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Vibrational circular dichroism: Chiroptical analysis of biomolecules

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Introduction

General aspects

Chirality is a fundamental feature of all living organisms at the molecular level as well as on the macroscopic scale. The high degree of preference for only one of two possible mirror images in nature, such as L-amino acids or D-sugars, often called biological homochirality, is a puzzling and not yet fully understood phenomenon [1]. The chirality of some known compounds can be "felt" by smelling or tasting small amounts of them. As often exemplified by the sodium salt of glutamic acid or the hydrocarbon limonene, many chiral odorants or flavor compounds cause different sensory properties depending on their stereochemistry. This is because the receptors are proteins, constituted of chiral amino acids, which interact with enantiomers to form diastereomeric molecular complexes that result in various smells and tastes [2]. Stereospecific differences have more serious consequences in pharmacology, as shown in studies on enantiomeric pharmaceuticals including thalidomide or ibuprofen [3, 4].

The use of a chiroptical technique, except for X-ray crystallography, comprise the sole method to nonempirically determine the absolute configuration of a compound [5]. X-ray scattering based on the Bijvoet method has been quite effective in determining chirality, but the requirement that samples be in crystalline form has frequently limited its applicability. On the other hand, the chiroptical methods have an advantage of an accessibility to the samples in a solution. Recently, a new chiroptical method, vibrational circular dichroism (VCD) spectroscopy was developed. VCD measures the circular dichroism in the infrared region usually between 4000 and 750 cm^{-1}. Since the advent of the first commercial VCD instrument in 1997, VCD is becoming one of the most powerful and convenient analytical tools for natural products and biomolecules due to the abundance of spectral information including some well-isolated peaks in the 1800–1500 cm^{-1} region. In this paper, we describe some fundamental VCD concepts, instruments, and applications.

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Abbreviations: ECD, electronic circular dichroism; **IR**, infrared; **VCD**, vibrational circular dichroism

Theory of VCD

Chiral molecules respond differently to left versus right circularly polarized radiation due to diastereomeric interaction. The measurements of this differential interaction relate to what is known as optical activity. The most familiar measurements are optical rotation, or optical rotatary dispersion when measured as a function of wavelength, and electronic circular dichroism (ECD). The electronic transitions of a chiral molecule give rise to circular dichroism (CD) in the visible ultraviolet (VIS-UV) spectral region. ECD spectrometers have been widely available for routine measurements since the early 1960s, whereas the measurement of CD in vibrational transitions, *i.e.*, VCD, has been available only for a limited number of researchers until the late 1990s.

The relationship between VCD and infrared (IR) absorption is the same as that between ECD and UV absorption (Fig. 1). VCD measures the differential absorption of left *versus* right circularly polarized IR incident light in the molecular vibrational transition.

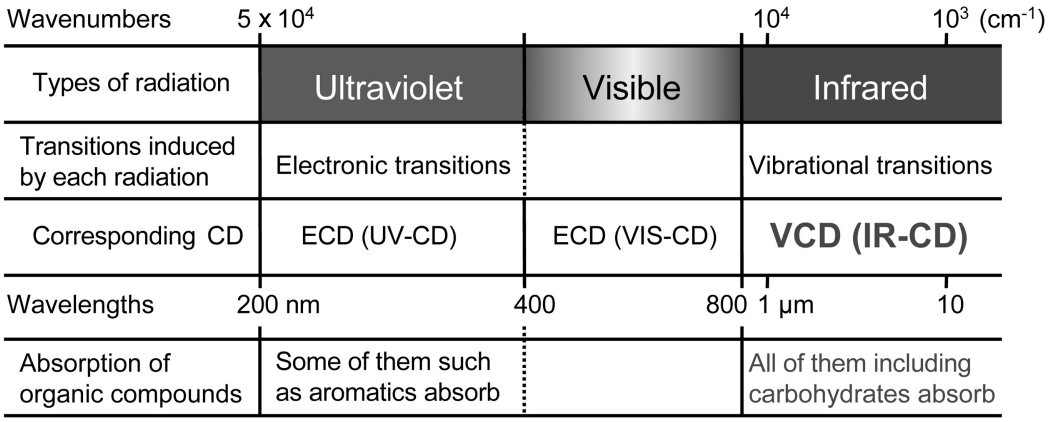

Figure 1. Type of radiation and corresponding CD. VCD is considered as an extended version of conventional ECD. The advantage of VCD comes from the spectroscopic detail of IR with the stereochemical sensitivity of CD.

