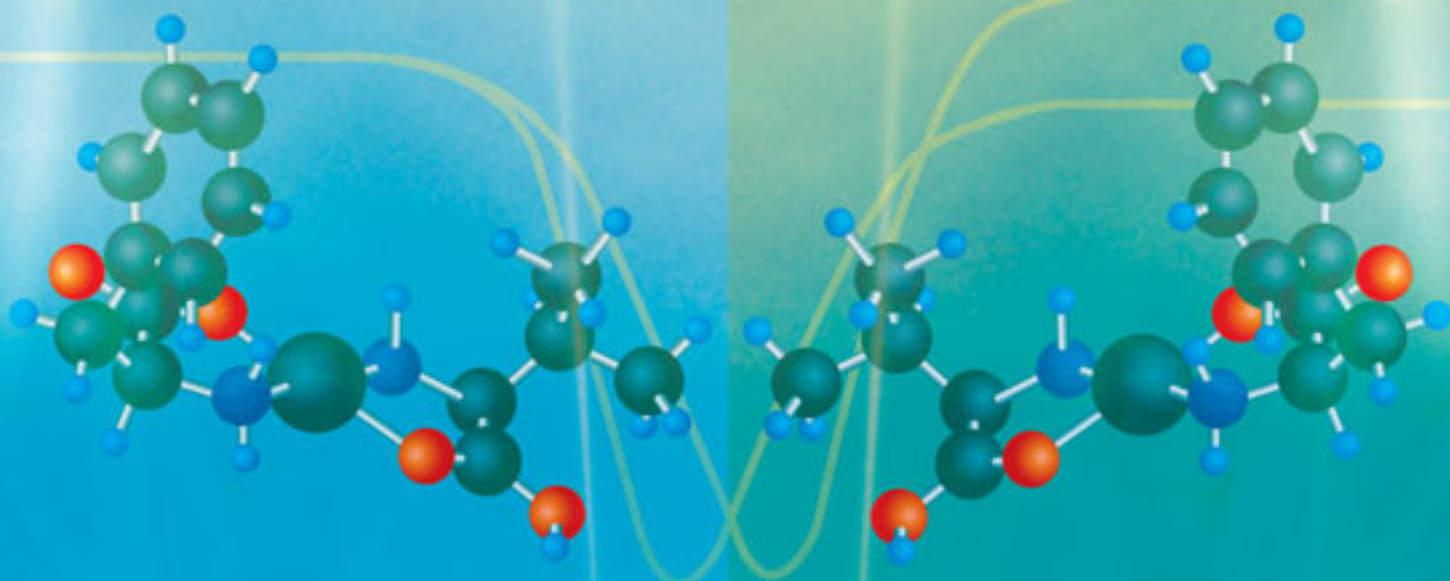


CHIRAL ANALYSIS BY MS



A new quantitative
method that is simple
and rapid and uses very
little sample.

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Few topics in the chemical, biological, and pharmaceutical sciences have attracted as much interest as chirality. Life itself has been under the constant influence of chirality, from the beginning of the evolutionary process right up through the present diversity of biological forms. Chirality represents an intrinsic property of the molecular building blocks—amino acids and sugars—and, therefore, of proteins, nucleic acids, and polysaccharides. An appreciation of chirality at the molecular level is essential to understanding molecular structure and reactivity.

The intrinsically chiral chemistry of living systems has evident implications for interactions of organisms

in tandem MS experiments (Figure 1) (10, 11). Complexes composed of CDs and protonated amino acids can be reacted with neutral alkylamines. The amino acid (A) is displaced by the amine (ref) in a guest exchange reaction to produce the CD-amine complex $[\text{CD-ref} + \text{H}]^+$. Chiral selectivity is the result of changes in the magnitude of the rate constants associated with changes in the chirality of the amino acid.

