II} species was observed to follow the order of neutral complex > mono cation > dication in the presence of SDS micelles, whereas the reverse order would occur on charge alone in the absence of SDS [146]. The explanation for these observations is that the positively charged 1+ and 2+ species "ride" the surface of the negatively charged micelles that move toward the positive anode in opposition to their own migratory attraction to the negative cathode and direction of electroosmotic flow [146]. Because electroosmotic flow (EOF) is greater than the electrophoretic flow on the miscelles, detection is made for all species at the cathode. An illustration of the separation scheme of Wenclawiak and Wollmann [146] is shown in Fig. 11.

Haddad et al.'s review [26] should be consulted for detailed information on the use of MEKC in the separations of a wide range of transition metal ions in various oxidation states as amplified by the use of metallochromic ligands such as PAR, and more standard chelates such as the edta-family and dithiocarbamates.

Lastly, in the area of recent developments concerning the application of CZE methods to transition metals, Timerbaev et al. have analyzed all the available CZE data for transition metal complexes in an attempt to obtain a quantitatively predictive structure–mobility correlation [147]. The main outcome is that CZE mobility is connected to the charge-to-size ratio of the migrating species. The composite influence of charge and size can be parameterized on the basis of metal electronegativity (a function of oxidation state), the donor atoms, the formal ligand charge and the nature of ligand functional groups, and the hydrodynamic radius of the complex. A linear relationship was obtained as the sum of logarithmic contributions plus a constant term in which the increasing charge and radius log components are opposed effects. The more important aspect for this review is that these researchers used migration data for a fairly representative and diverse set of metal chloro, bromo, cyano, 2,2′-bipyridine, *o*-phenanthroline, $edta^{4-}$, $cydta^{4-}$, metallochromic agents (PAR, DHABS, and HQS) amines (ethylenediamine and ammonia), and $C_2O_4^{2-}$ complexes.

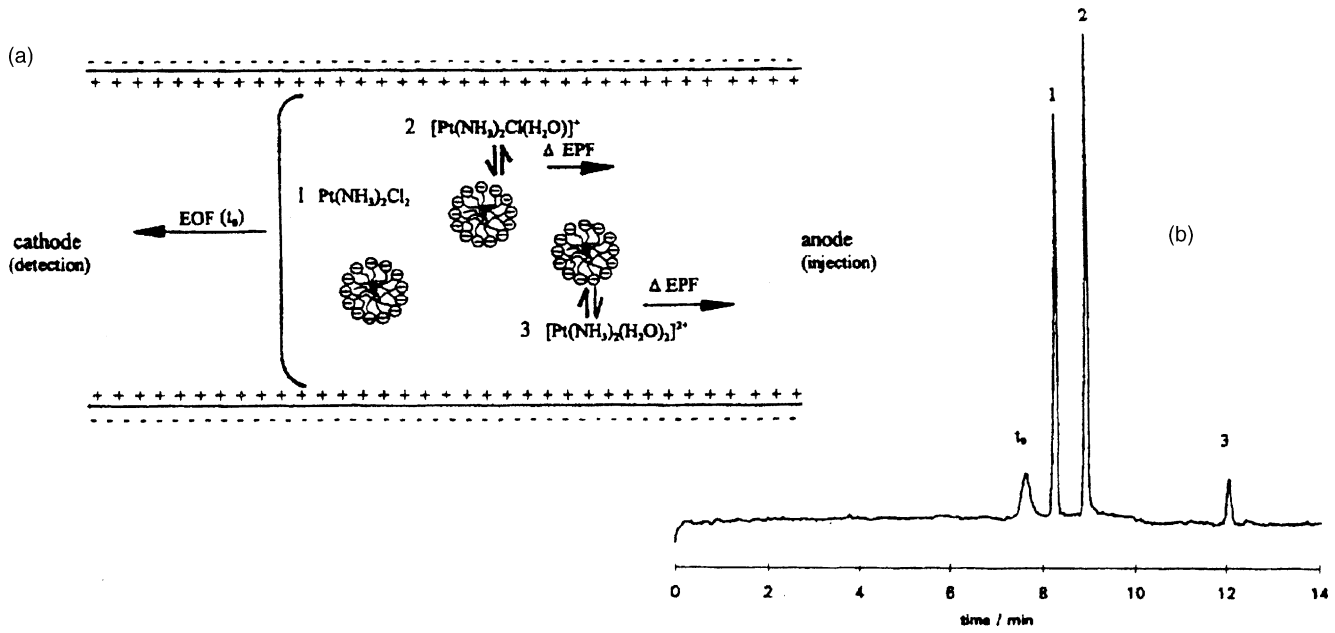


Fig. 11. MEKC separation of complexes in the hydrolysis of cisplatin: diagram of the MEKC procedure and the experimental electropherogram (reproduced from [146] with permission of the copyright holders).

Regression analyses between the log of ion mobilities and the parameterized charge and radius functions gave a reasonable fit for almost all families of complexes, with the edta^{4-} giving poorer than desired linear behavior as M^{n+} was varied. But good linearity is found for the $[M(cydta)]^{(n-m)-}$ analogues, suggesting that local solvation and ion-pairing provide differences that are not easily incorporated in a simple spherical point charge model. This is certainly not unexpected. In most cases, failures of complexes to fit linear relationships between the observed mobility and the charge and size parameters were those of complexes of the following types: (1) those known to add additional buffer ions in solution, altering the charge; (2) those which make ion pairs; or (3) those which deviate significantly from approximately spherical ions. An example of a non-spherical complex is one obtained via chelation with Alizarin complexone. Those complexes have a charged metal–iminodiacetate binding site and a large, appended hydrophobic attachment that renders these complexes asymmetric.

6. Electrochemical detection of substrates coupled to $[Ru(bpy)_3]^{3+/2+}$ redox cycles

The advantage and high sensitivity of electrochemical detection of species, along with the ease of automation of analytical equipment for electrochemical detection, is a strong motivating force in analytical chemistry developments. The problem with many biochemical samples, particularly in the analysis of peptides and many organic substrates is that their redox chemistry is by a slow two-electron step that prevents easy reversible Nernstian electrochemical changes at an electrode detector surface.

$$R_{ox} + 2e^- + 2H^+ \Leftrightarrow R_{red}H_2, \quad E^0_{organic}$$

One approach in making the analysis of sought after, two-electron oxidizable organic species is to trade their presence for two moles of a one-electron reversible reagent that is amenable to rapid electrochemical detection. The oxidized form of the electroactive agent is generated at high potential. It carries out the oxidation of the desired analyte within a flowing stream of chromatographically separated substrates. The reagent of choice has been the substitution inert $[Ru^{III}(bpy)_3]^{3+}$ complex ($E^0 = 1.33$ V) that can oxidize many organic groups such as tyrosine, tryptophan, hydroxylamines, nitrosamines, oxalate, ROH of sugars, and so on.

The amount of $[Ru^{II}(bpy)_3]^{2+}$ is then determined downstream for each slowly oxidized analyte species (Fig. 12). The amount of the analyte is one-half the amount of $[Ru^{II}(bpy)_3]^{2+}$ that is detected for that chromatographically separated species. In some cases where the oxidation by $[Ru^{III}(bpy)_3]^{3+}$ is rapid, the Ru^{II} product is formed in an excited state, $[Ru^{II}(bpy)_3]^{2+*}$, that can be detected by luminescence. The technique is then one of electrochemically-generated chemiluminescence (ECL), with the emission intensity proportional to the analyte concentration. The ECL proportionality is established with standard solutions of analyte.

Three reviews have been given recently on the ECL detection of $[Ru^{II}(bpy)_3]^{2+*}$ that is produced by oxidation of analyte substrates [27,148,149]; the latter is for HPLC separations of analytes [149].

Weber and co-workers have used laser-induced fluorescence detection of $[Ru^{II}(bpy)_3]^{2+}$ formed in the oxidation of analyte peptides containing tyrosine or tryptophan amino acid sites in a short sequence of AAs [150] and chemiluminescence detection of $[Ru^{II}(bpy)_3]^{2+*}$ obtained from hydroxylamine or nitrosamine substrates [151]. Wang and Bobbit have used the ECL detection of amines and amino acids separated by the MEKC method [152].

ECL detection of $[Ru^{II}(bpy)_3]^{2+*}$ has been carried out to analyze for the following substrates: glutathione [153], ascorbic acid [154], $C_2O_4^{2-}$ at a sol–gel matrix electrode carrying imbedded $[Ru^{II}(bpy)_3]^{2+}$ that can be electrochemically oxidized to the Ru^{III} form that reacts with solution phase $C_2O_4^{2-}$ that emits proportional to the analyte concentration [155], thiamine (Vitamin B-1) [156], amines and amino acids separated first by CE [157], amines and amino acids (in nanoliter volumes) separated by CE [158], sugars and carboxylate acids including sucrose, fructose, mannose, glucose, glycerol, ascorbic acid, citric acid, tartaric acid, monofunctional alcohols such as ethanol, and polyols [159], β-blockers (oxprenol) [160], $C_2O_4^{2-}$ and proline by flow injection of $[Ru^{III}(bpy)_3]^{3+}$ [161], ascorbic acid in soft drinks [162], $C_2O_4^{2-}$ in urine for prediction of gout [163].

Replacement of the 2,2′-bipyridine ligand of $[Ru(bpy)_3]^{2+}$ with 4,7-diphenyl-1,10-phenanthrolinedisulfonate makes a $[RuL_3]^{4-}$ complex that coats an electrode surface as an insoluble salt. This adsorbed complex has provided an

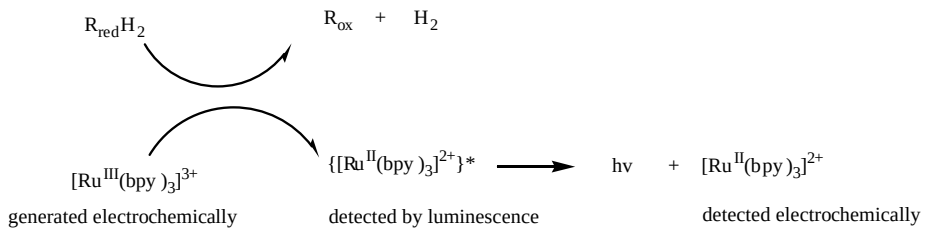


Fig. 12. Schematic of luminescence or electrochemical detection of substrates oxidizable by $[Ru(bpy)_3]^{3+}$.

alternate ECL detection scheme for $C_2O_4^{2-}$ at low pH and proline at high pH [164].

Alternative biosensors that respond to ROH mono-alcohols have been prepared using an "electrode sandwich" of a Pt electrode to allow electrogeneration of $[Ru^{III}(bpy)_3]^{3+}$ in an adjacent layer. A "Ru^{III} layer" in Nafion polymer is covered by a third layer of support for a dehydrogenase enzyme that reacts with the available analyte ROH, generating NADPH that is reoxidized by the $[Ru^{III}(bpy)_3]^{3+}$ [165]. ECL detection quantifies the amount of alcohol that is available, based on standard solutions that allow for the proportionality of emission versus radiationless decay to be calibrated per mole of substrate. Nafion polymer/$[Ru^{II}(bpy)_3]^{2+}$ films on Pt electrode backings for oxidation to Ru^{III} have also been used by Downey and Nieman as an ECL method to analyze for $C_2O_4^{2-}$, RNH_2, or NADH samples [166].

The photo-luminescence detection method has been transferred to the determination of $M^{II} = Cu^{II}$ or Co^{II} [167]. A dithiocarbamate ligand is prepared from the reaction of emetine and CS_2. The resultant dithiocarbamate (L) is added to a sample containing the M^{II} analytes. Formation of the CuL^+ or CoL^+ occurs. Upon chromatography and addition of $[Ru(bpy)_3]^{2+}$, electrochemical oxidation to $[Ru(bpy)_3]^{3+}$ promotes the oxidation of the $M^{II}L$ dithiocarbamate complex. The amount of $[Ru(bpy)_3]^{2+*}$ is determined by luminescence measurements, and this concentration is related to the amount of the 1:1 ML complex (and hence to the $[M^{II}]$) in the sample.

7. Electrospray mass spectrometry of transition metal complexes: connections with chromatography

Characterization of transition metal complexes has most generally been accomplished on solid materials (X-ray methods) and in solution (electronic spectroscopy, NMR methods). Less frequently, the gas phase species are examined or utilized. GC separation of metal acacs has a long history going back to pioneering studies by Sievers. These are neutral species which because of their robust nature survive elevated temperature. But for many transition metal complexes the species are charged ions which do not readily transport into the gas phase. Studies of organometallic complexes that are neutral complexes have more often been subjected to MS characterization in order to get the molecular weight of the parent ion, and of ligand dissociations that create simple fragmentation patterns. But the injection of aqueous solutions into mass spectral cavities was virtually absent prior to 1990. The ability to detect charged ionic species that have been stripped of the solvent shell is a major step forward in the characterization of charged transition metal complexes.

The subject of gas phase characterization of complexes by ESI-MS has been reviewed in its application toward the identification of all types of inorganic species by Stewart [32]. The application of ESI-MS to metallated biomolecules followed by ICP for metal detection and identification is discussed by Szpunar [33]. Another review of ESI-MS applied to metallated biomacromolecules has been presented by Przybylski and Glocker [168].