$$\Delta A = A_L - A_R \quad (1)$$

where A_L and A_R are absorbances for left and right circularly polarized radiation, respectively. The ordinary IR absorption, A, is given by the average of A_L and A_R, namely,

$$A = 1/2\,(A_L + A_R) \quad (2)$$

and

$$A = -\log_{10}(I/I_0) \quad (3)$$

where I and I_0 are the average transmission with and without the sample. Assuming the Beer-Lambert law is valid and in the case when the pathlength and concentration are known, VCD can be expressed in terms of the difference in molar absorptivity as

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R = \Delta A/cl \quad (4)$$

where c is the molar concentration and l is the pathlength in cm. In this paper, measured VCD and IR spectra are expressed by $\Delta\varepsilon$ and ε, respectively.

The anisotropy ratio, g, the quotient of the intensity of VCD to that of IR, is another significant parameter which is also specified in publications.

$$g = \Delta\varepsilon/\varepsilon = \Delta A/A \quad (5)$$

The anisotropy ratio g, which is meaningful between ~10^{-3} to 10^{-5}, could be a criterion for judging whether a vibrational mode can cause large or small VCD absorption. For instance, C=C=C antisymmetric stretching of a chiral allene shows strong VCD at around 1945 cm^{-1} in spite of relatively small corresponding IR absorbance (g = ~10^{-3}) [6], while C=O stretching of 3-hydroxyoxindole at around 1730 cm^{-1} has intense IR absorbance but small VCD (g =~10^{-5}; see Fig. 3). The question of whether a vibrational mode exhibits large VCD or not is ultimately ascribed to the magnitude and direction of both electronic- and magnetic-dipole transition moments [7, 8]. The anisotropy ratio g is also important in terms of analytic calculations because it relates theoretical spectra to experimental ones by the following equation:

$$g = \Delta A/A = 4R/D \quad (6)$$

where R and D are called rotatory strength and dipole strength, respectively. For a small molecule, both R and D can be calculated using Gaussian 98 or 03 [9]. A more complicated theoretical background of VCD is not addressed here, but more detailed descriptions are offered in other publications [7, 8].

Experimental methods

There are currently a few commercially available factory-aligned Fourier transform (FT)-VCD instruments. Chiralir, released 1997 from Bomem/BioTools, Inc., is the most widespread of these. Also, JV-2001, provided by JASCO Co. since 2001, has been popular. Both VCD optical systems can be described by the same simple block diagram (Fig. 2). In both systems, IR radiation from a light source first directs through a Michelson interferometer and then passes through an optical filter to isolate the spectral region of interest. The IR beam continues through a linear polarizer that defines a single state of polarization and then on to a ZeSe photoelastic modulator (PEM) that modulates the polarization between left- and right-circularly polarized states. A chiral sample in an appropriate IR sample cell is placed directly after the PEM and the beam is focused with

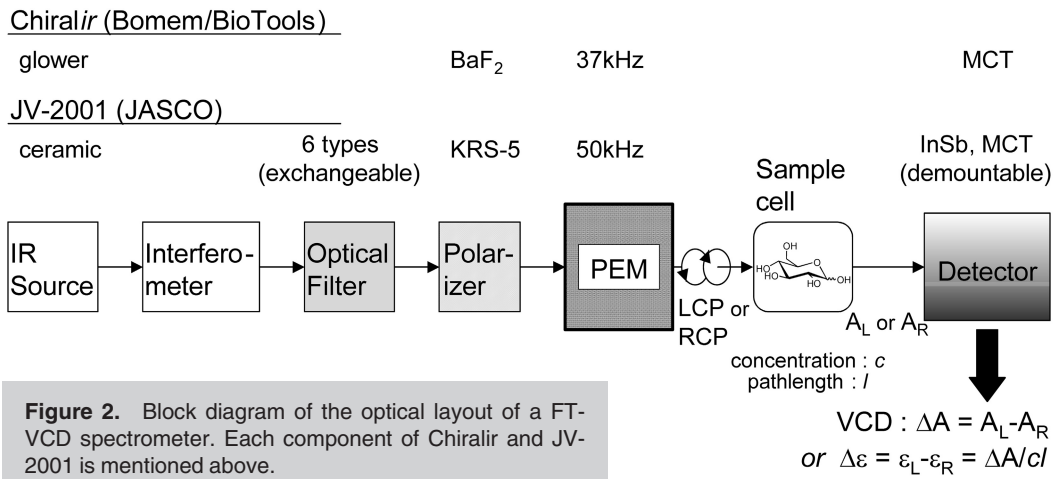

Figure 2. Block diagram of the optical layout of a FT-VCD spectrometer. Each component of Chiralir and JV-2001 is mentioned above.

