Determination of Enantiomeric Compositions of Amino Acids by Near-Infrared Spectrometry through Complexation with Carbohydrate

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A new method has been developed for the determination of enantiomeric compositions of a variety of compounds. The method is based on the use of the near-infrared technique to measure diastereomeric interactions between an added carbohydrate compound with both enantiomeric forms of an analyte followed by partial least-squares analysis of the data. The fact that the method works well with all three macrocyclic carbohydrates with different cavity size (i.e., α-, β-, and γ-cyclodextrin) as well as with sucrose, which is a linear carbohydrate, clearly demonstrates that it is not necessary to have inclusion complex formation in order to produce effective diastereomeric interactions. Rather, a simple adsorption of the analyte onto a carbohydrate is sufficient. Since inclusion complex formation is not a requisite, this method is not limited to the amino acid studies here but is rather universal and sensitive as it can, in principle, be used to determine enantiomeric compositions for all types of compounds with only microgram concentration and enantiomeric excess as low as 1.5%, in water or in a mixture of water and organic solvent. Furthermore, it does not rely on the use of rather expensive carbohydrates such as cyclodextrins but is equally as effective even with a simple and inexpensive carbohydrate such as sucrose.

Analysis of chiral compounds is an important subject in science as well as in technology. Enantiomeric forms of many compounds are known to have different physiological and therapeutic effects. Very often, only one form of enantiomeric pair is pharmacologically active. The other or others can reverse or otherwise limit the effect of the desired enantiomer. However, despite this knowledge, only 61 of the 528 chiral synthetic drugs are marketed as a single enantiomer while the other 467 are sold as racemates. Recognizing the importance of chiral effects, the FAA in 1992 issued a mandate requiring pharmaceutical companies to evaluate effects of individual enantiomers, as well as to verify the enantiomeric purity of chiral drugs that are produced. Furthermore, even if the drug is to be marketed as a single enantiomer, the pharmaceutical properties and toxicity must be established for both enantiomers. It is thus hardly surprising that the pharmaceutical industry needs effective methods to determine enantiomeric purity and chiral separations.

Currently, methods used to determine enantiomeric purity are based on either separation or spectroscopic techniques. Separation-based methods include HPLC, GC, and CE. Circular dichroism, NMR, and MS are some of the spectroscopic methods that are widely used. While these methods have proven to be effective, they all have some drawbacks including being time-consuming (separation-based methods), requires adding of reagent(s) (NMR), destructive (MS), and relatively low sensitivity (circular dichroism). More importantly, none of them is truly universal; namely, they cannot be used for all types of compounds. A truly spectroscopic method that is capable of quickly and sensitively determining enantiomeric purity of all types of compounds is, therefore, needed. A near-infrared (NIR) technique can offer a solution for this problem.

NIR spectrometry has been used extensively in recent years for chemical analysis and characterization. The popularity stems from the advantages of the technique including its wide applicability; namely, the NIR region covers the overtone and combination transitions of the C-H, O-H, N-H, and C=O groups, and since all organic and most inorganic compounds possess at least one or more of these groups, they all absorb NIR light and hence can be detected by an NIR technique. Additionally, NIR light can penetrate a variety of materials and there is no need for pretreatment of samples for NIR measurements. As a consequence, the NIR technique is noninvasive and nondestructive and has real-time and on-line capabilities. A chiral analysis method based on the NIR technique is therefore desirable as it is universal and


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can be used for all types of compounds. Unfortunately, in spite its potentials, to date, an NIR-based chiral analysis method has not been developed.

To facilitate analysis of a chiral compound, the addition of a chiral reagent (selector) to promote diastereomeric interactions between the reagent and two optical isomers of the enantiomeric analyte is required. A variety of chiral reagents have been developed and successfully used for chiral analysis.\(^2\)–\(^{15}\) However, all of the reported methods are based on analysis of changes in either the UV or the visible spectra of analytes upon addition of chiral reagents.\(^2\)–\(^{15}\) As a consequence, not only that they are based on the use of rather expensive chiral reagents but also are limited to reagents or analytes that absorb in the UV or visible region, or both. Such limitation can be ameliorated by replacing UV–visible with the NIR technique. This is because, in principle, the NIR technique can be used to detect any changes induced by interactions between analyte and added chiral reagent, regardless of the types of analytes and reagents. In fact, we have recently demonstrated that adding chiral macrocyclic compounds such as cyclodextrins (CDs) into solution of various substrates led to changes in NIR absorption spectra of the latter and such changes can be used to determine binding constants between the CDs and the substrates.\(^12\)–\(^{18}\) More importantly, the NIR results indicate that this method is so sensitive that it is not limited to complexes formed by inclusion complex formation between CDs and analytes but rather to all types of complexes formed by other interactions including electrostatic interactions.\(^17\)–\(^{18}\)