The applications of inorganic electrospray mass spectrometry as applied to organometallic complexes has been reviewed by Colton et al. [169] and by Henderson et al. [170]. A succinct review of the applications of ESI-MS to characterization of transition metal complexes appears in a 2001 report by Slocik et al. [171]. The use of ESI-MS to provide speciation of radionuclides in the environment [172], and its application to determining the composition of polyhedral complexes that form by self-assembly in solution [173] have been recently discussed.

The applications of ESI-MS to characterizations of Co^{III} amines, Co^{III}, and other M^{II} polypyridyl complexes ($M^{II} = Cu^{II}$, Ni^{II}, Ru^{II}), $Ru^{II}(bpy)_2L_2$ complexes, Ru^{II} and Fe^{II} nitrosyls, ruthenium carbonyls and carbenes, and Pt^{II} and Pd^{II} halo complexes are summarized in Slocik et al.'s report [171]. In Stewart's review [32] references are cited to the application of ESI-MS to peroxovanadium clusters, Cu^{I} complexes, metal alcoxides, diasteromers of $Re(CO)_2(\eta_3\text{-}\phi_2P(CH_2)_2P\phi(CH_2)_2P\phi_2)Cl$, Cu^{I}, Ag^{I} and Au^{I} complexes of phosphathia ligands and dimethylthiophosphoryl hydrazones, Pt^{II}, Pd^{II} and Ni^{II} thiosalicylates, Pt^{II} and Pd^{II} thiourea/olefin and sufathiazole complexes, Re N_2S_2 macrocyclic chelates, thioether complexes of $Re(O)^{3+}$, nitrido and imido complexes, phosphile ligands, β-diketonates of alkaline earths, Pt^{II} and Pd^{II} amide chelates, metal polyether-pyridyls, $Ru^{II}(bpy)_2(diamines)^{2+}$, Ru^{II} carboxypyridine complexes, Ru^{II} bipyridine and quinoxaline complexes, Na^+ adducts of $M(bpy)_n$ complexes, lanthanide diethylpyridine-2,6-dicarboxylates, and dicarboxamides.

In ESI-MS experiments, neutral complexes will sometimes appear in the gas phase as protonated species at $m/z = (M + 1)$ or as adducts of Na^+ at $m/z = (M + 23)$. The H^+ or Na^+ are associated with lone-pairs from ligands that are not protonated or metal-associated in the solution phase. As the solvation shell is stripped under gas phase conditions of the mass spectral cavity, the exposed lone-pairs that interact with solvent in the solution phase are "uncovered" in the desolvated ion in the gas phase. These then form associations with ions able to polarize the lone-pairs, forming $(M + H)^+$, $(M + 2H)^{2+}$, $(M + Na)^+$ and so on.

Metal–halo complexes have a tendency of eliminating HX from the formula, forming ions of lower mass than the parent ion, as in the loss of HCl from $[Pt^{II}(dien)Cl]^+$ to make $[Pt^{II}(dien\text{-}H)]^+$ [174], or $[Ru^{II}(NO)(dpa^-)_2]^+$ from $[Ru^{II}(NO)Cl(dpa^-)(dpaH)]^+$ [171].

Formation of ion pairs during the desolvation process is also common. For example, when $[Ru^{II}(bpy)_2(NO^+)Cl]^{2+}$ is characterized by ESI-MS from solutions of its chloride salt, the main starting ion is indicated by a isotopic bundle of mass spectral lines centered about $m/z = 514$ for the 1+ ion, $\{[Ru^{II}(bpy)_2(NO^+)Cl]^{2+}, Cl^-\}^+$ rather than at $m/z = 239.5$ for $\{[Ru^{II}(NO^+)Cl(bpy)_2]\}^{2+}$ [175]. Significantly to the above-mentioned loss of HX from

metal halo species, the $m/z = 514$ ion-pair loses HCl by deprotonation of an *ortho* hydrogen of bpy to make $\{[Ru^{II}(NO^+)Cl(bpy)(bpy\text{-}H)]\}^+$ of $m/z = 478$. The author points out that the present discussion is a simplified one because the eight isotopes of Ru and the two isotopes of Cl created bundles of m/z lines centered on the values of 514, 239.5 or 478 given for simplicity of discussion. Such "isotope bundles" are a big advantage to the experimentalist in determining the speciation and numbers of metal centers that are present within a correct formulation of the ions by ESI-MS. There are useful programs to calculate the theoretical distribution of mass spectral peaks having elemental contributors with substantial amounts of more than one isotope. A program of M. Winter of the University of Sheffield, available at http://www.shef.ac.uk/chemistry/computer/isotopes.html is highly recommended.

Recent applications that amplify points made in the previous paragraphs include a study by Colton and co-workers on the ESI-MS of square-planar M^{II} Schiff base complexes such as the ethylenediamine Schiff base from acetylacetone ($M^{II} = Ni^{II}$, Cu^{II}, Pd^{II} or VO^{2+}) [176]. The resultant complexes should be neutral ML species. However, from solutions having sources of Na^+, Ca^{2+} or Mg^{2+} acetate salts, species of the formulations $(LMNa)^+$, $\{(LM)_2Na\}^+$, $\{(LM)_3Na\}^+$, $(LMH)^+$, and $\{(CH_3CO_2)M^{II}(LM)\}^+$ are readily detected.

In the analysis of neutral sugar molecules including anomers of glucose, mannose, galactose, and talose that differ in the relative orientation of the OH groups at C-2 and C-4, the ESI-MS of the sugars could be observed if $Fe^{II}Cl_2$ is added to the solution phase prior to ESI-MS injection. The ions of composition $[M + FeCl]^+$ are detected. Since the population of the $Fe^{II}Cl^+$ adducts vary with the amount of chelation provided by the sugar OH groups, and this is a function of the C-2 and C-4 orientations, the identities of the sugars can be made. This identification is based on ion intensity and fragmentation patterns that are matched with authentic samples of the anomers of the sugars [177].

ESI-MS spectra can be obtained for negative ions. Thus, the amount of M^{II} species such as $NiCl_2$ in HCl can be detected from the ESI-MS spectrum as $NiCl_3^-$ [178]. Simple Fe^{3+} and Cu^{2+} samples can be analyzed by adding NaX salts or HX to the solution phase. Upon ESI-MS injection, the forced ion pairing created $Fe^{III}X_4^-$, $Fe^{II}X_3^-$, $Cu^{II}X_3^-$ and $Cu^{I}X_2^-$ species that may be quantitated [178]. Ions such as ReO_4^- and $HMoO_4^-$ can be observed directly by ESI-MS [178].

Gianelli has reported that seven Cu^{II} tetradentate complexes are reduced by the solvent during desolvation under ESI-MS conditions in a manner that correlates with the ionization energy of CH_3CN, CH_3OH, C_2H_5OH, C_3H_8OH, and C_4H_9OH [179]. This illustrates the general situation that experimentalists must be aware of reduction pathways which can alter the m/z value for a complex in the gas phase, yet that do not occur in solution with the same solvent. The gas phase reduction may alter fragmentation pathways by changing the central metal's oxidation state and ligand labilities.

Callahan and co-workers have attempted to obtain structural insights and coordination number data for tren and tren$(CH_2py)_3$ complexes of Ni^{II} and Mn^{II} in the gas phase by examining what ligand fragments are displaced during ion–molecule bombardment under electrospray conditions [180]. These workers also observed ion pairs of the general formula ML_nX^+ for M = Mn^{II}, Fe^{II}, Co^{II}, Ni^{II}, Cu^{II}, and Zn^{II}; $X^- = Cl^-$, NO_3^-, $acac^-$, ClO_4^-, NCS^-, and $CH_3CO_2^-$; L = *o*-phen, bpy, en, dien, and cyclam [181]. Speciation of piperazine-bridged pyridyl chelates, L = pip(CH_2pyR), of Co^{II}, Cu^{II}, Zn^{II}, and Cd^{II} in forming $[MLX]^+$, $[M(L–H)]^+$, $[MM'(L)X_3]^+$ species wherein the piperazine portion of the chelate adopts a boat configuration for the 1:1 complexes, and the chair arrangement for the 2:1 complexes has been described [182]. Binuclear complexes of M^{II} species with formulas of $[M_2L(X)_2]^{2+}$ and $[M_2L(X)_3]^+$, $X^- = Cl^-$ and I^- with L = 3,6,9,16,19,22-hexaazatricyclo-[22.2.2.2(11,14)]-triaconta-11,13,24,26,27,29-hexaaene were similarly identified by ESI-MS [183].

Henderson and co-workers have applied ESI-MS to characterization of Pt–V clusters such as $[Pt_2(P\phi_3)_4(\mu_3\text{-}S)_2VO(OCH_3)_2]^+$ that are obtained in the reaction of NH_4VO_3 with $Pt_2(P\phi_3)_4(\mu\text{-}S_2)$ [184].

Lanthanide ions (Sm^{III}, Yb^{III}, Dy^{III}, and Lu^{III}) have been separated by capillary electrophoresis as their 1,4,7,10-tetra-azacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetate ($dota^{4-}$) complexes due to differing mobilities; the $[Ln(dota)]^-$ complexes were identified by ESI-MS [185].

ESI-MS is a rapidly expanding field that is finding many new applications in inorganic synthesis characterization and in bio-inorganic studies. The technique appears to be in the earliest phase of its development as a standard characterization tool.

8. Size exclusion methods for metal complexes

Many separation methods are of value in biomedical research, and developments of the method, are found in that field before its application is made to inorganic chemistry. Gel filtration (gel permeation chromatography) and size exclusion chromatography are methods that separate mostly by size. Often as in gel electrophoresis, ionic components in the sample are forced to migrate within channels of a polymer material in an electric field. These are methods that have "hopped" into the inorganic repertoire from molecular biology. A gel polymer such as a polyacrylamide gel has many channels in which molecules can move, and more interior regions near the polymer that can attract and hold back the migration of molecules. The larger molecules that cannot invade the polymer region are thus "excluded" and tend to migrate more rapidly than those that perform excursions of motion into the polymer interior zone. Thus, the

name of SEC is used to describe separations under these conditions.

Many lower molecular weight, metal complexes are too small to be "excluded", and migration rates of smaller species can be very slow—invalidating the use of SEC as a particularly useful technique. However, if the metal centers are attached to a large molecule, as on labeled biopolymers, including proteins, then separation by SEC is a favorable experimental approach. Therefore, many applications for separation of complexes involve protein-bound metal complexes or metal ions bound to ligand carriers.

Haraguchi and co-workers have provided two reviews that describe the application of SEC to the metallated biomolecules [15,16] that are taken from biological and geological sources, particularly blood samples and groundwater testing. The SEC separation is followed by analysis of the metal species by AA, by ICP-MS, or by ICP-AES. Yang and Ni have reviewed the separation by SEC adapted to the HPLC format [186]. They state that the combination of HPLC (SEC) followed by ESI-MS is the best pairing of techniques that provide speciation and metal identification.

A number of recent reports concerning inorganic applications of SEC involve labeled peptides that contain an introduced metal center. One cyclic peptide which has four cysteine amino acids, L = [glu-cys-gly-val-cys-gly-lys-cys-ile-ala-cys-lys-] binds metal complexes as $[M^{II}(SR)_4]$-type complexes. Several M^{II} = Cu^{II}, Zn^{II} were found as dimer M_2L_2 species, whereas $Co^{II}L$ and $Fe^{II}L$ were pseudo-tetrahedral, and $Ni^{II}L$ is distorted square-planar [187]. These complexes were separated by SEC which showed the migration rate of the ML species was slower than the M_2L_2 dimers. Metallothionein dimers have been examined by nanospray mass spectrometry after separation by SEC as $Cd^{II}{}_7L$ and $Zn^{II}{}_7L$ species [188].

Phytochelatins are thiol-containing plant peptides which are made in response to exposure to toxic metals, and act much like metallothioneins. These peptides possess structures of formula gamma-$(Glu\text{-}Cys)_n$-Gly. Complexes of Pb^{II} and Cd^{II} of the stoichiometry ML and ML_2 were separated using SEC [189].

Plant proteins with histidine-rich sequences that are used to bind Zn^{II}, Ni^{II}, Co^{II}, Cu^{II}, Cd^{II}, or Hg^{II} were separated by SEC, followed by IMAC as a secondary separation scheme [190]. The number of metal ions per metal binding protein have been determined by SEC separations followed by ESI-MS; particularly, the number of weakly coordinating Ca^{II} ions per protein were successfully determined by this combination of separation/detection procedure [191]. SEC has very recently been used to separate a range of metal binding proteins by the Rodriguez and co-workers [192].

Zn^{II} complexes of human milk proteins were fractionated by adding Zn^{II} salts to the milk prior to SEC separation [193]. Zn^{II} complexes of casein, albumin, lactoferrin, metallothionein, and citrate were separated [193]. SEC-HPLC, followed by ICP-MS detection has been used by Keppler and co-workers to investigate Pt^{II} and Ru^{III} antitumor drugs which are bound to serum proteins during transport in the blood [194].

Laplaca and Holm have used SEC to separate a model of carbon monoxide dehydrogenase in which a central peptide spacer that also binds to an Fe_4S_4 cluster is held between two regions of alpha-helical peptides that hold Ni activating sites [195].

