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In the Classroom

Analytical Applications of Chiroptical Detectors

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Of the three chiroptical detectors, only laser-based polarimetry and CD have the sensitivity capabilities … to measure traces of one isomer in an excess of the other.

Circular dichroism (CD) spectropolarimetry is one of only three analytical detector devices (sensors) capable of recognizing enantiomeric forms of optically active (chiral) molecules. The majority of naturally occurring substances that see significant applications as drug molecules are optically active. An intense interest in preparing chiral forms of drugs as single enantiomers has emerged with the rapid developments that are being made in the biotechnology industry. When a chiral drug is synthesized in the laboratory without the specificity normally provided in nature by enzymatically controlled reactions, the products are equimolar mixtures of both enantiomeric forms (racemic

In the past 20 years the number of instrumental techniques available to the chemist has grown exponentially. In order to help our readers keep up with this rapidly growing field, tutorial articles on chemical instrumentation will be a regular feature of *The Chemical Educator*. The articles are designed to serve as a brief introduction to emerging instrumental techniques with an outline of the underlying principles and major applications.

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mixtures). It too often happens that only one of the optical isomers has therapeutic value leaving the other as inert ballast at best or toxic at worst. Over the past decade or so, the preferred order of business in chiral drug synthesis has been to prepare the racemic mixture and either separate the enantiomers by derivatizing them with a third chiral species to prepare diastereoisomeric forms, which are separable, or by enantiomeric enrichment processes in which one is converted to the other. The technology associated with both of these processes is very difficult. As a result, in the United States, the Food and Drug Administration (FDA), which is in favor of marketing enantiomeric forms of drugs, has proposed a reward of an extended patent lifetime to those manufacturers who commit to developing enantioselective synthetic methods of drug production.

An equally, if not more difficult task in the manufacture of pure enantiomers, is to develop a quality control protocol. Two levels of purity determinations must be satisfied: (a) chemical purity, for which numerous methods are already available, and (b) enantiomeric purity. There are two experimental choices for the latter tests. Either a chiroptical detector with the necessary sensitivity to discriminate small percentages of one enantiomer in the presence of a huge excess of the other must be found, or the enantiomers must be chromatographically separated and the purity determined using a conventional detector. The FDA requires that on the submission of a new chiral drug form an analytical protocol must be submitted for both enantiomers. The development of an enantiomeric purity test is particularly difficult because there are no reference standards available for many enantiomeric forms.

Of the three chiroptical detectors, only laser-based polarimetry and CD have the sensitivity capabilities that are necessary to measure traces of one isomer in an excess of the other.

Chiroptical Detectors

The physical property that is unique to chiroptical detectors is their sensitivity towards the direction and magnitude of the angle of rotation of an incident linearly polarized light beam after its interaction with a chiral medium or chiral molecule. Rotational data are applied to (a) structural (or conformational) analysis of the chiral medium and (b) analytical determinations for chiral analytes.

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An expectation made in the 1960s, when CD instrumentation first became available, was that rotational data alone was sufficient independent information to arrive at the unequivocal *absolute* conformation for any chiral molecule. This expectation was quickly shown to be false. The very best one can ever expect to achieve is a *relative* molecular structure arrived at through the use of sophisticated mathematical algorithms, and justified by making comparisons between the best mathematical model and the absolute conformation for a reference material or molecule that has been determined by an independent experimental method such as single crystal X-ray analysis.

The chiroptical procedures used for making analytical determinations are entirely analogous to other spectroscopic methods. A measured signal, for example optical density in the case of absorption spectrophotometry, is presumed to correlate linearly with the molar concentration of the absorber. The correlation is expressed by Beers' Law:

$$A = \varepsilon C d \tag{1}$$

where A is the measured absorbance of a solution, C is the molar concentration, d the sample thickness or pathlength, and ε is the absorbance value for a 1.0 M solution when d = 1.0 cm. An equivalent equation that describes the correlation of the measured rotation with concentration is obtained by substituting the rotation quantity α (in degrees) for A and Φ (the molar rotation) for ε:

$$\alpha = \Phi C d \tag{2}$$

Polarimetry

The incident light beam in polarimetry is linearly polarized, which is conceptually described as the trigonometric resultant of two circularly rotating components propagating in phase. The angular rotations are directionally opposed. On their passage through an optically inactive (achiral) medium, the components remain in phase and the exiting linearly polarized beam lies in the same vector plane as the incident beam, that is, the angle of rotation is zero degrees.