The information presented is indeed provocative and clearly demonstrates that diastereomeric interactions between both enantiomeric forms of an analyte with either a macrocyclic compound such as cyclodextrin or a linear compound such as sucrose should produce changes in NIR spectra. The chiral composition of the analyte can then be determined by analyzing the spectral changes. Such consideration prompted us to initiate this study, which aims to develop the first universal chiral analysis based on synergistic use of the NIR technique to measure diastereomeric interactions of both enantiomeric forms of an analyte with added cyclic or linear sugar (cyclodextrins or sucrose), followed by analyzing the changes in the NIR spectra using a multivariate method for enantiomeric purity. Preliminary results on chiral analysis of various amino acids together with possible mechanism for chiral recognition will be reported in this article.

### EXPERIMENTAL SECTION

All d- and L-amino acids (alanine, valine, leucine, isoleucine, phenylalanine, cysteine, tryptophan) were purchased from either Sigma-Aldrich or Alfa Aesar and were used as received. α-, β-, and γ-cyclodextrins were a gift of Cargill Corp. (Hammond, IN). NIR spectra were taken on the home-built NIR spectrometer based on an acousto-optic tunable filter. Additional information on this NIR spectrometer is detailed in our previous papers.\(^17\)–\(^{20}\) Normally, each spectrum of a sample (in 5-mm-path length cell) was an average of 50 spectra taken at 1-nm intervals from 1450 to 2450 nm. The standard deviation of the absorption spectrum calculated from an average of 12 spectra was found to be \(1.1 \times 10^{-3}\).

Multivariate analysis of data was performed using Unscrambler version 8.0 (Camo ASA) similar to the procedures previously reported.\(^19\)–\(^{20}\)

### RESULTS AND DISCUSSION

The NIR spectrum of a solution of 170.0 mM of L-Phe in water, taken using a 0.1-mm-path length cell, is shown as solid line in Figure 1. The spectrum with the dashed line is that of water taken in the same cell. As illustrated, absorbance of Phe in this region is not only very small but also is very similar to that of water. For clarity, the Phe spectrum was remeasured in two regions where amino acids are known to absorb: 1600–1800 and 2100–2400 nm using a cell with a relatively longer path length (5 and 1 mm for the 1600–1800 and 2100–2400-nm regions, respectively). The spectra obtained, with background absorption of water subtracted, are shown as insets A and B in Figure 1. While it is relatively difficult to completely and unambiguously assign these bands, based on our previous study\(^9\)–\(^{10}\) and others,\(^12\)–\(^{22}\) the band at 1689 nm and the shoulder at \(\sim 1750\) nm can be assigned to the overtones and combinations of the \(\text{C–H}\) groups; the binary combinations of vibrations of \(-\text{NH}_2^+\) and \(\text{C–H}\) groups may be responsible for the bands at 2154, 2175, and 2296 nm.

It was found that adding α-CD to a solution of Phe led to changes in the position and bandwidth of Phe absorption. Since α-CD is chiral, it is expected that its interactions with L-Phe will be different from those with d-Phe. Accordingly, a set of 12 solutions of 100.0 mM Phe in 200.0 mM α-CD were prepared. The enantiomeric compositions of these solutions are listed in Table 1. As listed, each of these solutions has the same total Phe concentration (100 mM) but with different enantiomeric composition. Figure 2A shows the NIR spectra of these solutions (with background absorption of water and α-CD subtracted). It is interesting to observe that changing the enantiomeric composition of Phe led to changes in its absorption spectrum. The magnitude