Coordination of metal ions to polysaccharides and carbohydrates are important in the bio-transport of metal ions into and inside cells. Szpunar et al. have studied the binding of polysaccharides from apples to Pb^{II}, Be^{II}, Sr^{II}, Ce^{III}, and B^{III} [196]. These researchers found the metal ions bound to a polysaccharide of greater than 50 kDa by ICP-MS detection of the complexes that were separated by SEC. Lower molecular weight complexes for Zn^{II}, Cu^{II}, and Mg^{II} carbohydrate complexes were also separated. A metal-binding carbohydrate identified as rhammogalacturanon(-II) a pectin polysaccharide from plants, could be separated by SEC after treatment of plants or fruit with pectinlytic enzymes, followed by metal salts [197].

Geological analyses and surface water studies are often assisted by SEC in determining the presence of metal ions being transported. When multiple coordinating ligands are present in such samples, addition of metal ion salts that coordinate to each ligand may be separated by SEC and detected by ICP-MS [198]. This allows identification of multiple ligand donors in an environmental sample.

Humic acids, which form by the decomposition of living materials, have been studied by SEC with ICP or MS detection. Two types of complexes are usually found, including metal ions bound to carrier ligands of >300,000 molecular weight, and another type of carrier of 5000–10,000 molecular weight [16]. A similar distribution of metal-bound species was found in bog water [199]. Complexes of Ag^{I}, Cd^{II}, Cu^{II}, Mo^{VI}, Pb^{II}, Tl^{I}, U^{VI}, W^{VI}, Zn^{II} and Zr^{IV} were separated by SEC from ground water and sewage sources [200], and in pond water [201–203]. The natural distribution of metal ions on humic acids was studied after SEC separations by Heumann et al. [204,205], who also found selenates and selenites bound to humic acids [204].

Cu^{II} and Fe^{III} complexes of poly-phenols have been separated by SEC from beer [206], showing that metal ions are often retained in biological or geological samples, being bound by organic polymers. This is an important feature of metal storage and transport in the environment.

Velten and Rehahn have made polymers with *o*-phenanthroline attachments that bind metal ions in the normal bidentate fashion for each *o*-phen site. By studying the molecular weight distribution, Cu^{I} and Ag^{I} complexes of the $M(phen)_2$ composition are obtained, but there are two types of these—those in which the metal center bridges two of the polymer strands in a co-parallel assembly, and those sites in which the linear polymer loops back around to supply both "*o*-phen" chelating units for one metal ion [207]. Cyclo-trimers of three metal ions and three polymer strands were also observed.

Chelating agents made from cyclic polyamines with side arm carboxylates in an N_4O_4 binding site connected by amido tethers to either a second, identical like "dota"-like binding site, or to an antibody, have been prepared by Rogers et al. [208]. The $^{64}Cu^{II}$-labeled complexes of these ligands were then checked for the resultant biodistribution after injection into colon tumors in mice, and into normal mice. Normal mice degraded the complexes by 85% in one day. The metabolized complexes were separated by SEC with radio-detection.

Hydrosilation of CpFe(CO)(-CO(CH_3)) by ϕSiH_3 has been followed using SEC to separate the resultant intermediates and four isomer products [209]. The last product in the sequence is CpFe(CO)CH_2CH_3.

9. IMAC protein separations via inorganic complexes

9.1. Basic IMAC strategy

Porath and co-workers developed a chromatographic technique for bio-separations in which a metal ion that is attached to a chromatographic solid phase by chelating groups, usually iminodiacetate (ida^{2-}) bound to cellulose through the nitrogen linkage (Fig. 13), can interact with ligands in a mobile phase [210]. Several recent reviews are available [39–42]. The equilibrium situation is depicted in Fig. 13 for a bound ligand, LL, having two heteroatom attachments, however other numbers of attachments are possible, and may depend upon the pH. Other metal-binding groups that are attached to the support material include functionalities similar to nta^{3-}, $hedta^{3-}$, the iminoethylamine linkage, dithiocarbamate chelation, 8-hydroxyquinolate and short peptide sequences [42].

Ligands that bind well are retarded from movement in the mobile phase, and can be eluted later with suitable buffers. The technique is termed IMAC chromatography, and it is applied widely to the separation of proteins, of antibodies, of oligonucleotides, or of any material that can be conjugated with histidine-bearing peptides. The retention of proteins on IMAC columns was first limited to proteins having adventitious surface-exposed histidyl groups or thiol groups that could bind to Cu^{II}, Ni^{II}, Zn^{II} or Co^{II} derivatized M^{II}(ida) columns [211–213].

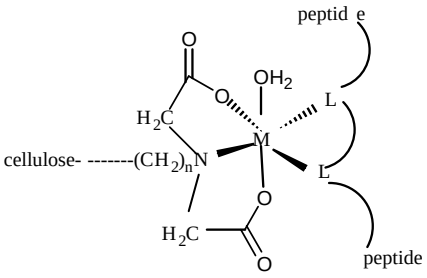
9.2. Affinity tails (tags)

However, the attachment of polyhistidine tails (His_n, n = 2–6), usually using an N-terminal fusion, has allowed separation of proteins that lack adventitious histidyl or thiol sidechains, and provides for multiple coordination anchors to the metallo-active site on the support phase which improves separation resolution [214,215].

A bio-engineering advance in protein tags (affinity tails) was achieved by Ataai and co-workers by means of a phage-displayed combinatorial library to optimize the fusion tag for protein separations [216]. Ataai's researchers showed that for Cu^{II}(ida) columns, a tag with the peptide sequence of Ser-Pro-His-His-Gly-Gly (SPHHGG) provides the best IMAC affinity tail, allowing for elution of the desired product protein or peptide under mild pH conditions between the pH = 4.0 (required of His_6 tags, wherein denaturation of proteins may occur), and below the pH 7.0 fraction that contains unwanted cellular proteins from recombinant sources such as *E. coli* cells.

9.3. Structure at the IMAC binding site

Until 1999 it was often debated as to the extent of coordination that occurs between a protein or peptide ligand and the IMAC metal center. Some chromatographers claimed as few as one histidyl linkage to a bound protein, while others believed that two or three contacts are necessary to bind the substrate. Shepherd and co-workers obtained the first definitive physical chemical evidence for a three-donor attachment using methyliminodiacetate as a coordination model for the IMAC binding site of M^{II}(ida)-IMAC columns [217,218]. This has led to understanding the coordination by affinity tags at Cu^{II}(ida)-IMAC sites at the molecular level. It was shown by NMR contact shifts in the diamagnetic [Pd^{II}(mida)(peptide)] complexes that three histidyl groups are coordinated for SPHHGG and $(His)_6$ affinity tails, the carboxylate donors from the $mida^{2-}$ (or the chromatographic support) being displaced to afford



L = side chain of his, asp, glu,cys,lys,etc.

M^{II}(ida)(LL) IMAC chelation site for peptides

Fig. 13. IMAC binding site structure for iminodiacetate-cellulose columns in peptide separations.

three square-planar coordination sites for the peptide. The results apply particularly to the Cu^{II}(ida)-IMAC columns wherein a square-planar coordination is favored for the d^9 metal center [218]. The [Cu^{II}(mida)(peptide)] complexes serve as models of IMAC sites, and have structures which are also consistent with three histidyl attachments when affinity fusion tails are used to isolate proteins as the UV and EPR spectra are matched by the three imidazole plus N donor from the ida binding site ligand [218].

A significant outcome of Shepherd and co-worker's studies is that the affinity tails were shown to make attachments at the first, third, and fourth amino acid side chain donors—skipping the second amino acid in the sequence. This arrangement affords the correct orientation of the histidyl donors to achieve the needed central bond angles of nearly 90° for square-planar coordination. Coordination of the peptide can be improved by providing a proline residue at the "turning" second position of the peptide chain.

Molecular modeling studies were performed to study the energetics of coordination of peptide affinity tails in comparison with monodentate donor ligands by Ward and Shepherd [219]. It was found that the peptide chain alters the favored donor set from one amine and two imidazoles for monodentate ligands to a set of three histidyl side chain donors when peptide chelation is involved. Energy minimized structures for the [Pd^{II}(mida)(SPHHGG)] and [Pd^{II}(mida)$(His)_6$] models of IMAC sites of the M^{II}(ida) type are shown in Fig. 14 as calculated using SPARTAN software that incorporates MMFF94 molecular mechanics methods [219].

Recently, Sharma and Agarwal have modeled the effects of pH and ionic strength of peptide binding at IMAC sites using a trp-gly-ala peptide binding with Ni^{II}(tren)$(H_2O)_2$ [220], however, few IMAC support columns utilize a tetraamine coordination. Rather, the iminodiacetate chelation is more common.

There are several earlier reviews of IMAC methods [39,212,213]. A recent advance is the use of four possible 12-mer tandum hexamer sequences obtained from combinations of SPHHGG and HPHHGG to afford fusion tails that can be tailored to allow elution of IMAC products in selectable 0.50 pH steps between pH of 5.0 and 7.0 [221]. Pasquinelli et al. used these 12-mer peptide fusion tags in combination with Zn^{II}(ida)-chelating Sepharose Fast Flow 6B columns to obtain the pH-selectable elution windows [221]. The IMAC action of the 12-mer tandum peptide affinity tags appears to be by accomplishing a two chromatographic site, near-neighbor attachment, on the IMAC column that enhances affinity on Zn^{II}(ida) columns. But the two-site binding to adjacent Zn^{II} locations is also significantly pH sensitive as to detachments which favor elution to the mobile phase. Thus, a careful balance between column affinity and pH-assisted removal is necessary for the most successful IMAC applications in bio-separations.

9.4. IMAC advances with polyacrylamide-based supports

The literature involving IMAC separations is rapidly increasing, and it would be impossible to mention all the proteins, peptides and bio-polymers that have been separated by IMAC. However, it is important to survey some of the advances in this field. Two commonly used support materials are polyacrylamide-derivatized gels and agarose-derivatized gels. Representative applications include separation of milk proteins, phosphopeptides, and casein on Sepharose columns [222], separation of β-amyloid protein, important in Altzheimer's disease research, on Ni^{II}(ida) columns [223], separation of three isoforms of superoxide dismutase on Cu^{II}(ida) columns [224], separation of human protamines from saliva which vary as to the number of histidines near the N-terminal position using Zn^{II}(ida) columns; some of the protamines bind weakly with only 0 or 1 histidine, but others with up to nine histidines bind tightly.

Human IgG immunoglobin factor has been separated using Cu^{II}, Co^{II}, or Zn^{II} on M^{II}(ida)-columns [225], and polymethacrylate ester with attachments of edta or 8-hydroxyquinoline using Ni^{II} [226]. The viral envelope

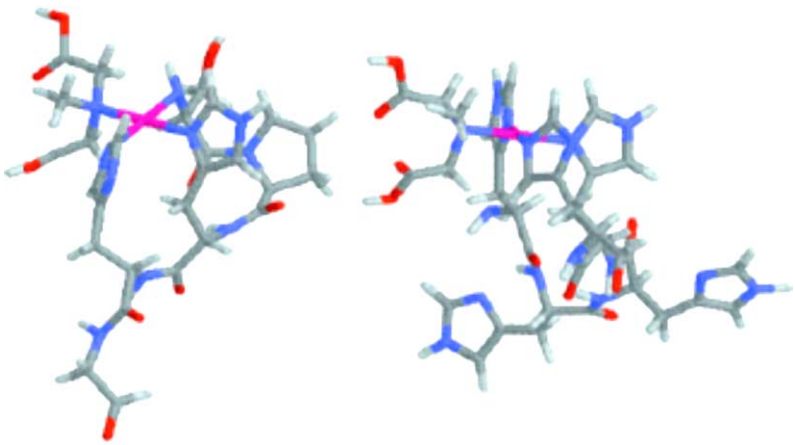


Fig. 14. Models of the IMAC binding sites from molecular mechanics calculated structures of [Pd^{II}(mida)(peptide)] complexes, SPHHG = ser-pro-his-his-gly and HHHHH = his-his-his-his-his (reproduced from [219] with permission of the copyright holders).

protein of the Dengue virus was only recently separated using IMAC procedures [227].

Beitle and co-workers have studied a green fluorescent protein (GFP_{UV}) that has five exposed histidines that bind well to Cu^{II} or Zn^{II} IMAC columns at three-His and a two-His binding sites [228]. The fluorescent-green fusion tag is useful in visualization of the location of its fusion proteins during IMAC separations or in the eluent solution, and was used in this manner by Ataai and co-workers in developing the 12-mer tandum SPHHGG/HPHHGG fusion tags [221]. Sloane et al. have used C-terminal fusion tags of sequence His-Gln-His-His-His (HQHHH) to separate a bacteriophage T4 lysozyme with M^{II}(ida) columns, showing a retention order of $Cu^{II} > Ni^{II} > Zn^{II}$ on the columns [229].

Schmidt et al. have used a three-histidine containing region derived from human carbonic anhydrase as a fusion tail to isolate serum retinol binding protein on Cu^{II}(ida) or Zn^{II}(ida) columns via a three-donor attachment to the metal center at an IMAC site [230]. The use of affinity tails has led to better characterization of ligand-IMAC column attachments. Presence of a binding tail in the structure transfers the point of protein binding to a more specific region of the protein. Usually, the activity of the protein is unaffected because the affinity tails are fused to the N- or C-terminal locations, away from enzyme action of the proteins.