Because of distinctly different interactions that occur between the opposite electromagnetic fields of the two circularly propagating components and the helical

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electronic motion in a chiral medium, two different refractive indices are presented to the incident coherent beam. Since the speed of light propagation through a medium depends upon its refractive index η, it follows that the two circularly polarized components travel at different speeds and are driven out of phase. The net result is that their trigonometric resultant, although still linearly polarized, has been rotated either right or left of the incident beam vector by a system-determined angle of α degrees. The magnitude of α is proportional to the *difference* in the refractive index values ($\eta_L - \eta_R$), a phenomenon referred to as birefringence. The subscripts *L* and *R* describe the rotations as being to the left or to the right of the incident plane. The slope of the line that defines the linear dependence of α on the concentration of the chiral analyte in solution is the value of Φ.

Optical Rotatory Dispersion (ORD)

Optical Rotatory Dispersion is the name that is given to the spectral variation of α with wavelength. ORD has no particular advantage over either polarimetry or CD for either conformational analyis or enantiomeric analysis, and is seldom if ever preferred. It is worth noting that the magnitude of α increases with decreasing wavelength, implying that the strongest rotations are seen in the UV to far-UV electromagnetic spectral range.

Circular Dichroism (CD)

Birefringence is but one property of CD activity. A second physical property associated with CD is the unequal absorbance (or dichroism) of energy from the two circularly propagating components of the linearly polarized incident light beam on its passage through a chiral medium that is also an absorbing medium. The absorbance requirement for CD detection means that the effect is only observed over the wavelength range of an absorption band. This has obvious analytical advantages in that the instrument baseline is set by the zero dichroism over ranges where absorption does not occur. To be more exact, CD bands are observed only over the range of an absorption band where the electronic transitions are associated with chiral chromophores. Although CD can be measured at a single wavelength, it is as a spectroscopic method that its analytical selectivity is derived.

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The added effect of unequal absorbances is to cause the normally linearly polarized transmitted beam to become elliptically polarized. The ellipticity ψ of the exiting beam is given by the expression $\psi = \tan^{-1}$ (minor axis/major axis) and is one physical property that is proportional to the absorbance difference or optical dichroism:

$$\Delta A = (A_L - A_R) \tag{3}$$

In plotting a CD spectrum, the variable can be either ψ (which was the quantity measured in the earliest CD instruments), or ΔA measured as a function of the wavelength. The analog of the molar absorbance ε is the molar ellipticity $\Delta\varepsilon = (\varepsilon_L - \varepsilon_R)$.

Although CD is a detector that is primarily selective towards chiral molecules that absorb, it is most easily and most accurately handled experimentally as a differential absorbance detector. Therefore CD is no more difficult to use or understand than conventional absorbance spectrophotometry, and

$$\Delta A = \Delta\varepsilon C d \tag{4}$$

is analogous in every way to the Beers' Law expression for absorbance.

One significant difference between absorbance and CD spectra is that while the signals are always positive in absorbance, they are both positive and negative in CD, the sign being determined by the relative values of ε_L and ε_R. Successive bands of opposite sign are separated by a crossover wavelength, which occurs when $\varepsilon_L = \varepsilon_R$, and can happen within the wavelength range of what is seemingly a broad single-absorbance band. All of these special spectral properties are responsible for the superior analytical selectivity that CD has over conventional absorbance detection.

Probably because of its direct affiliation to absorbance detection, CD has become a partner in hyphenated analytical techniques, for example, fluorescence and CD detection (FDCD), liquid chromatography with CD detection (LCCD), and capillary electrophoresis with CD detection (CECD). The extrapolation of adding CD detection as an integral part of these systems might appear to be elementary because of its similarity to absorbance detection. There are, however, some experimental constraints that will only be resolved as technology is advanced.