of these changes is, as expected, relatively small. However, they are certainly larger than experimental error. This conclusion was made by comparing the spectra shown in panel A of Figure 2 with that in panel B, which shows an average spectrum together with standard deviations of sample 7, obtained from 12 measurements. As illustrated, the standard deviation is $\sim 1.1 \times 10^{-3}$, which is much lower than the variations among spectra in (A). The diastereomeric interaction between both enantiomeric forms of Phe and $\alpha$-CD can be observed by comparing spectra in (A) with those in (C), which shows absorption of a second set of Phe solutions that are identical to the first set (i.e., same total Phe concentration of 100 mM and different enantiomeric compositions as listed in Table 1) except that they are in pure water without any $\alpha$-CD. Since $\alpha$-CD was absent in the second set, it is expected that solutions in this set should not have any differences in their absorption spectra. The observed small differences among these spectra are similar to that shown in Figure 2B and are due to the experimental errors including drifts and instability of the NIR spectrometer. Clearly, the differences among spectra of Phe solutions with $\alpha$-CD (Figure 2A) are much larger than those without $\alpha$-CD (Figure 2C). They are, therefore, due to the diastereomeric interactions between $\alpha$-CD and both enantiomeric forms of Phe. It is evident from Figure 2A that spectra of Phe are dependent on the enantiomeric composition of the solution. There may be a correlation between the spectra and the enantiomeric composition of Phe in solutions. However, it was not possible to use univariate calibration for analysis because not only are the spectral variations small but also there are other effects including instability and drift of spectrometer. Accordingly, the multivariate method of analysis (i.e., partial least-squares method (PLS)) was used to develop calibration models for subsequent determination of enantiomeric purity of unknown samples. Calibrations were performed on NIR spectra of the 12 samples of 40.0 mM Phe in 80.0 mM $\alpha$-CD from 1555 to 1870 nm using the partial least-squares analysis and the full cross-validation method with the Unscrambler Chemometric software package (version 8.0). While these samples have the same enantiomeric compositions as those of 100.0 mM Phe in 200.0 mM $\alpha$-CD, whose spectra are shown in Figure 2A (enantiomeric compositions are listed in Table 1), their relative concentrations are 2.5-fold lower. Rather than using samples containing 100.0 mM Phe, lower concentration samples were used to demonstrate the sensitivity of the NIR technique. Results from the PLS cross-validation show that calibrations for 12 models require a relatively small number of factors for optimal performance (5 for L-Phe and 7 for D-Phe). The root mean standard error of prediction (RMSEP) values are 0.119 and 0.127 for L- and D-Phe while the standard error of prediction (SEP) values are 0.120 and 0.128 for L- and D-Phe, respectively. PC factors for this set of samples as well as for sets of other amino acids were found to be as low as 2 and as high as 7 with an average of of $\sim 5.0$.

To evaluate the effectiveness of this method, seven samples of Phe (in 80.0 mM $\alpha$-CD) with the same Phe concentration (40.0

Table 1. Compositions of Solutions Used for Calibration

<table>
<thead>
<tr>
<th>sample</th>
<th>mole fraction of L-amino acid</th>
<th>mole fraction of D-amino acid</th>
</tr>
</thead>
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<tr>
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<tr>
<td>12</td>
<td>0.90</td>
<td>0.10</td>
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</table>

Figure 2. (A) Spectra of a set of 12 solutions of Phe in 200 mM $\alpha$-CD. Each solution has the same total Phe concentration (100.0 mM) but with different enantiomeric compositions. Compositions of the solutions are listed in Table 1. (B) Background absorption by $\alpha$-CD and water was subtracted from the spectra. (C) spectra of a set of 12 solutions of Phe in water with the same Phe concentration (100 mM) but with different enantiomeric compositions. Compositions of the solutions are the same as those in (A). Background absorption by water was subtracted from the spectra.
but different enantiomeric compositions were prepared, and the concentrations of D- and L-Phe in each sample were calculated using the calibration models. Results obtained are shown in Figure 3A, where the calculated concentrations of D- and L-Phe in seven samples were plotted against actual concentrations (detailed information on enantiomeric compositions can be found in Table S1 in the Supporting Information). To illustrate the accuracy of the method, calculated concentrations of L-Phe (in seven samples) were plotted separately from those of D-Phe (of the same samples). As expected, the calculated concentrations for both L- and D-Phe

Figure 3. Predicted enantiomeric composition versus actual composition for 40.0 mM Phe in 80.0 mM α-CD. Filled circles, L-Phe; open circles, D-Phe. (A) Predicted L-Phe values were plotted separately from D-Phe with their corresponding plots of regression coefficients shown in the insets; (B) L-Phe and D-Phe were plotted together.