IMAC methods can be applied to any bioconjugate that carries an affinity fusion tag, or to substrates that will coordinate with IMAC binding sites on polymers. Pehlivan and Yildiz have bound triscarboxymethylethylenediamine to a natural Sporopollenin polymer form *Lycopodium clavatum* to make an "$hedta^{3-}$" binding site for Cu^{II} or Ni^{II} [231]. The natural polymer was bound to CDAE (cellulose). These workers obtained better separation of nucleosides and nucleic bases on the Cu^{II}(hedta)-derivatized polymer than upon Chelex-100 resins.

Often a pH gradient or an imidazole gradient is used to elute biopolymers from their IMAC binding sites. Ivanov et al. have made an imidazole-containing polymer of 11,000 molecular weight and ca. 11% imidazole by weight, by combining *N*-vinylcaprolactam and vinylimidazole [232,233]. The imidazole polymer is useful to displace IMAC-bound proteins at Cu^{II}(IMAC) sites so that the isolated protein does not have free imidazole contamination.

Advances in the use of polyacrylamide as a support material for IMAC separations are numerous. Since polyacrylamide supports are also used in bio-separations such as blotting methods in connection with electrophoresis, there are studies that have been aimed at staining or detecting the location of the separated biomaterials on the columns or on the blotting polymer sheets. Recently, Eu^{III}(bathophenathroline disulfonate)$_3$ complexes have been used by Patton and co-workers as luminescence markers for the location of IMAC-bound or blotting sheet-bound proteins [234]. The Eu^{III} complex adheres only to the supported protein and not to the support phase, and can be located by the luminescence at 590–615 nm of the Eu^{III} complex. The Eu^{III} can be removed with edta solution in order to isolate pure proteins without Eu^{III} contamination, if necessary.

Patton's group has also used ferrozine in the ferrous form and $Fe^{III}(CN)_6^{3-}$ to stain proteins electroblotted on polyvinylpyrrolidone [235]; the iron staining complexes may also be removed by treatment with 0.02 M edta to afford protein separation of the located materials. A fluorescent protein containing Ru^{II}, SYPRO Ruby IEF Protein Gel Stain, that emits at 302 and 470 nm has been used to detect proteins bond on polyacrylamide or agarose IMAC locations [236].

Gupta et al. have separated soybean trypsin as a model for all protein separations using Zn^{II} on alginate beads without additional derivatization as per the M^{II}(ida)-type supported columns as a cheap, quick support medium for IMAC [237].

Other representative use of IMAC separations on polyacrylamide of interest to bio-inorganic chemists include Pillai's studies of supported triethyleneglycoldimethacrylate–ethylenediamine conjugates as metal support sites wherein a chelate between amido-nitrogen and the terminal amine of the bonded en unit at the dimethacrylate sites can occur with the metal ion center. Catalase activity of supported metal ions followed the order $Mn^{II} > Cu^{II} > Fe^{III} > Cr^{III} > Pb^{II}$ for the decomposition of H_2O_2 [238]. Spectroscopic and EPR studies by Pillai and co-workers allowed them to conclude that d^9 Cu^{II} is supported as distorted tetragonal complexes, but d^3 Cr^{III} and d^5 Fe^{III} or Mn^{II} are octahedral [239,240]—patterns well known to inorganic chemists. These results imply that IMAC binding sites are similar to normal transition metal chelate complexes for the metal of interest. This allows inorganic chemists to apply rationale design to IMAC sites, and to use molecular modeling and molecular mechanics techniques to advance the utility of IMAC separations as discussed in a later section of this review.

Representative separation on polyacrylamide-derivatized with M(IMAC) binding sites include the separation of human prolactin on Ni^{II}(IMAC) [241], phosphoprotein on Fe^{III}(IMAC) columns [242], a human capase-3 protein conjugated to a C-terminal $(His)_7$ affinity tail [243], and $[Rh^{III}(o\text{-phen})_2Cl_2]$-treated HIV transcriptase proteins which were labeled by the Rh^{III} $(o\text{-phen})_2$ moiety at the G base locations [244].

Pillai and co-workers have prepared polyacrylamide cross-linked with 4% divinylbenzene or with *N*,*N*′-methylene bisacrylamide, and connected this via amide bond to glycine to make an amido and carboxylato chelating terminus for supporting metal ions as per IMAC resins [245]. These workers have reported that when such resins are treated with Co^{II}, Ni^{II}, Cu^{II}, or Zn^{II} that coordination occurs. But after leaching out the metal ions with buffers, the Cu^{II} columns are claimed to retain "memory" of their Cu^{II} partners. New solutions containing mixtures of Co^{II} and Cu^{II} showed preferential binding of Cu^{II}, according to Pillai and co-workers [245]. The *N*,*N*′-methylene bisacrylamide-ethylenediamine-treated resins may be

further treated with CS_2 to yield polymer-supported dithiocarbamate terminal groups which bind metal ions in the affinity order of $Hg^{II} \gg Cu^{II} > Zn^{II} > Ni^{II} > Co^{II}$, similar to the anticipated order of sulfur donors toward M^{II} ions [246].

IMAC separations are valuable to the bio-inorganic chemistry community for isolation of metal complex–peptide conjugates. Groves and Kady have prepared 2,6-pyridinedicarboxylate and 2-picolylamine conjugates of 5′-TCGCCTTGCAGC-3′ oligonucleotide [247]. Binding of Fe^{II} at the Groves' pyridyl chelate affords a highly specific DNA cleavage agent that cuts DNA of a plasmid pUC9 only at a complementary binding site of the 12-mer oligonuleotide. Separations are made on SDS–PAGE gels (SDS–PAGE = sodium dodecysulfate–polyacrylamide gel electrophoresis) that combine features of the size exclusion chromatography, and of ligand affinity toward oligonucleotides [247].

Ruthenium complexes which are attached to histidyl units at one face of a 30-residue peptide that makes a self-assembled coiled dimer structure with Ru centers residing far apart have been studied by Ogawa and co-workers [248]. With attachments of $[Ru^{II}(trp)(bpy)]$ at one side of the coiled protein and $[Ru^{III}(NH_3)_5]$ as the electron acceptor site on the opposite protein coiled face, electron transfer could be studied over many angstroms separation. The hetero-labeled Ru complexes were prepared and separated by IMAC and identified by SDS–PAGE methods [248].

Photolysis of $[Ru^{II}(phen)_2(dppz)]^{2+}$ (phen = orthophenanthroline; dppz = dipyridylphenazine) promotes the photo-oxidation of G bases of DNA; if histones are present within the groove of DNA, a trapping of the oxidized DNA creates a protein-like–DNA conjugate. These histone–DNA conjugates have been established by analysis of the products using IMAC separation and SDS–PAGE [249]. It was shown that the amount of the histone conjugate depends upon the lifetime of the Ru^{II} photo-excited state. Presence of quenchers, added to the DNA/Ru photo-oxidant solution, decrease the yield of the histone–DNA conjugate that is separated [249]. It was also established that only G base regions are susceptible to the photo-oxidation; e.g. (dA-dT) polymers do not photo-oxidize, but (dG-dC) polymers behave as normal DNA.

Barton and co-workers have separated DNA metallo-intercalators of Λ-$[Rh^{III}(phi)_2$(bpy-protein conjugates)] that can be isolated from reactant materials using SDS–PAGE or IMAC strategies [250].

9.5. IMAC advances with agarose-based supports

Agarose is another support medium for IMAC methods that competes almost equally with polyacrylamide-based supports for IMAC applications. Oligonucleotides may be separated on agarose derivatized with a Reactive Green 19 dye as the binding site group [251]. Certain sequences such as AACCGGCGTTCGGGGGGTA that form structured units in the presence of K^+, but are stem-loop structures in the presence of Li^+ can be separated on Reactive Green 19-agarose columns [251].

Attachment of carboxymethylaspartate to agarose affords a column known as "Superflow". Co^{II} Superflow allows IMAC separations at flow rates of 5 cm/min for preparative separation of lactate dehydrogenase [252], and was used with a 19 amino acid containing a polyhistidine run for N-terminal attachment to recombinant human proteins to achieve >95% purity with 77% recovery in 1 h [253].

Uses of ida-derivatized agarose include separations of glucose oxidase on Ni^{II} or Zn^{II} columns [254], of myelin nerve proteins on Cu^{II}(ida)-agarose [255], of enkephlins on Fe^{III}(ida)-agarose [256], of hexokinase with an $(His)_6$ affinity tail on Ni^{II}(nta)-agarose [257], and of human T-cell leukemia envelope protein with an $(His)_6$ fusion tail on Cu^{II} or Ni^{II} sepharose and Ni^{II}(nta)-agarose [258]. A detoxification protein of schistosomiasis may be separated on Ni^{II}(nta)-agarose [259], as were proteins synthesized using polymerase chain reaction (PCR) technology on Ni^{II}(nta)-agarose [260], a human atriel naturetic peptide [261], and β-galactosidase on Cu^{II}(hedta)-agarose or Cu^{II}, Zn^{II} or Ni^{II} ida-agarose columns [262].

Various proteins have been separated on a peptide supported-agarose with a GHHPH binding site for metal ions; the agarose-GHHPH-Cu^{II} derivative performed best [263]. Phophopeptides have been separated on agarose containing adsorbed Fe^{III} or Ca^{II} ions [264].

Abudiab and Beitle have described an interesting method in which agarose that has been aged with iron salts, forming entrapped ferromagnetic particles (Fe_3O_4?). The aged Fe-agarose was then derivatized to make magnetic agarose-M^{II}(ida) support materials, which can be used to separate proteins with affinity tails [265]. The proteins bind at the IMAC sites in the magnetic agarose beads. After loading with the proteins, the particles are separated from the reaction phase in a batch method by means of a suspended magnet to which the IMAC-bound proteins adhere. Removal, washing and elution provide the desired protein separation.

9.6. IMAC advances with alternative supports

Cu^{II} or Zn^{II} Sepharose-ida and polyethyleneglycol (PEG-5000) supports for proteins have been used as separation phases in electrophoresis [266]. Mobility in the media decreases with the number of available surface histidyl groups on the proteins.

Polyethyleneglycol and polyethyleneglycol linked to ida chelate sites have been used for separations using Cu^{II} as an IMAC procedure [267,268]. It is reported that supported salicylaldehyde-Cu^{II}(amino acid) IMAC sites are more selective than Cu^{II}(ida)-IMAC sites [268].

Porphyrins with metal centers of Cu^{II}, Zn^{II}, Ni^{II}, Fe^{III}, In^{III}, and Sn^{IV} have been attached to silica supports for the separation of fullerene hydrocarbons via differing π to substrate π interactions, for the separation of anionic species

(particularly the In^{III} and Sn^{IV} derivatives), and of peptides with histidyl or tryptophan side chains [269]. It is claimed that the porphyrin–silica peptide separations have an advantage over Cu^{II}(ida)-IMAC columns as the porphyrin–silica systems undergo little metal leaching that is a contamination problem for M^{II}(ida)-columns.

Galaev et al. have described isopropylacrylamide/styrene co-polymers with ida derivatization [270]. The same workers prepared a polymer derivatized by 1-vinylimidazole groups as binding sites for Cu^{II} [270]. The Cu^{II}–vinylimidazole-polymer was used to separate protein inhibitors from cereals by means of the ternary Cu^{II}(vinylimidazole)(peptide-histidine) adducts in IMAC fashion [270].

Raska et al. have identified phosphopeptides using MALDI-MS/MS methods after separation of the phosphopeptides on IMAC polymer beads [271].

10. Chromatography of bio-inorganic complexes and environmental samples

Many studies of metal complexes of biochemical relevance have been discussed in the previous sections for IMAC and SEC methods. However, ions and their complexes that are found in natural environmental sources, or in synthetically prepared materials for biomedical purposes, are also often separated by chromatography techniques. Some of these were given as examples within the previous sections of this review, such as the separation of metabolites from cisplatin-related and ruthenium antitumor drugs [142,194,272].

Additional information of interest to the bio-inorganic community for separation methods may be found in recent reviews. These include Timerbaev's review of inorganic biological analysis by capillary electrophoresis (151 references) [273], a review of DNA cleavage agents, peroxynitrite reactions and Fenton DNA nicking pathways covering 437 references by Burrows and Muller [274], a review by Sandier et al. on ICP and AA detection of metal protein complexes separated by gel filtration [275], and Szpunar's review (38 references) on the HPLC-ICP-MS hyphenated technique for analysis of organoselenium, organoarsenic, metal–polypeptide, and metal–polysaccharide obtained from plant and animal sources [276].

Bio-inorganic complexes are being increasingly utilized in drug therapies for many different diseases. Ruthenium-based antitumor agents are being developed for many tumors such as colon cancer that cannot be treated by Pt^{II} reagents. Keppler and co-workers have used HPLC-MS methods to examine the speciation and biodistribution of complexes derived from the administered $\{trans\text{-}[Ru^{III}Cl_4(L)_2](HL)\}$ agents (L = imidazole or indazole) [272]; various aquated species involving loss of Cl^- or of the L ligand were identified. The more active indazole complexes are slower to hydrolyze, but become $[Ru^{III}Cl_4(H_2O)_2]^-$ which are found in isolated fractions. Tetracycline antibiotics were detected at ppb levels in milk and blood plasma using laser fluorescence detection of Mg^{II} tetracycline complexes after separation methods [277].