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The conception that a strong absorbance will engender a strong CD signal because they have a common origin holds without exception for electronic transitions in the far-UV range, but is quite erroneous for bands at longer wavelengths. From the author's experience, the most lucrative ranges for finding strong CD bands to maximize the analytical selectivity and sensitivity of CD are the near-UV and visible ranges, where the lower probability $\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$, and metal ion $d \rightarrow d$ electronic transitions are prevalent. The explanation for this apparent inconsistency should be obvious. It is not the absolute value of the absorbance that establishes the magnitude of the CD signal, but the difference in absorbances between the two circularly polarized components.

The most ubiquitous chromophores in organic molecules that absorb in the near-UV are aromatic rings and carbonyl groups, both of which are structurally planar. Planarity is not synonymous with chirality. In order to "self induce" CD activity, a molecule must have a chromophore in close juxtaposition with an asymmetric (chiral) or disymmetric center in its structure. In general, it is a relatively easy matter to identify a molecule that will satisfy this requirement, but one has to be aware that an enantiomer and its racemic mixture can not be discriminated by the written molecular formula, which will explain why a suspected chiral analyte is in fact CD inactive.

From symmetry reasons, enantiomers of the same molecule with the same chemical purity have CD spectra with bands of equal intensities but opposite signs, so that in an exact racemic mixture the net signal is zero over the entire spectral range. The same is true for molecules with more than one chiral center that internally compensate to form a meso-isomer. Molecules with two chiral centers will theoretically have four chiral isomers, although not all four may be available materials. Two of the four are enantiomers and two are diastereoisomers. The four CD spectra divide into two identical pairs of opposite signs; so, the absolute discrimination between an enantiomer and the diastereomer with the identical spectral is not an easy exercise.

Induced CD Activity

The structural origins of CD activity intrinsic to a single molecule, described as self-induction in the previous paragraph, apply equally well to intermolecular interactions. In this context, chirality is centered on one molecule and the chromophore on another. This extrinsic CD activity is induced through some physical interaction, for example, a complexation equilibrium or chemical derivatization reaction, by which the active

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centers are brought into the proper mutual environment. Numerous examples of this type of CD induction are to be found in the literature. Some generic examples that might be mentioned here are: (a) the complexation of an achiral aromatic molecule within the lumen of a chiral host such as β-cyclodextrin; (b) the association between dye molecules and macromolecules such as oligomeric and polymeric carbohydrates or between oligopeptides and proteins, which provide the means to probe the gross structural properties of the host molecule and the structural details of the reactive site; (c) the association of achiral as well as chiral analytes to chiral metal complexes by ligand exchange.

The reciprocity of the interactions imply that extrinsic CD induction can just as easily be described as a chirality induction on a chromophore process, or as a color induction on an asymmetric center process.

Applications

Structure Determinations

The most prevalent uses of CD detection in the study of molecular structures are in the area of biomacromolecules such as carbohydrates, lipids, lipoproteins, proteins, and nucleic acids. The useful spectral range stretches from the vacuum-UV to the near-IR. Recent innovations in the near-IR include vibrational optical activity (VOA), Raman optical activity (ROA), and vibrational circular dichroism (VCD). For technological reasons the development of instrumentation for the IR range postdated that for the UV-visible by almost 30 years. The early promise of a greater sensitivity to modeling structural parameters in the IR has already been fulfilled. At the other extreme of the electromagnetic spectrum, the first CD spectra have been measured in the range of soft X-rays. It has been speculated that the “holy grail” of structural analysis, namely the indisputable determination of the *absolute* molecular conformation of a chiral molecule, might at last have been discovered.

In the area of proteins, for which the literature is by far the greatest, the focus of CD has been the discrimination among various bulk or three-dimensional-solution structural forms of globular proteins, for example, α-helix and β-sheets, and how to calculate the proportions of each in the structure for a particular macromolecule. Studying the bulk solution properties of other macromolecules is a major ongoing

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effort, as is the investigation of the particular details of the stereochemical changes that are involved in the binding of small molecules (ligands) to large biomolecules.