Several reviews summarize chiral recognition by MS (4, 12). This article focuses on quantitative chiral analysis by MS and emphasizes an MS method based on the competitive dissociation of metal ion-bound cluster ions (13, 14). It borrows from ligand-exchange chromatography the idea of creating diastereomeric complexes by binding the chiral analyte to a chirally ligated metal center (15). It adapts the kinetic method to convert extremely small differences in energy into relatively large differences in fragment ion-branching ratios (16, 17). (The kinetic method is widely used for determining thermochemical properties and is capable of differentiating processes with energy differences of <1 kJ/mol.) The result of this combination of concepts is a method that allows accurate, quantitative measurements of optical purity (18). It does not involve wet chemistry, isotopic labeling, or chromatography and therefore may be particularly useful for fast analysis and for circumstances in which chromatography and derivatization are ineffective or inconvenient. The results show interesting parallels with condensed-phase chiral selectivity data obtained by chromatography. Not only are the measurements simple and rapid, they use standard ESI MS and tandem MS on commercial instruments and require very small amounts of sample.

Chiral recognition

Chiral recognition and quantitation are based on the reactions in Figure 2. The chiral analyte (enantiomer A_R or A_S) and chiral reference compound (ref*) are complexed with a transition-metal ion (M) to generate high-order metal ion-bound cluster ions; in this particular case, trimeric cluster ions $[\text{M}(A_R)(\text{ref}^*)_2]^+$ or $[\text{M}(A_S)(\text{ref}^*)_2]^+$ (three chiral ligands in one complex comprising 1 molecule of the analyte and 2 molecules of the ref*). Divalent transition-metal ions such as Cu(II), Zn(II), and Ni(II) are among those that can be used to form suitable complexes. In these cases, the singly deprotonated ions $[\text{M}^{\text{II}}(A_R)(\text{ref}^*)_2 - \text{H}]^+$ and $[\text{M}^{\text{II}}(A_S)(\text{ref}^*)_2 - \text{H}]^+$ are usually generated in the MS experiment. These $[\text{M}^{\text{II}}(A)(\text{ref}^*)_2 - \text{H}]^+$ ions are generated efficiently in the gas phase by simply mixing A, M, and ref* in aqueous methanol and

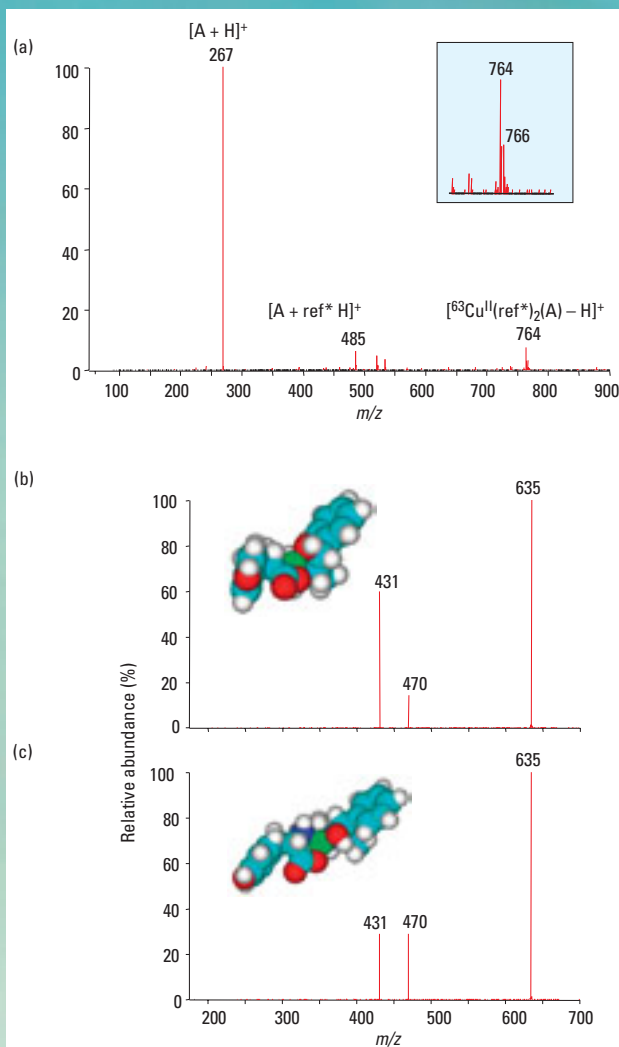


FIGURE 3. Mass spectra of chiral complexes.