Eu^{III} and Pr^{III} ions assist the transport of Cl^-, Br^-, tropolonate, 8-hydroxyquinolate, and trifluorodithioacetylacetonate through membranes and Nafion films, as shown by separation methods for their detection [278]. Aminothiol complexes of $Re^{V}O^{3+}$ and $Tc^{V}O^{3+}$ were separated by chromatographic methods; these MO(aminothiol) complexes have been used as mimics of steroid hormones at receptor targets, and as imaging agents for the dopamine receptor by Katzenellenbogen and co-workers [279]. Deutsch and co-workers have used HPLC to examine the biodistribution of 23 $[^{99}Tc^{III}(PR_3)L]$ complexes used in organ imaging [280]. Samples were separated at 5 and 60 min times to show the increase or decrease in Tc concentration from various organs. ^{186}Re, ^{188}Re, and ^{166}Ho radiopharmaceuticals have been prepared from radioisotopic precursors using 1-hydroxyethylenediphosphonate as a chelating agent to provide a charged ion that may be separated by CE [281]. After separation of the purified isotopes, the chelate complexes may be further derivatized by selective transport ligands to provide selectively absorbed radiopharmaceuticals for imaging or organ irradiation.

Vanadium peroxo complexes are being tested as insulin mimics for diabetes therapies. Their effects on other organs and enzymes are of further interest. Therefore, Zhou et al. isolated a bovine heart enzyme complex of vanadium complexes administered as the oxalate, the bipyridyl, *o*-phenanthroline, and picolinate of $[VO(O_2)L]^{n-}$ which adds to the BHPTPase of the bovine heart tissue [282]. Enzyme adducts inhibit tyrosine phosphatase, and were separated using DEAE-cellulose of sephadex-G-75 columns.

Polytungstates are now known to be inhibitors of HIV-1 viral entry into cells [283]. Pope and co-workers have separated $[PW_{11}O_{39}RhL]^{n-}$ (L = dmso, Cl^-, Br^-, I^-, CN^-, py, etc.), prepared by the reaction of $[PW_{11}O_{39}]^{7-}$ with $RhCl_3$ at 150 °C for 20 h, and separated the complexes on sephadex [284]. Krotz and Barton have made a series of DNA intercalating agents of $Rh^{III}(A_4)L$ formulation where A_4 represents amines such as trien and L = phi = 9,10-phenanthrenequinone diimine. HPLC was used to separate intercalators of defined chirality on C-18 reversed phase columns [285].

Many separations of metallothioneins have been carried out recently, followed by speciation—usually by ESI-MS methods as discussed earlier in this review. Two additional examples are the studies of metallothionein–metal complexes using several hyphenated techniques (RP-HPLC-ICP-MS, CZE-ICP-MS, RP-HPLC-ESI-MS, CZE-ESI-MS) by Lobinski and co-workers [286]. RP-HPLC will separate several isoforms of metallothioneins as their Hg^{II}, Cd^{II} or Zn^{II} complexes. Best detection according to these workers is by RP-HPLC-ESI-MS. Chassaigne and Lobinski also studied Cd^{II}-labeled rabbit metallothioneins [287].

Separation techniques have been applied to many bio-inorganic samples and samples directly from

environmental sources for speciation. Representative examples include the separation of Co^{III} acac, tren and amino acid complexes on sephadex [288,289]. HPLC was used to separate the a and b pheophinates at the ppm range on Silasorb (hexane/butanol 96:4) or on Nucleosil C-18 with 40:40:16 CH_3CN:C_2H_5OH:CH_3COOH [290]. Metal carbohydrate complexes of Li^+, Na^+, K^+, Rb^+, Cs^+, and Co^{2+} have been identified using tandum MS techniques [291].

Analysis of the metal ion content and associated carrier ligand species are an important part of environmental analytical procedures. Azo dyes added to complex Cu^{II} and Fe^{III} in pondwater, followed by chromatography and electrochemical detection, allowed analysis at below the μg/l level [292]. The amount of Co^{II}, Cd^{II}, Cu^{II}, or Zn^{II} in waste water has been analyzed at the $6–8 \times 10^{-8}$ M level by RP C-18 column concentration of their M(edta) complexes [293]; the separated species can be identified by the order of migration, and their amounts are measured post column by displacement of various Ms using Fe^{III}. UV-Vis detection of the $[Fe^{III}(edta)]^-$ complex then provides for quantification of the amount of M(edta) that was present. [294]. Waste water content of Pb^{II}, Zn^{II}, Cu^{II}, Cd^{II} at 1×10^{-5} M level was also achieved by forming 8-hydroxyquinolate post column adducts of their M(edta) chelates which were separated by ion chromatography [294]. $M^{II/III}$(edta) complexes with Cu^{II}, Pb^{II}, Cd^{II}, Al^{III}, or Fe^{III} are readily detected as the protonated species that are produced as gas phase cations in an ESI-MS analysis [295].

Haraguchi and co-workers have determined 41 different elements in pondwaters [296]. In their procedure, gel filtration is used to preconcentrate the metal complexes which are transported on humic acids of two types (>300,000 molecular weight and one of 10,000–15,000 molecular weight). The identity of the metal ions are found using ICP-MS after separation on columns. Pondwater in Argentina was separated by gel filtration and analyzed to examine Cu^{II} and Cr^{III} movement on humic acids. After gel filtration, the complexes were separated on sephadex 15 [297].

Tap water metal ion content for Co^{II}, Ni^{II} or Cu^{II} can be achieved by separation of their $[M^{II}(Et_2dtc)_3]^-$ complexes (Et_2dtc^- = diethyldithiocarbamate) using RP-HPLC columns, and eluting with hexadecyltrimethylammonium bromide added to the eluent phase to alter the anion mobilities [298]. HPLC of solutions of Pb^{II}, Cd^{II}, Ni^{II}, Co^{II}, Cu^{II}, and Hg^{II} with added RS^- salts was recently achieved, with $M^{II}(SR)^+$ detection being made amperometrically, using a wall-jet flow cell with a graphite detector electrode [299].

Airborne metal contamination has been studied by the amount of metals taken up by proteins in oak leaves on their metallothioneins, using electrophoresis and ion chromatographic separations [300]. Lobinski and co-workers have described microwave-assisted extraction of analytes in environmental samples, followed by multi-capillary GC separations. The techniques have been applied to the analyses of Se and As contaminants, metallothioneins and enzymes from plant and animal sources [301].

Metal cyano complexes represent a significant problem from exposure of old toxic waste and industrial sites. The metal cyano complexes are readily transported to ground waters. $[M^{II}(CN)_4]^{2-}$ (M^{II} = Zn^{II}, Cd^{II}), $[Cu^{II}(CN)_3]^-$, and $M(CN)_6{}^{n-}$ (M = Fe^{II}, Fe^{III}, Co^{II}, and Ni^{II}) were separated by ion chromatography and analyzed at the 10 μg/l level [302].

Planar chromatography is used less by inorganic chemists than their organic colleagues. However, plates covered with a double hydroxide layer have been used by Ghoulipour and Husain to separate 26 inorganic cations and 17 anions in mixtures, as well as for the separation of Co^{III}(aminocarboxylate) complexes [303].

11. IMCOS separations of organic materials

There are advances in the separations of organic compounds that have been carried out using metal complexes supported on a solid phase. The approach is much like IMAC separations for hydrophilic biomaterials, but rather more hydrophobic molecules are separated. Much of the pioneering work has been done in the laboratories of Wasiak and co-workers in Warsaw, Poland. Wasiak and co-workers have presented a very good overview of the field [304]. An earlier review by Rykowska and Wasiak is also available [44]. It should be recognized that a number of reviews by Schurig of studies of the separation of enantiomers by GC methods using supported chiral complexes that were mentioned in sections related to chiral separations and organometallic separations also contribute to the field of IMCOS [12,13,37].

Separations on modified silica supports has been reviewed by Biernat et al. [6]. The newer report of Wasiak and co-workers report [304] compares the chromatographic behavior of trimethoxysilylpropyldiethylenetriamine which was attached to a silica support to other silica-supported ligands for Cu^{II} and Cr^{III}. The metallated silica column materials are then used to separate a wide variety of aromatic, linear and branched hydrocarbons, thioethers, ethers, and organics containing nucleophilic heteroatom substituents. The column binding site can be envisioned as shown here with the dien-like unit forming coordination complexes with available metal species. In Wasiak and co-workers studies, usually $CuCl_2$ and $CrCl_3$ are used to supply the metal chelation. To keep the electrostatic charge neutral, the anionic ligands (Cl^-) are retained with the metal center, and this is particularly important for separations in non-polar media.

silica-O-Si$(OCH_3)_2(CH_2)_3NH(CH_2)_2NH(CH_2)_2NH_2$

Much earlier work on supported silica phases were performed using silica attached to propylthiol, propylnitrile, or propyldiphenylphosphine moieties as the metal ion attachment points. These silica-derivatized functionalities containing Cu^{II} bound via the RSH, RCN, or RPϕ_2 attachments have been used to separate C-6 branched, saturated hydrocarbons and olefinic hydrocarbons [305].

Thermodynamics of the adsorption of substrates has been evaluated [305].

Bruce et al. have stated that when non-specific interactions occur at the supported metal complex sites on silica-derivatized columns, e.g. that coordinate chemical bonds to the metal center are precluded by the absence of Lewis base donors as in the case of most aromatics and hydrocarbons toward a hard Cu^{II} complex, that the main source of substrate affinity resides in H-bonding interactions with the oxo groups of the silica support, assisted by hydrophobic contacts in the region of the supported metal complex [306]. Wasiak has presented data on the ΔG, ΔH, and ΔS for the interaction of numerous olefins, hydrocarbons and aromatics with silica–Cu^{II} supported material with the metal bound by RSH, RCN and $RP\phi_2$ attachment ligands [307]; separations on the Cu^{II} polysiloxane-thiol or nitrile supported phases for ketones, ethers and thioethers were described [308].

The attachment of amine ligands to silica supports has followed the progression from monoamine terminus (one N donor) to a supported ethylenediamine or propylenediamine (two N donors), to the supported dien type (three N donors). The supported dien-Cu^{II} and -Cr^{III} separations were mentioned above [304]. Wasiak and Urbaniak prepared the monoamine supported materials by reacting the silica surface with aminosilane or generated an en-like binding site using *N*-(2-aminoethyl)-3-aminopropylmethoxysilane to derivatize a silica surface [305]. The Cu^{II} derivatives of the silica-aminosilanes are useful in separations of hydrocarbons, ethers, and nitroalkanes [309]. A propylenediamine-supported version makes complexes with $CuCl_2$ or $CrCl_3$ that separate hydrocarbons, ethers, thioethers and esters [310].

Another type of metal attachment with silica is readily created using a Schiff base adduct of acetylacetone with amine-supported phases. This creates a binding pocket of the imino-acac type (Fig. 15). Wasiak and co-workers describe two types of Cu^{II} coordination sites on the silica surface [311] that are as represented here via the synthetic steps.

Supports of the mono-iminoacac variety shown in structure (A) and bis-iminoacac in (B) have been prepared using 2,4-pentanedione and the trifluoro analogue [312] for the separation of hydrocarbons, ethers and thioethers. A similar $Cu(acac)_2$-derivatized column, but with a longer spacer between the silica phase and the metal complex region, has been prepared by Barroso et al. in order to achieve separations of di- and tri-substituted benzenes [313].

Wasiak and Rykowska prepared Co^{II} and Ni^{II}(acac)-supported silica columns for the separation of saturated hydrocarbons, aromatics and olefins [314,315]. They report that olefin donation to the supported $M(acac)_2$ chromatographic sites is stronger (showing higher retention) than for the same olefins with silane-diphenylphosphine(M) binding sites, and that for the $M(acac)_2$-supported columns, the alkane elutes ahead of the alkene with the same chain length. This shows that the olefins form η^2 complexes with the metal centers, retarding their passage through the column, even for relatively hard Cu^{II} and Cr^{III} complexes.