a lens to a liquid nitrogen-cooled detector. The detector signal is processed by subsequent electronics to yield IR and VCD spectra. In both spectrometers, detector sensitivity (or polarizer transmittance) prohibits measurements in the spectral region below 750 cm^{-1}. Besides these two VCD instruments, other companies (*e.g.*, Bruker) provide VCD accessories to be used in conjugation with FT-IR spectrometers.

To examine the accuracy of a VCD instrument, optically pure α-pinene or a CCl_4 solution of camphor are widely used as standards because their VCD spectra can be obtained at high signal-to-noise (S/N) ratios in relatively short scan times. The latter is particularly a good standard for the hydrogen stretching and the carbonyl stretching region.

If a pair of enantiomers does not adequately exhibit sufficiently opposite VCD spectral patterns, a raw VCD can be corrected by the VCD spectrum of its enantiomer. A half-difference spectrum is also calculated simply to obtain a more accurate VCD spectrum:

$$\Delta A(S)_{\text{corrected}} = 1/2\,[\Delta A(S)_{\text{raw}} - \Delta A(R)_{\text{raw}}] \quad (7)$$

However, enantiomer correction can be performed only in the case where both enantiomers are available. Therefore, most sugars and proteins are inaccessible by this approach. An alternative and general way to correct a spectrum is solvent correction where the solvent spectrum is subtracted from the raw sample spectrum:

$$\Delta A_{\text{corrected}} = \Delta A_{\text{sample}} - \Delta A_{\text{solvent}} \quad (8)$$

Unless the sample is neat liquid, an IR spectrum also has to be corrected for the IR spectrum of the solvent.

To obtain a VCD spectrum with higher accuracy in the desired wavenumber region, a solvent should be carefully selected as well as concentration, sample cell, and instrumental conditions. CCl_4 or $CDCl_3$ have been widely used for small chiral molecules but they are not suitable for most biomolecules that are intrinsically hydrophilic. Instead, water, deuterium oxide or DMSO-d_6 have been frequently chosen to analyze biomolecules. These solvents might form hydrogen bonds with the sample molecules and then affect the VCD shapes, in part, by altering sample conformation. Such solvent contribution is not always undesired and, in practice, many biological molecules have been measured using such solvents [10]. However, in case the solvent contribution must necessarily be supressed, VCD can be measured in the solid state. Thus, some solid state VCD spectra for peptides have been measured in halocarbon oil [11], and VCD spectra for carbohydrates in KBr were recently reported [12].

When water or deuterium oxide is selected as a solvent, which is uncommon in a normal IR measurement, an ordinary NaCl or KBr sample cell must be avoided. This is typically replaced by a CaF_2 or BaF_2 sample cell. Though a little expensive, these are used in almost all VCD measurements. CaF_2 windows preclude measurement below 1000 cm^{-1}. However, BaF_2 affords a wider spectrum up to ~750 cm^{-1} (see Fig. 5) but care must be taken regarding storing and handling because of the slight deliquescence of BaF_2. In fact, solubilities of NaCl, BaF_2, and CaF_2 in 100 g H_2O at 0°C are 35 g, ~0.2 g, and ~0.002 g, respectively.

IR absorbance adjusted to around 0.4 is optimal for VCD measurement, which will need a sample on the mg order. The high sample requirement is unfavorable for the measurement of rare biomolecules. Another disadvantage of VCD is, due to the small magnitude of VCD signals, the necessity for longer collection time, usually 1 h or more. The aforementioned α-pinene is an exception. It offers VCD spectra in sufficiently high S/N ratios even in only 5 min scanning time. The problem of low S/N is being improved through new instrumental developments [13].