Analytical Determinations

In spite of the fact that it was identified as a priority area as early as 1963, the application of CD to qualitative and quantitative analysis has just reached adolescence in the last 15 years. Methods developments took off in two general directions: (a) direct analysis using CD detection and (b) the use of polarimetry and CD as detectors in the separation sciences.

Direct CD Detection

In this context direct, analysis means that no prior sample workup is done, which puts a greater demand on the selectivity capabilities of the detector. CD brings more selectivity to the table than any other method that is based upon electronic spectral data. If a mixture is to be analyzed for a particular CD-active analyte, the only potential interferences are other CD active constituents. Substances that absorb but are not chiral and substances that are chiral but do not absorb do not interfere. Added to this is the fact that CD spectra offer better “resolution” than other methods because positive and negative bands are possible.

The greatest hindrance to direct CD analysis is the presence of strong absorbers in the substrate, which adversely affect the signal-to-noise quality of the transmitted signal because of the need for excessive electronic pre-amplification. The problem is minimized by operating at wavelength ranges longer than 240 nm, which is roughly the upper limit of the most intense $\pi \rightarrow \pi*$ transitions. Results from numerous direct determinations that employed intrinsic as well as extrinsic CD detection are summarized in the works of this author. Illustrative of these are the determinations of opium alkaloids in unseparated poppy extracts; S-nicotine in tobacco leaf extracts; cannabinoids in marihuana leaf extracts; vitamin C in fruits and vegetables; cocaine, heroin, and LSD in unseparated confiscated street drug forms; quinine and quinidine diastereoisomers; carbohydrates complexed with linear dye molecules; and di- and tripeptides complexed with chiral complexes of Cu(II) ion.

The spectroscopic methods recommended for measuring enantiomeric purity or enrichment use either multiple-wavelength-polarimetry detection or CD detection. Data analyses are done using multivariate methods such as principal-component

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analysis, or principal-component regression, for which software programs are both readily available and user-friendly. For the four ephedrines, enantiomeric purities were measured at better than 99.0% accuracy for prepared mixtures of reference materials using both chiroptical detectors. A reason for preferring CD over polarimetric detection is that sample-size requirements are smaller by a factor of ten, unless a laser source is used in the polarimetric method.

Chiroptical Detection in Separation Sciences

Enantiomers have identical retention times on achiral stationary phases. They are separable if they are first derivatized to diastereoisomers in reactions with a third common chiral species. The derivatizing species is either included in the mobile phase, or it is adsorbed on or is an integral part of a chiral stationary phase. Given the assumption that a mixture that is wholly or partially racemic is completely separated in enantiomeric terms, conventional detectors can be used if the order of enantiomeric elution is known. Of the two chiral detector choices, polarimetry has the broadest range of application because absorbance is not a prerequisite. The relative insensitivity of polarimetry, however, makes laser source illumination necessary.

As a single-wavelength detector CD is just an expensive polarimeter that is limited to chiral absorbing species, although it does have a selectivity advantage if coelution is unavoidable. Tandem single-wavelength detection, for example, absorbance and CD arranged in series, was the earliest experimental setup used to measure enantiomeric ratios or purities. In this configuration the absorbance signal is proportional to the sum of the enantiomer concentrations, and the CD signal is proportional to their difference. Simultaneous solution of the two equations gives the enantiomeric ratio. When one enantiomer is in very large excess over the other, the necessary arrangement is to elute the lesser component first. Compared to multivariate CD and polarimetric spectroscopic methods, the errors in measured enantiomeric purities are unacceptably high (~200%) for mixtures that are above a 90% enantiomeric excess.

The emphasis of CD has always been to extort structural information from reluctant molecules. This will continue as the focus is directed more and more towards the spectral fringes of the electromagnetic spectrum, namely, the IR, far-UV, and soft X-ray regions. The technological advances that are needed to make data collection more routine will be developed in relatively short order, and CD could reach a status that is almost equivalent to NMR. The methods however will be complementary rather than

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competitive because they operate on different time scales. As an analytical detector, the future of CD is as assured as that for absorbance spectrophotometry.

FURTHER READING

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