(a) ESI mass spectrum of Cu(II)/atenolol/L-abriner solution with major cluster ions. The trimeric ion ($m/z = 764$) is used in MS/MS experiments for chiral analysis. The spectrum was recorded using a commercial ion trap mass spectrometer with a 50/50 water/methanol solution of 10 μM atenolol (A), 10 μM L-abriner (ref*), and 2.5 μM $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$. (b, c) Competing dissociation pathways are examined in the MS/MS spectrum of a deprotonated complex ion $[\text{Cu}^{\text{II}}(\text{L-Trp})_2(\text{ephedrine}) - \text{H}]^+$ ($m/z = 635$) that contains Cu^{2+} , two L-Trp, and (b) (+)-ephedrine or (c) (-)-ephedrine. Energy differences between the products ($m/z = 431$) containing (+)- and (-)-ephedrine result in differences in their abundances relative to the reference product ion ($m/z = 470$). Molecular models show distinctive structures and steric interactions in the diastereomeric product ions ($m/z = 431$).

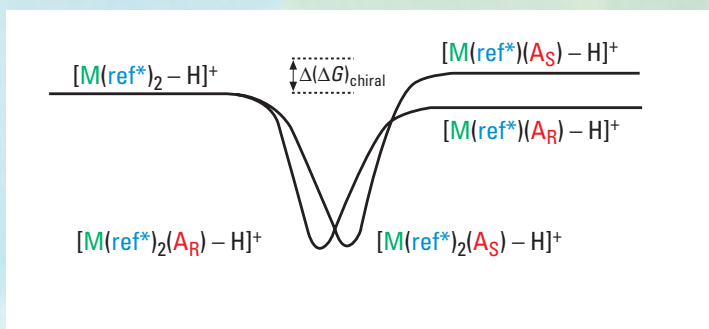


FIGURE 4. Energy diagram for competitive dissociations of metal ion-bound cluster ions of two deprotonated trimeric cluster ions that differ in the chirality of one ligand.

The two cluster ions yield identical products by one fragmentation route and diastereomeric product ions with energies that differ by $\Delta(\Delta G)_{\text{chiral}}$ by competitive ligand and loss fragmentation. This free-energy difference is due to differences in steric interactions because of the chirality difference. The energy difference is proportional to the log of the product ion abundance ratio, which is therefore a measure of enantiomeric composition.

creating the gaseous ions by ESI. A typical example of the resulting ESI mass spectrum is illustrated in Figure 3a for a Cu(II) salt/abrine/atenolol mixture. The M is used to create multipoint interactions between the A and the ref* to facilitate chiral recognition.

Dissociation of $[M^{\text{II}}(\text{A}_R)(\text{ref}^*)_2 - \text{H}]^+$ is achieved in an MS/MS experiment and occurs by competitive ligand losses to produce dimeric ions: $[M^{\text{II}}(\text{A}_R)(\text{ref}^*) - \text{H}]^+$ and $[M^{\text{II}}(\text{ref}^*)_2 - \text{H}]^+$; by contrast, dissociation of $[M^{\text{II}}(\text{A}_S)(\text{ref}^*)_2 - \text{H}]^+$ generates $[M^{\text{II}}(\text{A}_S)(\text{ref}^*) - \text{H}]^+$ and $[M^{\text{II}}(\text{ref}^*)_2 - \text{H}]^+$. Each of the dimeric ions has an associated ion intensity, I , as indicated in Figure 2.