Determination of absolute configuration

Since the first VCD measurements reported for crystalline samples in 1973 [14], and for neat liquids in 1974 [15], VCD has been applied in many fields. Absolute configuration determination of synthetic or natural compounds may be the most common application. Similarly, VCD can predict an enantiomeric excess in pharmaceutical samples [16]. Figure 3 shows an example of the determination of the absolute configuration of natural products, where two cruciferous phytoalexin-related metabolites, dioxibrassinin (**1**) and 3-cyanomethyl-3-hydroxyoxindole (**2**), were studied [17]. 3-Hydroxy-3-methyloxindole (**3**) was prepared as a model compound. Note that the VCD spectra of a pair of enantiomers are mirror images of each other. Generally, to determine the absolute configuration of a compound, an enantiomerically pure sample needs to be prepared and its spectrum measured. Also, the theoretical VCD spectrum has to be calculated for one or more lowest energy conformer(s) derived by conformational analysis. If two or more conformers are considered, the final spectrum is created by superposition of the Boltzmann weighted populations. Conformational analysis must be performed with great care, and one must strictly avoid overlooking or disregarding an energetically lower conformer since even small conformational differences may dramatically change the VCD features. With respect to this point, VCD has been utilized to analyze not only configuration but also conformation. Conversely, the requirement to analyze multiple conformers makes it difficult to calculate the VCD spectrum of a conformationally flexible molecule. In the case of a flexible compound, the superposed VCD spectrum of several lower energy conformers, the sum of Boltzmann weights of which covers over 95% of those of all conformers will afford better agreement between a calculated spectrum and the observed one. The stereochemistry of a calculated molecule is arbitrarily selected as (*R*) or (*S*) before computation. Finally, a calculated VCD spectrum is compared with the observed one. The correspondence of the sign and frequency pattern between an observed spectrum and the theoretical one or its mirror image indicates that the stereochemistry of the measured compound is the same as the theoretical one or is its opposite, respectively.

In the study of Fig. 3, all spectra were calculated at the B3LYP/6-31G(d,p) level using Gaussian 98 arbitrarily for the (*S*)-enantiomers. The observed spectra of **1**, **2**, and **3** have three characteristic bands near 1730, 1620, and 1470 cm^{-1}, which are also exhibited by the calculated spectra. Signs of these three bands showed reasonable agreement between the observed and calculated VCD with slight difference of their wavenumber frequencies. These comparisons allow to conclude that the absolute configurations of **1** and **2** are both *S*. This result indicated that VCD could be useful in determining the absolute stereochemistry of tertiary alcohols.

Stereochemical analysis is very helpful for biosynthetic pathway studies and biological activity investigations. Thus, VCD can be applied to these studies. The effectiveness of VCD is demonstrated by its application to investigations of natural products like gossypol, a polyphenolic terpenoid aldehyde found in cotton plants [18], or to synthetic molecules like mirtazapine, a well-known pharmaceutical ingredient with antidepressant therapeutic effect [19].

Peptides and proteins

VCD has also been used for the conformational analysis of peptides and proteins in an empirical manner. The secondary structure of a peptide or protein, such as α-helix, β-

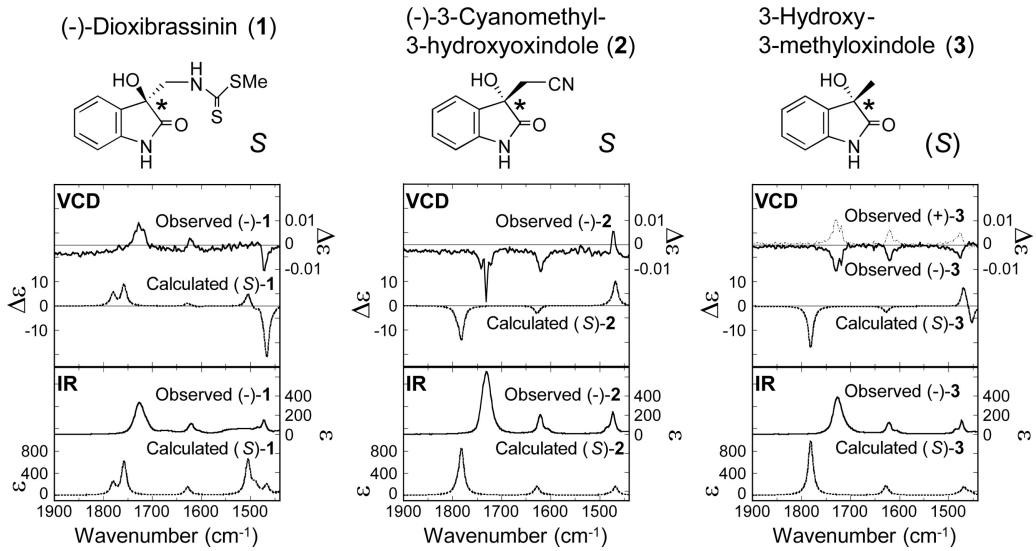

Figure 3. Comparison of observed IR and VCD spectra of two phytoalexin-related metabolites (−)-**1**, (−)-**2** and a model compound (−)-**3** with their calculated spectra.