Figure 4. Predicted enantiomeric composition versus actual composition for 40.0 mM Phe in 80.0 mM concentrations of either β- or γ-CD (A and B, respectively) and of 40.0 mM Ala in 80.0 mM concentrations of either α-, β-, or γ-CD (C–E, respectively). Filled circles, L-amino acids, open circles, D-amino acids.
Figure 5. Predicted enantiomeric composition versus actual composition for 40.0 mM Leu in 80.0 mM concentrations of either α-, β-, or γ-CD (A–C, respectively) and of 40.0 mM isoleucine in 80.0 mM concentrations of either α-, β-, or γ-CD (D–F, respectively). Filled circles, L-amino acids; open circles, D-amino acids.

Figure 6. Predicted enantiomeric composition versus actual composition for 40.0 mM Val in 80.0 mM concentrations of either α-, β-, or γ-CD (A–C, respectively) and of 40.0 mM Cys in 80.0 mM concentrations of either α-, β-, or γ-CD (D–G, respectively). Filled circles, L-amino acids; open circles, D-amino acids.
are linearly related to actual concentrations. Furthermore, the linear relationship obtained for L-Phe ($y = (1.06 \pm 0.06)x - (0.03 \pm 0.04)$) is, within experimental error, the same as that for D-Phe ($y = (0.95 \pm 0.06)x + (0.02 \pm 0.02)$). In fact, both concentrations of L- and D-Phe fit well into a single equation (Figure 3B, $y = (1.01 \pm 0.02)x - (0.00 \pm 0.01)$ with correlation coefficient of 0.9938). The effectiveness of the method can also be seen from the corresponding plots of regression coefficients for L-Phe and D-Phe, which are shown as insets in the figures.

The effectiveness of this method stems from the diastereomeric interactions between the chiral macrocyclic α-CD with both enantiomeric forms of Phe. One possible type of interaction is the inclusion complex formation. This is because Phe is known to form inclusion complexes with α-CD. It is, however, also possible that Phe may externally adsorb onto α-CD, and if such external adsorption is strong, it may produce measurable differences between D- and L-Phe with α-CD. To evaluate whether inclusion complex formation is the main interaction, similar experiments were performed in which α-CD was replaced with cyclodextrins, which have relatively larger cavity, namely, β- and γ-CD. Results obtained are shown Figure 4A and B, where the calculated concentrations of a set of seven solutions of Phe in β-CD (A) and in γ-CD (B) were plotted against actual concentrations (and also listed in Table S1 in the Supporting Information). As illustrated, similar to α-CD, calculated concentrations agree well with actual concentrations for both β- and γ-CD. The results seem to suggest that diastereomeric interaction is not dependent on the type of cyclodextrin used or, more accurately, on the cavity size of the CD. Therefore, it is possible that inclusion complex formation may not be the only or the dominant factor responsible for the interactions between CDs and both enantiomeric forms of Phe.

Additional information on the interaction mechanism can be obtained by replacing Phe with other amino acids that are either smaller or larger and have substituents such as Ala, Val, Leu, Isoleu, or S–H group (Cys). Results obtained, plotted as calculated concentrations against actual concentrations, for Ala, Leu, Isoleu, Val, and Cys with all three cyclodextrins, α-, β-, and γ-CD, are shown in Figures 4C–E, 5A–F, and 6A–F (and listed in Tables S2–S6 in the Supporting Information). As illustrated, enantiomeric compositions for all seven amino acids can be accurately determined by this method, regardless of the size and type of the

\[ y = (1.00 \pm 0.07)x + (0.01 \pm 0.04) \]

**Figure 7.** Predicted enantiomeric composition versus actual composition for 40.0 mM Try in 80.0 mM α-CD in 20:80 methanol/water mixture. Filled circles, L-Try; open circles, D-Try.

\[ y = (0.66 \pm 0.05)x + (0.19 \pm 0.03) \]

\[ y = (0.81 \pm 0.09)x + (0.10 \pm 0.05) \]

\[ y = (0.92 \pm 0.07)x + (0.00 \pm 0.04) \]

**Figure 8.** Predicted enantiomeric composition versus actual composition for 40.0 mM alanine in 80.0, 120.0, 180.0, 240.0, 320.0, 360.0, and 420.0 mM sucrose (A–G, respectively). Filled circles, L-Ala; open circles, D-Ala.
functional group of the amino acid and the type of cyclodextrin added. The fact that this method can provide accurate enantiomeric compositions for a group of amino acids with diverse size and shape and with all three different size CDs again confirms that inclusion complex formation is probably not the only or the most important factor in the diastereomeric interactions responsible for its effectiveness. More importantly, it clearly demonstrates that this NIR method is rather universal as it can, in principle, be used to determine enantiomeric compositions for all types of amino acids.