The small differences in steric interactions in the diastereomeric cluster ions $[M^{\text{II}}(\text{A}_R)(\text{ref}^*) - \text{H}]^+$ and $[M^{\text{II}}(\text{A}_S)(\text{ref}^*) - \text{H}]^+$ are recognized by easily measured differences in branching ratios for dissociation of the complexes containing the *R*- and *S*-enantiomers of the analyte. The chiral selectivity R_{chiral} is defined as

$$R_{\text{chiral}} = \frac{I_{\text{R}}/I_{\text{ref}^*(1)}}{I_{\text{S}}/I_{\text{ref}^*(2)}} \quad (1)$$

in which $I_{\text{ref}^*(1)}$ and $I_{\text{ref}^*(2)}$ refer to the $[M(\text{ref}^*)_2 - \text{H}]^+$ ion intensities from dissociations of $[M(\text{A}_R)(\text{ref}^*)_2 - \text{H}]^+$ and $[M(\text{A}_S)(\text{ref}^*)_2 - \text{H}]^+$, respectively. R_{chiral} serves as a numerical indication of the degree of chiral distinction achieved in a particular system and is the ratio of the individual ratios of fragment ion abundances. The farther R_{chiral} is from unity, the higher the degree of chiral recognition. When $R_{\text{chiral}} = 1$, no chiral discrimination occurs, which means that the particular combination of M and reference ligand fails to create stereochemically dependent interactions with the enantiomers under the observation conditions used.

Chiral recognition of the stimulant ephedrine using the kinetic method is illustrated in the MS/MS data shown in Figures 3b and c. In this case, the ESI mass spectrum (not shown) is analogous to that shown in Figure 3a. The trimeric cluster ion $[\text{Cu}^{\text{II}}(\text{L-Trp})_2(\text{ephedrine}) - \text{H}]^+$ ($m/z = 635$) is generated, mass-selected, and allowed to dissociate by collision-induced dissociation in a tandem MS experiment. Dissociation yields dimeric complexes $[\text{Cu}^{\text{II}}(\text{L-Trp})(\text{ephedrine}) - \text{H}]^+$ and $[\text{Cu}^{\text{II}}(\text{L-Trp})_2 - \text{H}]^+$ by competitive losses of L-Trp and the ephedrine, respectively. The branching ratio of these fragment ions depends strongly on the chirality of the analyte drug. When the analyte is pure (+)-ephedrine, the branching ratio, R , is $I_+/I_{\text{ref}^*} = 3.8$, and for pure (–)-ephedrine, it is $R = I_-/I_{\text{ref}^*} = 0.91$, which results in $R_{\text{chiral}} = 4.17$.

The above discussion deals with the recognition of chiral pure compounds by the kinetic method. In cases in which the analyte is a mixture of the *R*- and *S*-enantiomers, R falls between 3.8 and 0.91 and is described by

$$R = \frac{I_{\text{R}} + I_{\text{S}}}{I_{\text{ref}^*}} \quad (2)$$

which is the sum of the intensity of the fragment ions containing the analyte divided by that of the fragment containing the ref*. The natural log of R is linearly related to the enantiomeric purity of the analyte, which allows quantitative chiral analysis (14, 18).

Quantitative chiral determination

The behavior of pure enantiomers (Figures 3b and 3c) indicates that when a chiral mixture is analyzed, samples with different enantiomeric excesses (ee) will show differences in R . The first step in the quantitative analysis of chiral mixtures, therefore, is to construct the calibration curve between R and ee. If a relatively simple relationship between R and ee can be established, then it is possible to rapidly determine the mixture components by measuring R in a single tandem mass spectrum. A linear relationship between $\ln R$ and ee has been observed in various systems and is predicted from kinetic method derivations (13, 14).

The behavior is typified by Clevudine (L-FMAU; 2'-fluoro-5-methyl- β ,L-arabinofuranosyluracil), a potent antiviral nucleoside against hepatitis B. Using *N*-Ac-L-Pro as the ref* and Co^{2+} as the metal ion, the data for various enantiomeric mixtures of FMAU display a linear relationship between $\ln R$ and ee with a correlation coefficient, r^2 , of 0.9995. This calibration curve is then used to measure the percent ee of various un-

known samples (19). The analysis of each sample requires only one R measurement in a single MS/MS spectrum. An average accuracy of 0.6 % ee was obtained for this particular case from four unknown samples.

Such a linear relationship between $\ln R$ and ee is expected, and a two-point calibration curve can be constructed simply by using two samples with known ee values, such as the pure R - and S - enantiomers or the racemic mixture and another sample. In the chiral analysis of the decongestant pseudoephedrine (ψ -ephedrine), a two-point calibration was used (18).