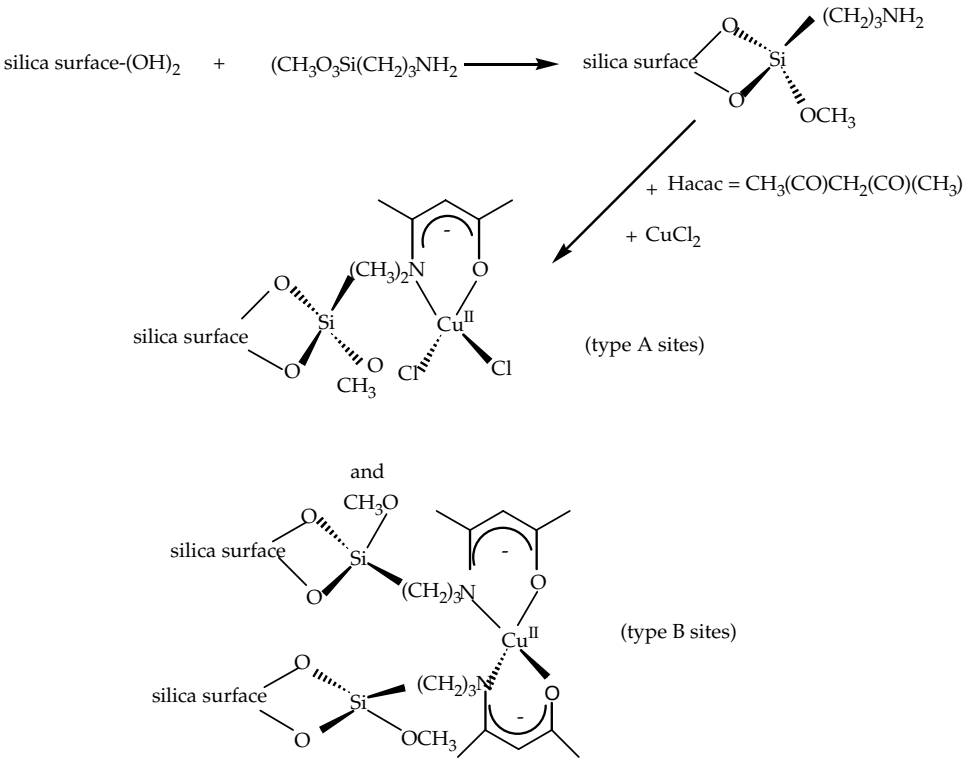


Fig. 15. Preparation of silica-supported imino-acac-Cu^{II} complexes for IMCOS separations as depicted in [311].

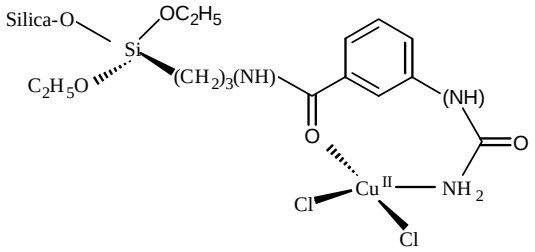


Fig. 16. Silica-supported phenylurea-based $Cu^{II}Cl_2$ complexes for IMCOS separations as depicted in [317].

Polysiloxane-hexane, polysiloxane-2-(isopropyl)acetonitrile and 2-(isopropyl)ethanethiol-supported silicas were prepared for the separation of aliphatic and aromatic hydrocarbons on coated glass capillaries [316]. Wasiak has also separated ketones, ethers and nitroalkanes on a silica-diethoxysilane(propylamide) of phenylurea metallated with $CuCl_2$, e.g. a structure as shown in Fig. 16 [317].

Other solid state supports have been used in addition to silica. These include a biosensor that is made by the adsorption of thiols on a gold electrode [318]. The thiol functionality resides a the end of a flexible chain of methylenes that connect to a chelator (such as ida^{2-}) for binding of Ni^{II}. The Ni^{II} complex at the solution phase terminus acts as an IMAC-like coordinator for substrates, including peptides. Binding of substrate is detected by changes in the electrode potential.

Co^{II} and Cu^{II} chelates of dithioxamide, linked via propylene tethers to silica (Fig. 17) were prepared by Akapo [319] who claims that the Co^{II} complex provides a better separation of olefins than for the Cu^{II} derivative.

Ohashi et al. have reviewed the adsorption of 8-hydroxyquinoline complexes that are immobilized on silica, on chelating resins, on chitosan, and on activated carbon (201 references) [320]. Some of these phases are suited to separation of aromatics and hydrocarbons.

Yeh et al. used a diamino spacer to create a supported dithiocarbamate chelation site on silica [321]. This support coordinated favorably to soft metal centers in the binding order of $Hg^{II} \gg Cu^{II} > Cd^{II} > Zn^{II}$. The Cu^{II} derivative successfully separates a thioether/hydrocarbon mixture,

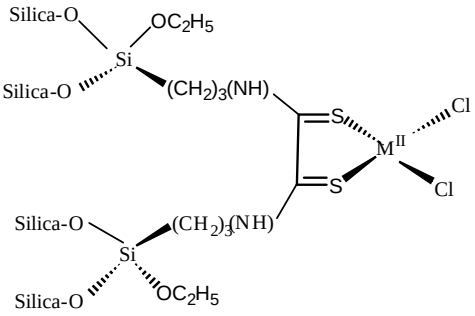


Fig. 17. Akapo's silica-supported dithioxamide complexes of Cu^{II} and Co^{II} for IMCOS separations a discussed in [319].

a process of value in removal of sulfur materials from synthetic gasoline. A similar sephadex–ethylenediamine conjugate was further linked via 2,2′-dihydroxyazobenzene to make an ethylenediamine-linked catechol metal binding site attached to sephadex [322]. This chelator will lower the Fe^{III} concentration to 1×10^{-16} M and Cu^{II} to 1×10^{-23} M. It will bind Fe^{III}, Cu^{II}, Zn^{II}, Pb^{II}, Mn^{II}, and W^{VI}. It was considered for harvesting of metal ions from seawater, but the higher affinity for Fe^{III} and for Zn^{II} swamps out its effectiveness in binding more economically interesting metal ions (e.g. Au^{III}, Ag^{I}, Pt^{II}, etc.).

Shin has prepared a silica column derivatized the leucine zipper protein GCN4. This hydrophobic protein support will separate oligonucleotide sequences such as AP-1 5′-TGACTCA-3′ which move as Group I salts in a form of RP chromatography of biomolecules on silica [323].

Harrison and co-workers have prepared Co^{II} complexes that are attached to polysiloxanes via linked tacn (1,4,7-triaminocyclononane). The six-coordination of the Co^{II} is completed by a free tacn capping ligand. When these supported $Co^{II}(tacn)_2$ complexes are coated on glass capillary tubes or spherical silica particles, the phase serves as a separation discrimination site for other complexes or substrates [324]. The authors have observed pH-independent osmotic flow with these derivatized columns. Harrison and co-workers have also used these Co(tacn)-supported complexes to separate alkylbenzenes [325].

12. Computer-aided chromatography involving transition metal complexes

There are numerous examples of chromatographers who have fit line width and retention data and flow rates in order to calculate binding constant of a substrate for a chromatographic support. Calculations of this type have been carried out by Haddad and co-workers on the equilibria between the mobile phase and in the stationary phase of micelles in the medium for the recently developed MEKC method (capillary micellar electrokinetic chromatography) [326]. In particular, Haddad and co-workers used metal complexes of Cu^{II}, Cd^{II}, Co^{II}, Ni^{II}, Pb^{II}, Bi^{III}, and Hg^{II} bound by $(HOCH_2CH_2)_2NCS_2^-$ (dihydroxyethyldithiocarbamate) and Fe^{III}, Ag^{I}, Tl^{I}, and Mn^{II} $cydta^{4-}$ complexes in an examination of how organic modifiers to the mobile phase influence ion transport [327]. Such calculations involve the competition between ion-pair formation and changes in metal binding constant as a function of the medium. But they do not readily address the fundamentals of coordination of the metal ion with ligating groups in a manner that is easily converted to improvements in the technique by means of altering the system at the fundamental molecular level.

Because of this limitation, this review will not attempt to extensively document this kind of mathematical manipulation of chromatographic data. Rather this section addresses a new application of computers, through molecular

mechanics methods, that can provide information at the molecular level in choosing substrate binding conditions that will optimize the binding and elution steps of the chromatographic method. There are very few reports of this type, but the future development of chromatography is sure to go in this direction. The concept is similar to the one used in pharmaceutical industries for the study of drug-receptor interactions. Two reviews by Lipkowitz address recent attempts to use molecular mechanics and statistical mechanics to the donor/acceptor (substrate-binding site) interactions that are of interest in chromatographic separations [46,47]. Extensions of these methods to include solvation behavior at chromatographic binding sites remains a difficult task.

A paper by Molina et al. has examined the binding of Zn^{II} and Cd^{II} PAR complexes on Sephadex QAE anion resin [328]. A combination of UV-Vis and infrared spectral measurements show that ML_2^{2-} are bound by the support phase where L = the deprotonated anion of PAR. These authors used SPARTAN molecular mechanics programs to predict that the lowest energy geometries of the $Zn^{II}L_2^{2-}$ and $Co^{II}L_2^{2-}$ species, e.g. whether these species are tetrahedral, octahedral, or some other coordination number. It was concluded that the $M^{II}L_2^{2-}$ (M = Zn^{II}, Co^{II}) are distorted octahedral in structure, and that these would be relatively unperturbed by electrostatic attraction to the anion resin phase because no net covalent attachment to the resin is anticipated for the quarternary ammonium groups of the resin.

The most detailed calculations of metal complexes that are involved in the binding sites of chromatographic separations are those of Shepherd and co-workers concerning the relative affinities of monodentate donors, and of affinity tags for IMAC chromatography with M^{II}(ida)-IMAC column sites [219,329,330]. The first study showed that anionic ligands would be preferred at the square-planar Cu^{II}(ida)-IMAC sites as modeled by a diamagnetic [Pd^{II}(mida)(peptide)] model system [219]. However, when peptide chelation is involved, it was shown that side chain coordination to neutral histidyl groups is modestly more favorable by 11.5 kcal/mol per histidyl donor than for coordination to amido anionic donors, accounting for the general observation that side chain coordination to a central metal ion is experimentally observed for peptides which have this option.

Shepherd and co-workers applied variations in the peptide sequence X_1-His-X_3-His-His in order to determine the optimal affinity tag for IMAC chromatography on Zn^{II}(ida)-agarose columns, considering the fact that a weaker binding ligand than the SPHHGG affinity tag would allow elution at a milder pH window [329]. This would provide some advantages in separation schemes for IMAC methods. MMFF94 calculations afforded the stability order on [Pd^{II}(mida)(peptide)] complexes as a model for IMAC binding to Cu^{II}(ida)-IMAC columns. The data for the [Pd^{II}(mida)(peptide)] complexes from most stable (lowest energy) to least stable (highest energy) as a function of the X_1 and X_3 amino acids were determined to be as follows: SPHHG (S = serine; H = histidine; G = glycine, 202 kcal/mol) < $(His)_5$ (205) YHYHH (Y = tyrosine, 249) < GHGHH (265) < WHWHH (W = tryptophan, 284) ~ GHPHH (P = proline, 286) < FHFHH-(F = phenylalanine, 311). These results suggest that the FHFHH IMAC affinity tail would be easier to remove than GHPHH (known to elute at pH 7.0–6.5) for Cu^{II}(ida) columns.

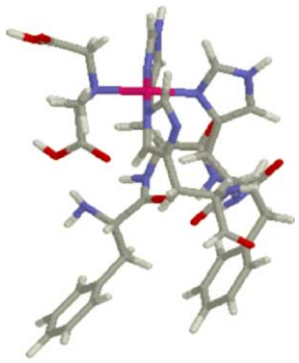


Fig. 18. π-Stacking internal interactions of the phenylalanines of the peptide FHFHH coordinated on [Pd^{II}(mida)] as a model of IMAC separations on IDA-cellulose columns (reproduced from [329] with permission of the copyright holders).

Several interesting structural features were uncovered by energy minimization using MMFF94 molecular mechanics calculations. The structure of the FHFHH complex according to the MMFF94 molecular mechanics calculations [329] revealed a π-stacked structure of the benzenoid rings of the phenylalanine groups, reminiscent of the π-stacking reported in the chromatography results for dansylated amino acids and D,L pairs of amino acids with Cu^{II} ternary complexes in Marchelli and co-workers' study of chiral molecular separations [108]. The [Pd^{II}(mida)(FHFHH)] complex's energy-minimized structure is shown in Fig. 18, wherein the ring orientation and π-stacking become obvious.

In the case of the tryptophan peptide complex, [Pd^{II}-(mida)(WHWHH)], there were two energy minima that involve a C–H to π-cloud interaction (Fig. 19, at the left)

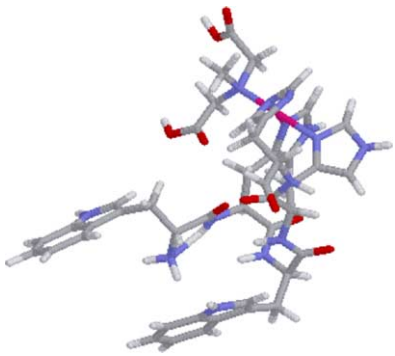


Fig. 19. H-bonding and π-stacking of tryptophans of the peptide WHWHH coordinated on [Pd^{II}(mida)] as a model of IMAC separations on IDA-cellulose columns (reproduced from [329] with permission of the copyright holders).

and the more stable π-stacked form (Fig. 19, at the right) which differ by ca. 39 kcal/mol, most of the difference residing in strain to produce the C–H to π H-bonded species [329].

Much information at the molecular level can be uncovered by computer modeling of chromatographic binding sites, and in modeling the local molecular interactions that can be exploited to improve chromatographic behavior. Many further use of computer modeling of the metal complex–substrate interactions are to be anticipated by others in this emerging field.