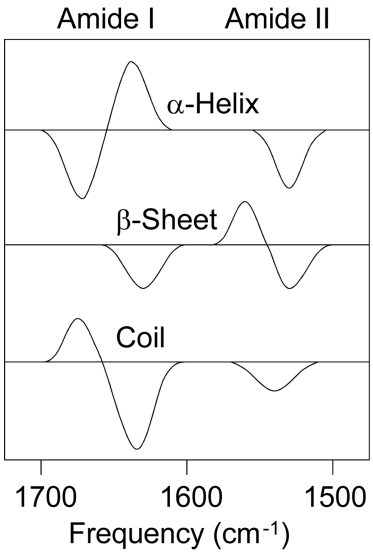

Figure 4. Model VCD shapes of α-helix, β-sheet, and random coil structure in the amide I and II regions depicted from hitherto reported data. According to the peptide sequence, some sample will have an additional peak or show weaker features. A deuterated sample exhibits different VCD patterns.

sheet, or random coil, is reflected in the VCD shape of the amide I (C=O stretch) and the amide II (N–H deformation and other vibration) regions (Fig. 4).

A VCD spectrum of a right-handed α-helical structure was first reported for the polypeptide poly(benzyl-L-glutamate) in $CHCl_3$ solution [20] exhibits a positive VCD couplet (a bisignate VCD couplet with a positive VCD and then a negative VCD with increasing wavenumber) in the amide I region and a small negative VCD in the amide II region. These VCD features of an α-helical structure can be observed in a protein containing a high fraction of α-helix such as hemoglobin [21].

A β-sheet structure exhibits distinctly different VCD features from an α-helix especially in the amide I region, where a β-sheet has a small negative VCD peak near 1600 cm^{-1}. In the amide II region, a VCD spectrum of a β-sheet often exhibits a negative VCD couplet (the opposite of a positive VCD couplet) with relatively small intensity. Concanavalin A, which has a high β-sheet content with almost no helical structure, exhibits a VCD band shape as shown in Fig. 4 [21, 22]. However, some of the β-sheet forming oligopeptides could assume a different spectral character particularly in the amide II region.

A random coil is an interesting case for VCD studies. In the amide I region the VCD spectrum has a negative VCD couplet in which a stronger negative peak appears at lower wavenumber and a positive one appears at higher wavenumber. Also, a weak negative peak is observed in the amide II region. The feature in the amide I region is opposite to that seen in a right-handed α-helix. Accordingly, a "random coil" is assumed to contain a substantial amount of local left-handed structure [23]. Although there are some differences in the frequency, it is noteworthy that VCD features observed in a "random coil" are similar to the VCD spectra calculated for a left handed poly-L-proline type helix [22]. Additionally, calculated VCD spectra for α-helix, β-sheet, and 3_{10} helix reveal good agreement with observed ones.

One problem in measurement of a peptide or a protein in aqueous solution is the solubility of the sample. Because of the strong IR absorption of the solvent (H_2O around 1650 cm^{-1}), the pathlength must be reduced to ~6 μm. Therefore, the sample solution needs to be prepared at a high concentration. Also, in some cases, the solvent IR absorption itself may interfere with a highly accurate measurement in the amide I region. D_2O provides an alternate possibility for measuring peptides or proteins at a lower concentration because a pathlength of 80 μm or more is acceptable. If D_2O is used, a sample should be doubly lyophilized following dissolution in D_2O before the VCD measurement to avoid expected noise caused by H–D exchange during the measurement. Deuteration confers slightly different spectral shapes than those observed for a non-deuterated sample. For example, a deuterated α-helical structure exhibits a three peaked negative-positive-negative pattern in the amide I′ (C=O stretch of a deuterated sample) region. In many cases, a VCD spectrum in the amide II′ region of a deuterated peptide or protein exhibits characteristically weak features [24].