Cu^{2+} was chosen as the central metal ion and L-Tyr was chosen as the ref* ($R_{\text{chiral}} = 2.05$). Using R -values for the pure enantiomers (ee = -100% and 100%), a calibration curve was constructed on the basis of the semilog relationship between R and ee. The corresponding linear equations were used to measure ee values of unknown samples. The measured average ee values of the mixtures also correlated well with the actual ee values. The overall correlation (r^2) is 0.9998 for measurements of pseudoephedrine. Accurately determining ee values is a straightforward process (the overall average error for pseudoephedrine is 2.3 ee%). It takes a few minutes to construct a calibration curve and seconds to analyze a sample. The calibration curve can be used for days, and the method can be applied to samples that contain only a few percent of one enantiomer. Quantitative enantiomeric determinations can also be made using methods other than the kinetic method, in particular, the host-guest exchange methods (6, 20).

Kinetics and thermodynamics

The success of the present experiments is due to direct interactions between the appropriate ref* and analyte in the mass spectrometer, which allow optimization of chiral recognition; the introduction of a transition-metal ion into the system, which provides multiple interactions for chiral recognition; and the use of the kinetic method to probe the formation of diastere-

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omeric ions from corresponding higher-order cluster ions, rather than investigating fragments of simpler diastereomeric ions. The method examines competitive dissociation channels of a mass-selected cluster ion, and even very small differences in critical energies for fragmentation will result in large changes in the respective rate constants and be reflected in the fragment ion abundances. This is the result of the logarithmic relationship between the relative ion abundances and energy that characterizes the kinetic method.

A free-energy diagram for the chiral analysis methodology is given in Figure 4. According to the kinetic method, the degree of chiral discrimination depends on the difference in the free-energy changes ($\Delta(\Delta G)_{\text{chiral}}$) of activation for dissociation to generate the diastereomeric complexes ($[\text{M}^{\text{II}}(\text{A}_R)(\text{ref}^*) - \text{H}]^+$ and $[\text{M}^{\text{II}}(\text{A}_S)(\text{ref}^*) - \text{H}]^+$). Dissociation of parent ions to generate dimeric ions is a highly endothermic process with a negligible reverse critical energy. As a result, the difference in stabilities of the diastereomeric complexes ($[\text{M}^{\text{II}}(\text{A}_R)(\text{ref}^*) - \text{H}]^+$ and $[\text{M}^{\text{II}}(\text{A}_S)(\text{ref}^*) - \text{H}]^+$), relative to the reference complex $[\text{M}^{\text{II}}(\text{ref}^*)_2 - \text{H}]^+$, is proposed to be the key factor in the observed chiral selectivity, assuming that the more weakly bound precursor complexes show only small differences in energy.

Table 1. Influence of reference ligand on chiral selectivity.

Analyte	Ref*	$[\text{Cu}^{\text{II}}(\text{ref}^*)(\text{A}) - \text{H}]^+ / [\text{Cu}^{\text{II}}(\text{ref}^*)_2(\text{A}) - \text{H}]^+$		
		D-isomer	L-isomer	R_{chiral}
Leu	L-Val	2.4	2.5	0.95
	L-Pro	0.099	0.11	0.87
	L-Phe	0.96	0.41	2.3
	L-Ser	9.5	10	0.91
Tyr	L-Pro	43	4.7	9.2
	L-Trp	0.21	0.020	11
	L-Met	2.8	0.90	3.1
	L-Glu	16	8.0	2.0
Met	L-Pro	60	33	1.8
	4-HO-L-Pro	59	33	1.8
	L-Trp	1.8	0.23	7.6
	L-Glu	27	18	1.5