Lastly, Ward and Shepherd have carried out computer modeling of M^{II}(ida)-IMAC column sites in which the metal center is allowed to vary in structure as octahedral with fac or mer coordination of the support ligation, tetrahedral, square pyramidal, trigonal bipyramidal, and square planar complexes (Fig. 20) [330]. Solvent or bonds to a ligand substrate make up the additional bonds to achieve the correct coordination number. The several possible geometries for an M^{II}(IDA)(peptide) coordination for a peptide of sequence X_1HX_3HH is illustrated here.

It has been shown that the "best" affinity tag of most stability or the affinity tag that might be easiest to elute at a higher pH for IMAC-like coordination is not the same as the geometry is varied [330]. For example, the FHFHH phenylalanine-based affinity is easiest to elute from a square-planar site as for Cu^{II}(ida)-agarose, but the WHWHH tryptophan-based affinity tag would elute most readily from an octahedral site of Ni^{II}(ida)-agarose. The most strongly bound X_1-His-X_3-His-His peptide or related peptide as determined from molecular mechanics interactions also varies by structure. For tetrahedral (T_d), square planar (D_{4h}), square pyramidal-mer and octahedral-fac, the HHHHH or SPHHG run binds most strongly. But for octahedral-mer and square pyramidal-mer coordinations the less hindered GHGHH binds best [330]. In general, the crowding of a binding site with increasing numbers of donor ligands, or by structural confinement to one face of the binding site as

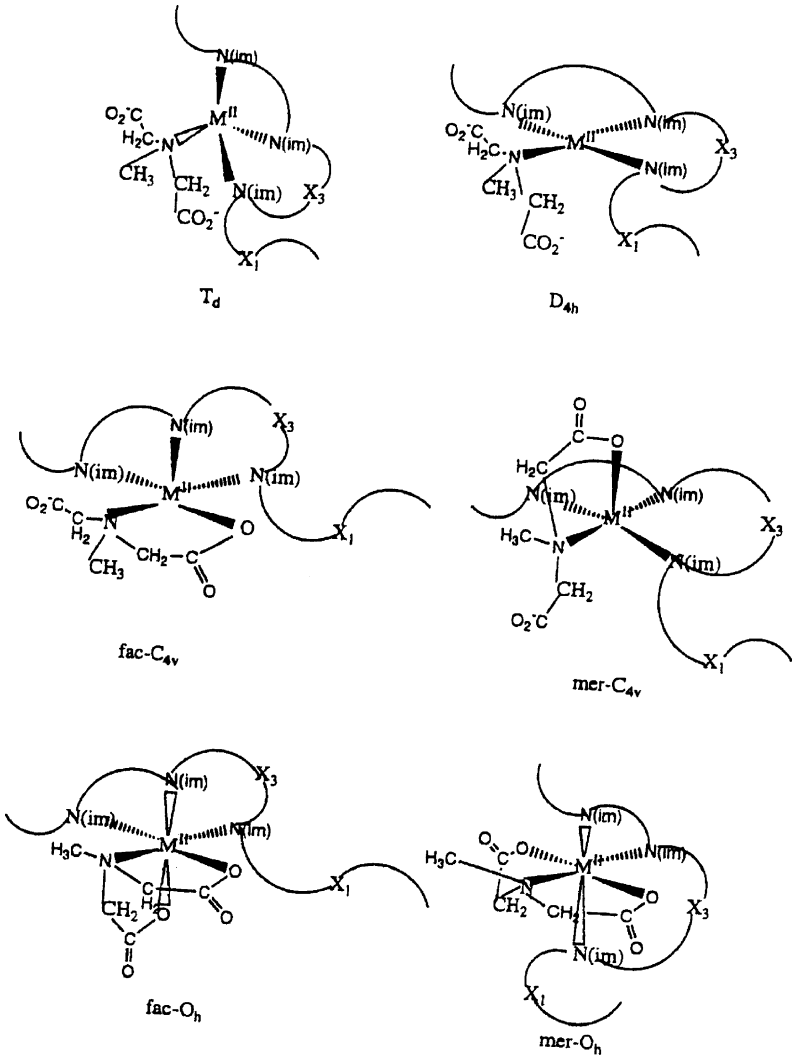


Fig. 20. Tetrahedral, square-planar, square-pyramidal and octahedral IMAC binding sites of IDA-cellulose columns (reproduced from [330] with permission of the copyright holders).

in octahedral-fac contributes to lower bonding stability and a higher energy for the complex.

Thus, an affinity tail that has the correct properties for separations on a square-planar Cu^{II}(ida)-column may not be the optimal tag for a tetrahedral Zn^{II}(ida)-column and so on. Thus, computer-based molecular mechanics calculations can be fine-tuned to optimize chromatographic results in advance of tedious laboratory work that is more time consuming. Chromatographic conditions may be selected, tailored to the kind of metal ion that is to be used on the IMAC column. The results of Ward and Shepherd show that the application of rationale design, using principles of coordination chemistry, offer much promise for the future design of binding sites for IMAC and IMCOS methodologies.

13. Mechanism of chromatographic separations

The key feature of a chromatographic separation is the differing affinities of substances in the mobile phase for the stationary support phase. These differences in affinities are the combined effects of (1) ionic attractions to charges on the stationary phase, (2) hydrophobic attractions to the support phase, and (3) special molecular recognition points provided by the surface of the stationary phase. Aspects of how these influence separations have been discussed throughout the previous sections as applicable there. However, some recent aspects of molecular recognition and ion-pairing as means to achieve separations will be mentioned here. A review on carbohydrate–carbohydrate interactions that are present in both cell adhesion, and in chromatography of carbohydrates, has been presented by Rojo et al. [331]. The complexation of substrates by cyclodextrins in the separations of pharmaceuticals by H-bonding interactions from protonated amines, and cyclodextrins as enantioselective agents in chiral separations have been recently discussed for CE methods [332,333].

Discussions concerning the separations of metal complexes are less common. Timerbaev et al. Have recently discussed structure–mobility relationships in modeling the electrokinetic chromatography of metal complexes [334]. They deduced that in some cases neither electrophoretic mobility or chromatographic affinity for the support phase dominates the separation efficiency. In past work, Timerbaev and co-workers [137,335] and Lee [149] have supported the view that for CE and CZE methods, the size to charge ratio, e.g. electrophoretic mobility, dominates many separations of metal ions coordinated by polyaminocarboxylate ligands of the $edta^{4-}$ family, cyano complexes, complexes of PAR and arsenate III ligands, etc. Ammann also has stated that the anionic attraction for the support phase (electrostatics that are related to the charge-to-size ratio) dominates over a minor hydrophobic attraction for the stationary phase in separations of $edta^{4-}$ family metal chelates [336]. However, Co^{III} complexes of the same $edta^{4-}$ family of ligands show that hydrophobic contacts are an essential part of the separation mechanism for $Co^{III}L$ complexes [57–59], as was concluded for the separation of α-amino acids on Cu^{II}(penicillamine)-supported columns [112a].

One may intuitive conclude that as the molecular shape of a complex departs more and more from a spherical point charge that H-bonding and hydrophobic contacts (molecular recognition for the stationary phase) becomes increasingly important in separations. However, deviation from a spherical symmetry also influence electrophoretic and diffusional mobility. The two main components that influence chromatographic separations are both a function of chemical structure. Therefore, it should be no surprise that many situations are in the intermediate regime, influenced by both factors [334].

In those cases where a metal complex has one or more labile coordination sites, or ligands that can be displaced by better donors from a support phase, the mechanism of forming ternary complexes at the stationary phase binding site will become the dominant mechanism that controls the “on–off” equilibrium that establishes separation of differing substrate species. This was noted in the separation of Pt^{II}, Cu^{II}, or Co^{II} salicilate complexes, ML_2, using RP-HPLC columns that were pre-derivitized by 2-(6-methyl-2-benztiazoylazo)-5-dimethylaminophenol (MBTAE) [337]. Ternary complexes, formed by displacement of one of the salicylate ligands, determines the separation order for a mixture of the complexes [337].

The use of butyrate anions as ion-paring reagents to alter the migration of $[M^{II}(o\text{-phen})_3]^{2+}$, $M^{II} = Fe^{II}$ or Ni^{II}, was recently described for a CE separation [338].

The mechanism of separation in IMAC chromatography, and often for IMCOS separations, is one of discreet ternary complexation wherein bond strengths and affinity of the mobile ligand for the metal of the stationary phase determines the separation process. In the case of IMCOS separations of hydrophobic organics, the additional affinity differences of the substrates for hydrophobic contacts with the support material is an increasingly important component of the mechanism of separation. These specific hydrophobic contacts achieve the ultimate separation of species, particularly those of relatively similar structure, that differ by small substituents or in the stereochemical configuration of the same functional groups.

14. Advances in molecular recognition via polymer imprinting

Ultimately, all the factors of molecular recognition are brought to bear in the separation of species by any chromatographic method. The selectivity factor for substrates in chromatography is akin to the selection of enzymatic surfaces for their molecular substrates by the summation of H-bonding, polar, and hydrophobic contacts. The design of coordination complexes capable of chiral discrimination of other ligands in homogeneous solution, or in the binding to biopolymers

such as DNA, are well underway in several laboratories. One example is the bis(picolyl)ethylenediamine-based series of ligands as tetradentate donors for Co^{III} and Ru^{II} complexes; these systems have been reviewed by Williams and co-workers [339]. The Λ-β-*cis* stereochemical coordination of [bis(picolyl)(R_2en)] or [bis(picolyl(R_2)hexanediamine)] ligands to Co^{III} and Ru^{II} provides the necessary chirality of the metal center to provide preferential affinity for separation of α-aminoacidates or the specificity in DNA major groove binding [339]. Ligands of this family are shown in Fig. 21a; molecular structures of how the Λ-β-*cis* Co^{III} complexes exhibit enhanced molecular recognition for phenylalanine and for tryptophan are shown in Fig. 21b that illustrates the additional π-stacking for the L-amino acids which would be absent for the D-amino acids having the side chain projected to the opposite side of the complexes and not near the pyridyl groups.

Although molecular recognition between partners in homogeneous solution is well recognized as a chromatographic advantage, the application to rigidly supported affinity sites for molecular separation of isomers is of much more recent development. Chromatographers have now begun the technique of molecular imprinting of polymer supports in the design of pockets for chromatographic separations and in the development of biosensor materials. The shape-selectivity of the metal complex is imposed on the polymer support material. This, in turn, is utilized to provide functional group selectivity or chirality specificity in the separation of organic substrates for chromatography applications, or in the quantification of the amount of substrate which can diffuse to the binding pocket in the case of the sensory application.

Mallik et al. have described the preparation of "synthetic antibodies" or protein receptors by using metal complexes of proteins to imprint polymers with cavities selective to the same metal ion–protein complex [340]. Another example that utilizes a transition metal-designed pocket has been described by Matsui et al. [341]. Polyvinylpyridine binding sites within polystyrene were created by polymerization of olefins around Co^{II}(vinylpyridine)$_2$(R_2acac)$_2$ complexes. The resultant pocket retains recognition of the diketonate ligands as a function of their "R" substituents, and has highest activity when Co^{II} is supplied to the active site instead of other, less active M^{II} ions such as Ni^{II} or Zn^{II}.

Polymers with imbedded *N*-(4-vinylbenzyl)-1,4,7-triazacyclononane groups arranged as a sandwich by either Hg^{II} or Zn^{II} in ML_2 complexes during polymerization of divinyl benzene have been used as ion-selective binding sites [342,343]. Imprinted polymers that have metal ion binding sites created by M^{II} complexes of bis-trimethoxysilylpropylethylenediamine polymerized in tetrachlorsilicate have been used to create binding sites

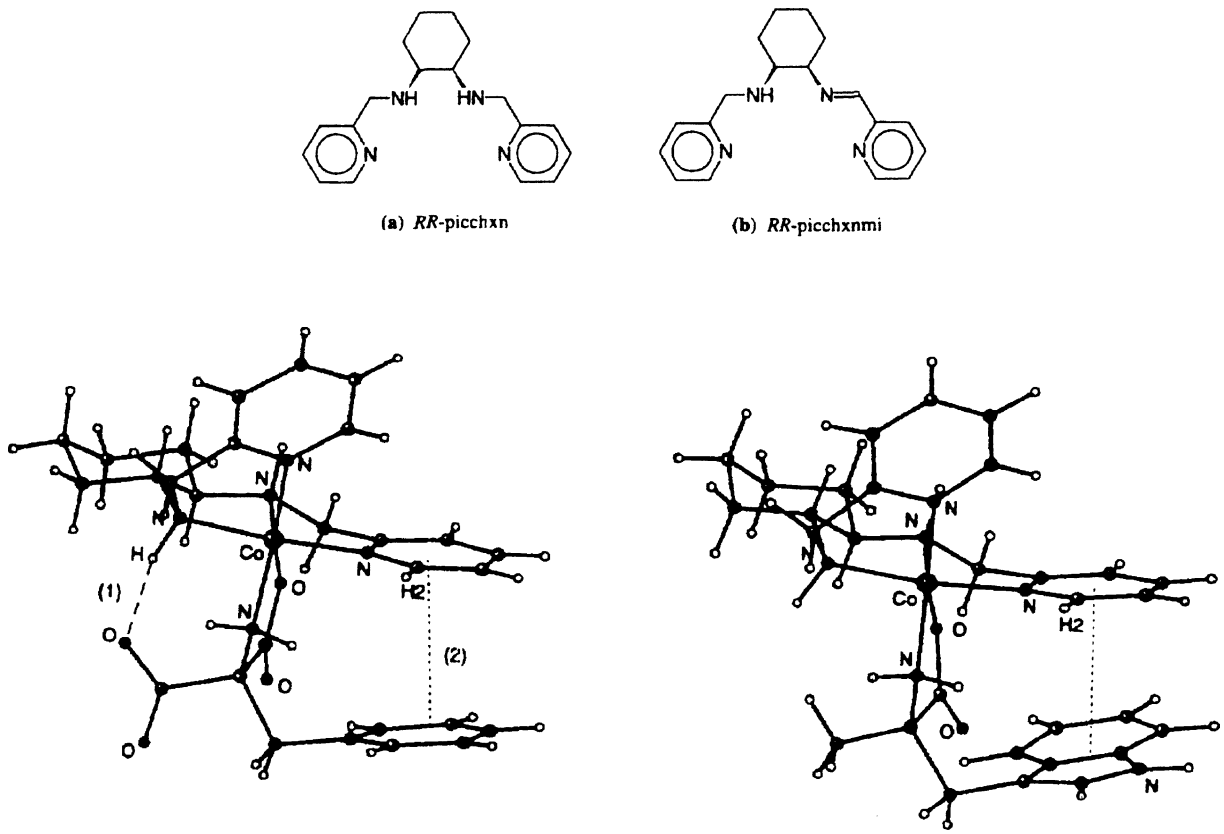


Fig. 21. Drawings of bis(picolyl)en-diamine and bis(picolyl)en-monoimine and the structures of the corresponding {Co^{III}[bis(picolyl)cyclohexanediamine](amino acid)} complexes, AA = phe and trp (reproduced from [339] with permission of the copyright holders).

with memory for the parent M^{II} ions (Cu^{II}, Ni^{II} or Zn^{II}) [344,345].