Conventional ECD has been a well-established and widespread technique for the analysis of the secondary structure of peptides or proteins in solution. In contrast, VCD data contain additional information relative to ECD data since the VCD is dominated by local chirality while ECD reflects global chirality for the sample of interest. In light of this, VCD plays a complementary role to ECD in peptide and protein conformational analyses.

Carbohydrates

Carbohydrates are important in synthetic research and industrial chemistry as "chiral pools". Also, carbohydrates in the form of glycoproteins or glycolipids play a significant role in various biological phenomena investigated in the field of glycobiology [25]. However, in many cases, carbohydrate analysis is a laborious task due to the complex nature of carbohydrates. Therefore, several methods including NMR, MS and HPLC have been complementarily combined to study complex carbohydrate sequence, configura-

tion, conformation and composition. Yet, further development of analytical methods is still greatly desired. Irrespective of their chirality, most of carbohydrate analyses are based on achiral theory, and chiral approaches have been of limited use. Optical rotation and optical rotatory dispersion provide indirect information about configuration and little information about conformation. ECD would be more informative, but multistep chemical derivatization to add chromophores is required since the electronic transitions of most carbohydrates, excepting acid or amino derivatives, occur below 185 nm [26]. However, carbohydrates have well defined IR absorptions. Therefore, detailed stereochemical information can be extracted by VCD. Furthermore, VCD is very promising as an analytical tool because the parent IR spectra of various carbohydrates from monosaccharides to polysaccharides have already been measured to analyze their configuration and conformation.

So far, only a few VCD applications for carbohydrates have been reported in contrast to the more widely studied peptides and proteins. Most of the published papers deal with monosaccharides solely in the mid-IR region [12, 27 – 30] or in the hydrogen stretching region [31, 32]. VCD spectra for some disaccharides in the mid-IR region appear in two publications including one review publication [33, 34]. The two publications also analyzed maltotriose and α-cyclodextrin. Observations of interactions between cyclodextrins and molecules or ions represent another area of interest [35, 36]. Nucleic acids and glycoproteins also contain a sugar moiety, but their VCD studies are not listed here. Except for such glycoconjugates, all these studies analyzed only simple sugars, and therefore available VCD peaks were concentrated at wavenumbers of 3800 – 2800 cm^{-1} and below 1550 cm^{-1} (Fig. 5). Actually, reported carbohydrate VCD spectra were restricted to 3100 – 2800 cm^{-1} for stretching vibrations of single C – H bond [31, 32], and restricted to 1550 – 900 cm^{-1} for stretching vibrations of single C–O and C – C bonds and for bending vibrations of various bonds [27 – 29, 33, 34]. In particular, numerous absorptions were observed below 1550 cm^{-1}, but most of these showed weak and broad features. To amplify VCD intensity and to extend the available spectral region, substitutions of hydroxyl groups could be effective. Polavarapu *et al.* [30] concluded that acetylated monosaccharides exhibited significant VCD peaks at ~1750 cm^{-1} (C=O stretches) and ~1370 cm^{-1} (bending of methyl groups). We also observed that benzoyl or other carbonyl-containing groups intensified the representative VCD signal about 10 times, but that other substituents such as benzyl groups did not result in a similar effect (unpublished data). Probably the introduction of carbonyl-containing groups constitute another possibility to analyze carbohydrates by VCD. Also, deuteration of hydroxyls by lyophilization from D_2O can afford different useful VCD spectral features [28].