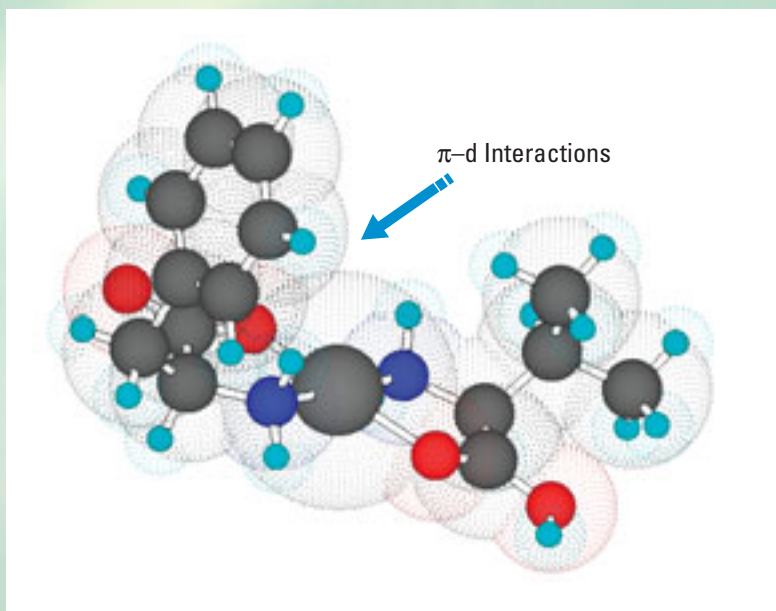


FIGURE 5. π -Cation interactions responsible for chiral distinction observed in the recognition of D,L-valine using Cu(II) with L-phenylalanine as the reference. Calculations show overlap between the π orbitals of the phenyl group in L-phenylalanine and the d orbitals of Cu(II) in $[\text{Cu}^{\text{II}}(\text{L-Phe})(\text{L-Val}) - \text{H}]^+$.

The linear relationship between $\ln R$ and relative energy is intrinsic to the kinetic method, but it also applies to the dissociation of a single cluster ion by two competing routes. When applying the kinetic method to chiral analysis, two cluster ions are considered. Their structures are chosen so that each gives one common set of fragments by its competitive dissociation channels. This reference fragmentation channel allows the normal kinetic method equation to be applied to the dissociation of the pair of cluster ions (Figure 2). Considering the kinetics and energetics of dissociation (14, 18), the normal kinetic method equation applies, with $\ln R$ being linearly proportional to $\Delta(\Delta G)$, as shown by

$$\ln R = \Delta(\Delta G)/RT_{\text{eff}} \quad (3)$$

in which R in the denominator is the gas constant, T_{eff} is the average (effective) temperature of the activated trimeric cluster ions, and $\Delta(\Delta G)$ is the difference in free-energy changes for the reaction $[\text{M}^{\text{II}}(\text{ref}^*)_2 - \text{H}]^+ + \text{A} \rightarrow [\text{M}^{\text{II}}(\text{ref}^*)(\text{A}) - \text{H}]^+ + \text{ref}^*$. The quantity $\Delta(\Delta G)$ is linearly proportional to the ee of A; therefore, $\ln R$ changes linearly with ee. The slope of the linear calibration curve, according to the derivation, is equal to half of $\ln R_{\text{chiral}}$, which is verified in various experiments. Therefore, larger chiral selectivity will result in a larger slope, which produces better accuracy for the ee determination.

Ion structure and intrinsic chiral interactions

MS, particularly tandem MS, allows studies of the intrinsic aspects of molecular and chiral recognition in a solvent-free environment. Moreover, comparisons of gas-phase and condensed-phase data provide a powerful means for understanding the role of solvent and counterions in determining the outcome of many reactions.

Structural studies of dimeric cluster ions, such as those produced in the reactions already discussed, reveal that two ligands are covalently bound to the metal ions through multiple binding sites, which provides the basis for efficient chiral distinction (14). In the case of amino acids, for example, two of the interactions between the two ligands are M(II)-mediated, resulting from the coordination of the amino and carboxylate groups to the central metal ion, whereas the third interaction involves the substituents at or near the asymmetric α -carbon atoms of each of the two ligands.