Carbohydrate-imprinted polymers have been used to measure differing binding constants for $[Cu^{II}(dien)]^{2+}$, $[Cu^{II}(2\text{-hydroxyethylethylenediamine})]^{2+}$ and $[Cu^{II}(4\text{-vinylbenzyldiethylenetriamine})]^{2+}$ complexes via the formation of ternary surface complexes of the carbohydrate unit and the $Cu^{II}L$ complexes [346].

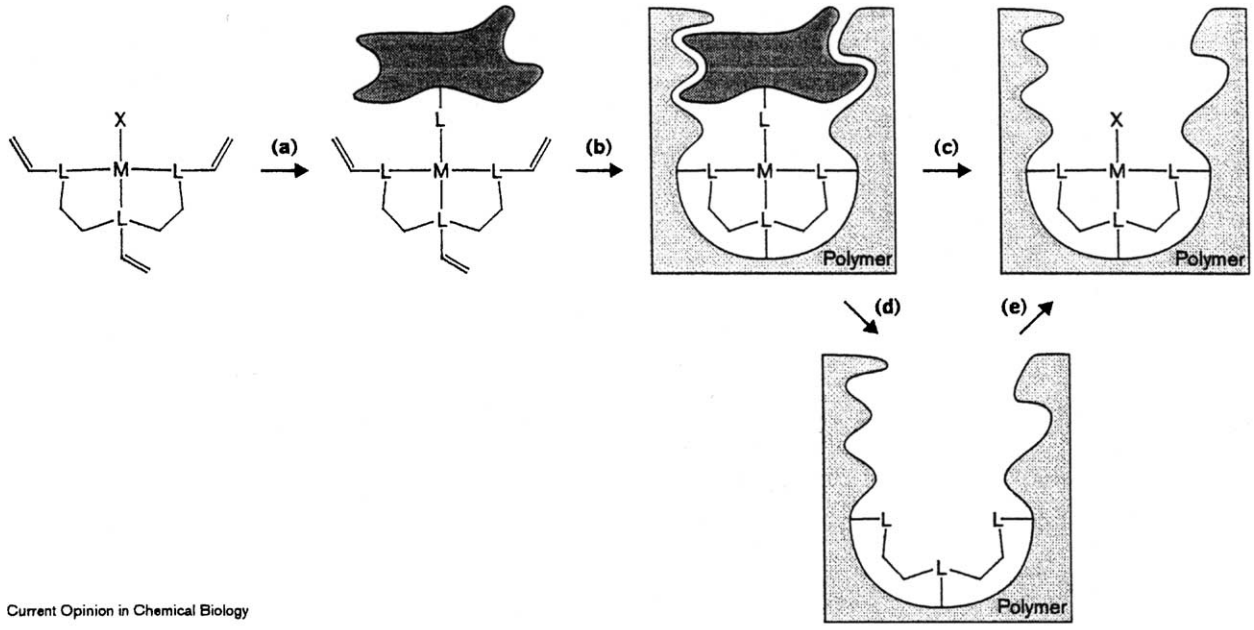
A electrode sensitive to M^{II} ions that are imprinted on a polymer film on gold with binding pockets created by cyclic and acylic polyethers has been reported [347], as has been a metal sensor for the 10^{-6} M range by templating a resin with Pb^{II} [348]. The concentration of Pb^{II} in unknowns is then evaluated by the quenching of fluorescence of a surface-bound [Pb(dye)] complex that forms on the imprinted binding sites [348].

A pocket designed to bind Fe^{II} via amine ligands on imprinted silica surfaces has been prepared using $\{Ru[(bipy\text{-}CH{=}NCH_2CH_2Si(OC_2H_5)_3)]_3\}$which upon hydrolysis during polymerization creates a three-amine receptor location [349].

A Zn^{II}-ion-selective chromatographic column was prepared using microspheres imprinted with Zn^{II} coordinated to carboxylate carriers during polymerization [350]. Such metal ion-sensitive microspheres have been generated in support materials created by polymerizations of divinylbenzene, styrene, butylacrylate and methacrylate polymers [351]. Related surface-supported sites that carry metal complexes for catalytic processes using Nb and Rh complexes are being prepared on SiO_2, TiO_2, Al_2O_3 and MgO surfaces [352].

The use of metal ion imprinted polymers has been used in the selective extraction of UO_2^{2+} from ore sources by polymers of chloroacrylic acid and ethylene glycol methacrylate imprinted with UO_2^{2+} [353,354]. Polymers with $edta^{4-}$ and $dtpa^{5-}$ functionalities linked to the polymer via one carboxylate as an amide have shown selectivity in the separation of Gd^{III} from La^{III} in lanthide separations [355].

The use of metal complex imprinted polymers to create immobilized transition metal catalysts the operate similarly to metalloenzymes has been reviewed by Severin [356]. In such systems, a complex which has a ligand structure that can be incorporated into the polymer matrix and which carries a temporary substrate ligand bound to the imprinting metal center is used (Fig. 22). After polymerization that creates a shaped cavity, the pseudo-substrate is removed by a bond cleavage that removes the pseudo-substrate or the ligand and cavity-inducing metal. If only the pseudo-substrate is removed, the bound complex serves as the catalytic reaction center. Alternatively, the removal of the pseudo-substrate and metal allows for insertion of a more reactive metal center to act in the catalytic cycle. This is shown in Fig. 22 adapted from Severin's presentation [356].



The basic concept of MI with transition metal catalysts. **(a)** A pseudo-substrate (dark gray) is coordinated to the active center of a transition metal catalyst having polymerizable side chains in its ligand periphery. **(b)** The conjugate is copolymerized with a large excess of a cross-linking monomer in the presence of an inert solvent to give a porous polymer. **(c)** Selective cleavage of the pseudo-substrate generates specific cavity in proximity to the active center. **(d)** Alternatively, the metal ion is also extracted and the active catalyst is **(e)** finally reassembled on the polymeric support.

Fig. 22. Synthesis of an imprinted polymer with a substrate-selective binding cavity for chromatography or catalysis (reproduced from [356] with permission of the copyright holders).

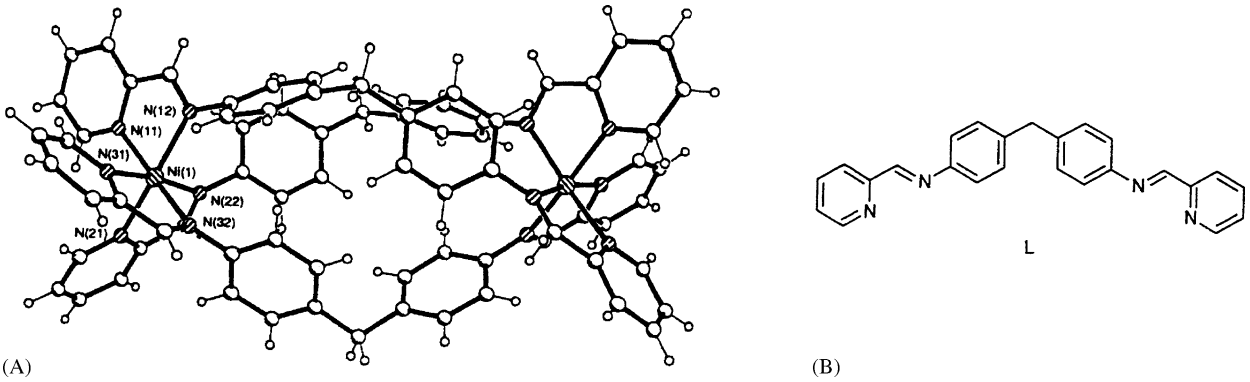


Fig. 23. One enantiomer of the chiral supramolecular helicate complexes, $[M^{II}{}_2L_3]$, $M^{II} = Fe^{II}$ or Ni^{II}, that binds to cellulose (reproduced from [360] with permission of the copyright holders).

It is possible to create chiral cavities by using chiral pseudo-substrate ligands in the assembly of the polymer sites. This approach was taken by Brunkan and Gagne, using a Pt^{II}(dppe)(BINOL) complex in the templating step [357]. This creates an imprinted polymer with multiple degrees of accessibility. Care in the polymerization conditions allows control of the more hindered and more selective imprinted sites which then can be used in synthetic reactions that afford up to 94% enantiomeric excess of one chiral product. Other arene-supported metal complexes for the purpose of selective recognition of similar substrates such as benzophenone versus acetophenone in transfer hydrogenation reactions, have been studied, but the kinetic advantage of one substrate for the templated cavity is often a factor of seven or less [358]. Zubieta and co-workers have reviewed the preparation of imprinted polymer pockets that utilize organic amine ligands for binding as hosts for guests of oxometalphosphates, transition metal oxides, transition metal halides, and transition metal pseudo-halides [359].

Improvements in the degree of selectivity of the imprinted binding sites are sure to follow soon, adding to the uses of inorganic complexes as IMCOS sites or improved catalysts.

Curiously, some of the most simple chromatographic support materials possess naturally "imprinted" structural motifs. Common cellulose chromatography paper or particles possess a groove structure on the surface that is generated by the parallel chains of β-glucose subunits polymerized head-to-tail. The chains aggregate by H-bonding to two other parallel chains forming a sheet with linear grooves. Hannon and co-workers have used the cellulose architecture to provide polymer binding sites for supramolecular helicate, rod-like, complexes of the general formula $[M_2L_3]^{4+}$, $M^{II} = Fe^{II}$ or Ni^{II} (Fig. 23B) where L is a two-sited pyridylimine ligand with the binding sites separated by a spacer unit (Fig. 23A) [360]. The similarity to the "Chirogen" ligands is apparent. However, the spacer is stiffer in the Hannon ligand, forcing coordination to two different metal locations rather than the "self-wrapping" of the chirogen ligands. Three ligands provide two octahedral binding sites for the M^{II} centers at opposite ends of a cylinder that is generated by a helical twist of the ligand structure along the main axis of the cylinder. This generates two enantiomers of the supramolecular helicate complexes.

The rod-like complex enantiomers exhibit affinity for the naturally imprinted groove of cellulose. Surprisingly, the enantiomers exhibit differing affinities for the linear groove of cellulose, and migrate at different rates upon elution using 0.020 M NaCl. This provides a separation of the enantiomers by a very simple chromatographic procedure that takes advantage of a naturally "imprinted" groove binding site [360]. Thus, the earliest forms of chromatography have utilized natural molecular imprinting. The current synthetic approaches introduce man-made polymer imprinted binding sites to discriminate in binding of substrates. This is intellectually an extension of the use of naturally occurring imprinted biopolymers as the earliest chromatographic support phase. Ironically, the cellulose stationary phase was first selected empirically (by accidental observation) at a time when molecular structural features of cellulose were yet unknown.

This represents the current directions of separation techniques—building support surfaces for the chromatographic stationary phase that retain their molecular shape as one that is suited to just one substrate, or one chirality of a desired molecule. Whereas chromatography evolved on a hit-and-miss basis by empirical processes in which it was found that a certain stationary phase would separate two or more species, chromatography as a field is now at the level where most progress for the future will be made by molecular design. This is true whether the binding site is designed to hold a shape by polymer rigidity by molecular imprinting, or whether computer modeling that simulates all the molecular contacts are considered is used to design the best separation scheme. The later approach has already been taken in IMAC separations [330].

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