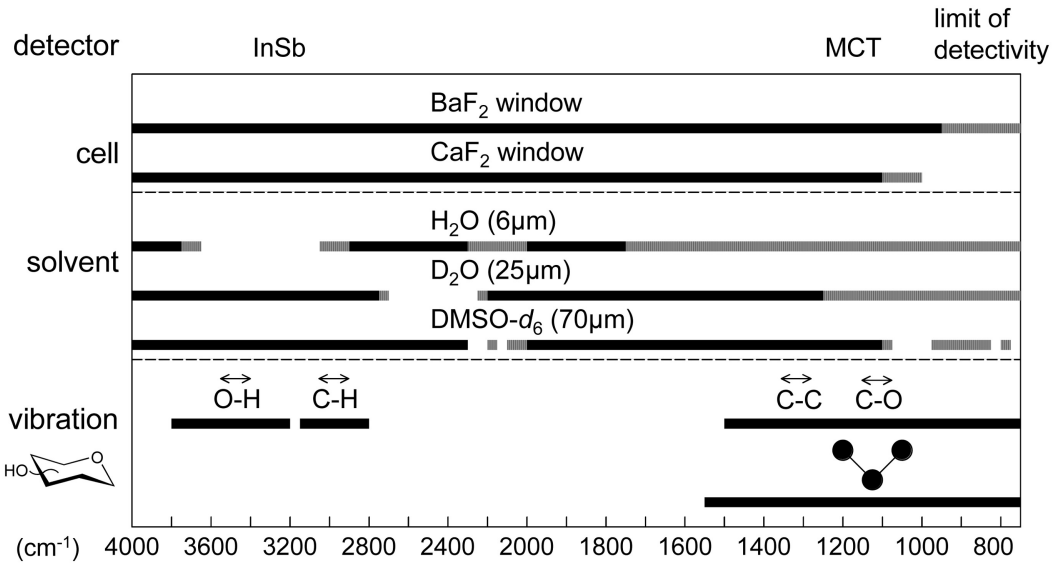

Figure 5. IR absorption of simple carbohydrates and the measurement conditions. Detector: InSb detector and MCT detector have to be adopted for the measurement of the hydrogen stretching region and the fingerprint region, respectively. Cell: representative two cell windows are shown. The solid lines indicate the available spectral region but the cell windows have a little absorption in the dotted zones. If a data in the dotted region is required, a solvent absorption has to be reduced as far as possible. Solvent: representative three solvents, which are sufficiently polar to dissolve a carbohydrate sample, are shown. They have little or no absorption in the solid lines but an extension of pathlength may preclude VCD measurement in the dotted regions. Vibration: simple carbohydrates show IR absorption, therefore VCD signals, in the regions displayed by the solid line. Many complicated vibrations exist in the fingerprint region.