Although relatively weak, it is this last interaction that is essential for determining chiral discrimination. The superior chiral recognition achieved when the reference ligand has an aromatic side chain (Table 1) suggests that π -cation interactions play an important role in the stereo-interaction. Evidence for such a π -cation interaction was observed in collision-induced dissociation spectra of dimeric cluster ions $[\text{M}(\text{ref}^*)(\text{A}) - \text{H}]^+$, in which one ligand is an aromatic amino acid and is supported by ab initio calculations (Figure 5). When an L-aromatic amino acid such as L-phenylalanine is used as the reference ligand, these interactions will be disrupted by the side group on the α -asymmetric carbon of the L-analyte, whereas the side-chain group in the D-analyte will have little steric effect on the interaction because it is located at the opposite side of the square planar structure.

This interpretation is consistent with the observation that the heterochiral dimeric fragment ions are more stable than their homochiral analogues in cases where an aromatic amino acid is used as either the ref^* or the analyte. The inhibition of such interactions increases as the size of the side-chain group on the analyte increases in the series alanine, valine, leucine, and isoleucine, and there should be an increasingly large preference for the heterochiral complexes over the homochiral complexes. As predicted, R_{chiral} increases in this series. Proline can be expected to provide a large steric effect, so D,L-proline is efficiently resolved (14).

The nature of the metal ion is expected to play an important

role in this method, and the experimental data indeed show intriguing effects (19). In the case of amino acids, for example, Cu(II) offers much larger chiral selectivity than does Zn(II) or Ni(II), which is due to the formation of a square planar structure (21). In contrast, copper gives the smallest chiral recognition for D,L-FMAU among several transition metal ions, presumably because it prefers to bind to the heteroaromatic ring, which is distant from the stereocenters (19).

Promises and challenges ahead

MS is a promising technique for quantitative chiral analysis. Applications are already wide-ranging (18, 19, 22–24) and useful for recognition and quantitative determination of various chiral molecules, such as amino acids, dipeptides, α -hydroxy acids, atenolol, propranolol, carbohydrates, norepinephrine, DOPA, and isoproterenol. Extensions to new classes of analytes and metal ions, including Au(I), Pd(II), and Ca(II), are of interest, in addition to the work underway on Ni(II), Fe(II), Zn(II), Cu(II), and Co(II). Any system that facilitates multipoint interactions is suitable, including host–guest systems. Improvements continue; for example, the kinetics has recently been simplified by selecting one of the ligands designated as a “fixed ligand”, so that it cannot be lost. In addition, the requirement that the analyte be available in two different enantiomeric compositions has been overcome. A single reference sample—for example, the racemate or either of the pure enantiomers—can suffice. In this case, the trimeric cluster ion selected is that which contains two molecules of analyte and one of reference; two measurements are made using the ref* in forms with different percent ee values in each case (25). Performance characteristics of this quotient ratio method are very similar to those described here for the single-ratio method.

Further advances are needed to fulfill the industrial requirement of quantitative ee analysis for trace amounts of enantiomeric contamination (<0.5%). Efforts to achieve this should concentrate on enhancing chiral recognition by selecting the best chiral ref* compounds, using instruments with better signal quality, extending the method to other ionization methods, developing alternative quantitative methods, and using enhanced data processing for more accurate measurements. Furthermore, a new variant on the kinetic method currently being developed uses the cluster ion $[M^{II}(L^{\text{fixed}})(A)(\text{ref}^*) - H]^+$ (which replaces one of the reference ligands with a fixed ligand). Only the analyte and reference ligand can be lost, the kinetics are simplified, and the chiral selectivity is increased.

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R. Graham Cooks is a professor at Purdue University. Several new types of mass spectrometers have been constructed in his laboratory, including hybrid sector/quadrupole instruments, ion trap instruments

capable of MS/MS experiments, cylindrical ion trap instruments, and arrays. He is also interested in desorption ionization and tandem MS methods for analysis of complex mixtures, measuring thermochemical quantities by kinetics, ion structures, fragmentation mechanisms, and inelastic and reactive ion collisions with surfaces. W. Andy Tao was a Ph.D. student under the direction of Cooks and is currently a postdoctoral fellow at the Institute for Systems Biology. Tao's research interests are biological applications using MS, including genomic and proteomic analyses, global phosphorylation analysis, and molecular recognition. Address correspondence about this article to Cooks at Department of Chemistry, Purdue University, West Lafayette, IN 47907 (cooks@purdue.edu).

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