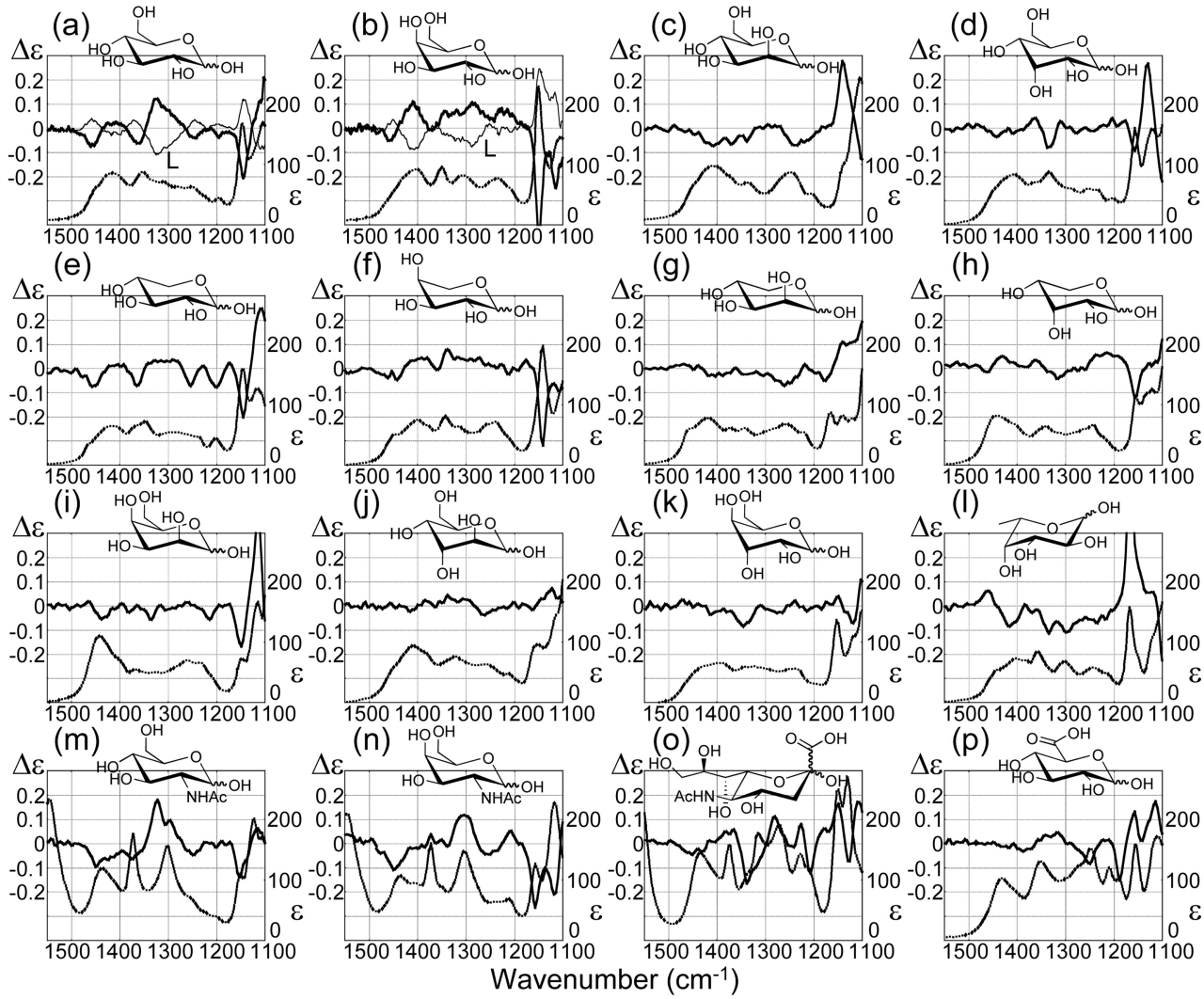

Figure 6. IR (dotted) and VCD (solid) spectra in DMSO-d_6 (*c* 0.16, *l* 0.72 μm) of (a) D- and L-glucose, (b) D- and L-galactose, (c) D-mannose, (d) D-allose, (e) D-xylose, (f) L-arabinose, (g) D-lyxose, (h) D-ribose, (i) D-talose, (j) D-altrose, (k) D-gulose, (l) L-fucose, (m) 2-acetamide-2-deoxy-D-glucose, (n) 2-acetamide-2-deoxy-D-galactose, (o) *N*-acetyl-D-neuraminic acid, and (p) D-glucuronic acid. The data collection time was 3 h. The resolution was 4 cm^{-1} for (a) and (b), and 8 cm^{-1} for others.

Some hitherto reported VCD spectra exhibited various patterns consistent with the presence of sugar species but structure prediction from spectra has been difficult. This is possibly due to paucity of VCD data resulting from inavailability of appropriate carbohydrate samples. Aiming at creating a novel methodology for carbohydrate analyses, a comprehensive VCD database on carbohydrates is being constructed by our group on the basis of knowledge about organic synthesis, enzymatic synthesis and biological manipulation.

To construct an extensive VCD database, we began by measuring the spectra for various reducing sugars. Excerpted spectra from the the database of free monosaccharides is shown in Fig. 6. Monosaccharides **6(a)**–**6(k)** are systematically gathered aldohexoses and aldopentoses. **6(a)**–**6(c)**, **6(e)**, **6(f),** and **6(l)**–**6(p)** are biologically important sugar units from mammals and plants [25]. One can observe that the inversion of even one chiral center offers a significantly different spectrum. This property is advantageous to discriminate carbohydrate families that are composed by several epimers. An acetoamido sugar, an uronic acid, and a sialic acid provide additional VCD peaks that are absent in the spectra of simple sugars. Any carbohydrate might be identifiable using the database once it has been suitably enlarged. Extended study of carbohydrate structure will benefit from further analyses using these VCD methods.

Concluding remarks

VCD, a new chiroptical spectroscopy method, has recently undergone rapid instrumental and theoretical development to become one of the most efficient analytical tools available to study the chirality of small molecules as well as macromolecules. As we have illustrated in the examples including plant metabolites, proteins, and carbohydrates, VCD is a useful technique for analyses of natural products. This paper highlights the application of VCD methods to the structural study of carbohydrates. The scope of applications for VCD has expanded to include analysis of biomacromolecules from proteins to nucleic acids. Although a requirement for relatively large sample quantity or long scannig time currently limit VCD studies, especially for rare or labile natural compounds, further development of VCD instrumentation, methods, and compound databases holds the potential to overcome current limitations in approaching some VCD analytical applications and will likely increase popular use and accessability to these techniques.

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