Interestingly, it was found that the method is not limited to water but is also effective for other solvents as well. To demonstrate this possibility, similar experiments were performed for 40.0 mM tryptophan and 80.0 mM \( \alpha \)-CD in a 20:80 methanol/water mixture. Results obtained are shown in Figure 7 (and also in Table S7 in the Supporting Information), where calculated concentrations of a set of seven solutions of Try were plotted against actual concentrations. As illustrated, similar to other amino acids in water, calculated concentrations of \( \text{L-} \) and \( \text{D-Try} \) in all seven solutions agree well with actual concentrations.

To gain more information on the mechanism of diastereomeric interactions, additional experiments were performed in which cyclodextrin was replaced with sucrose. Similar to cyclodextrins, sucrose is also a carbohydrate. However, since sucrose is a linear carbohydrate molecule composed of one glucose and one fructose, it cannot form inclusion complexes. Shown in Figure 8A–G (and also listed in Table S8 in the Supporting Information) are results obtained for Ala with seven different concentrations of added sucrose: 80, 120, 180, 240, 320, 360, and 420 mM. It was found that at the three lowest concentrations (80, 120, and 180 mM), the concentrations of \( \text{D-} \) and \( \text{L-Ala} \) predicted by this method do not agree with the actual concentration. Relative error can be as large as 68%. Also, the slopes of the straight line of the plot of

![Figure 9](image-url)

**Figure 9.** Added sucrose (dashed line) and \( \alpha \)-CD (solid line) versus slopes of straight line of calculated enantiomeric composition against actual composition. See text for detailed information.

![Figure 10](image-url)

**Figure 10.** Predicted enantiomeric composition versus actual composition for 40.0 mM Ala in 100.0, 160.0, 240.0, and 320.0 mM \( \alpha \)-CD (A–D, respectively). Filled circles, \( \text{L-Ala} \); open circles, \( \text{D-Ala} \).
diastereomeric interactions with D- and L-Ala, which led to accurate determination. Such analysis is possible because both enantiomeric forms of an amino acid are differentiated through their diastereomeric interactions. Rather a simple adsorption of the analyte onto a carbohydrate is sufficient. Since inclusion complex formation is not a requisite, this method is not limited to the amino acid studies but also very sensitive. It can accurately determine samples with concentrations as low as micrograms having an ee value as high as −90.00% (or +97.00%) and as low as 1.5%. Furthermore, even at an ee as low as 1.5% the relative error was only 0.66%

### Table 2. Actual and Calculated Enantiomeric Excess (ee) of Solution of 2.0 mM Ala in 4.0 mM α-CD

<table>
<thead>
<tr>
<th>sample</th>
<th>D-Ala (mole fraction)</th>
<th>L-Ala (mole fraction)</th>
<th>actual ee (%)</th>
<th>calc'd ee (%)</th>
<th>rel error (%)</th>
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<td>97.00</td>
<td>92.53</td>
<td>4.60</td>
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</table>

a Defined as ee (%) = \[ \frac{(L \text{-} Ala \text{--} D \text{-} Ala)}{L \text{-} Ala + D \text{-} Ala} \] × 100.
b Defined as relative error = (actual value − calculated value) × 100.

The observation that sucrose is effective at a concentration of 240 mM or higher but not lower may be due to the fact sucrose has only one glucose and one fructose molecule whereas the smallest CD (α-CD) has six glucose units. As a consequence, to produce a similar effect, sucrose must be used at a concentration at least three times that of the α-CD. Additional evidence for this explanation can be found from the results of Ala with four different concentrations of added α-CD: 100, 160, 240, and 320 mM. It was found (results presented in Figure 10 and also listed in Table S9 in the Supporting Information) that at all four α-CD concentrations, calculated concentrations of D- and L-Ala agree very well with the actual concentrations. In all five cases, relative errors were as low as 0.0% The slopes of the plots of calculated versus actual concentration are, within experimental errors, unity for all five concentrations (0.94 ± 0.06, 0.98 ± 0.06, 0.93 ± 0.07, and 0.97 ± 0.07). In fact, as illustrated in Figure 8 where the slope was plotted against concentration of added carbohydrate, different from sucrose, in the case of α-CD, the slope remains at a constant value of 1 regardless of concentrations of added cyclodextrin.

As stated above, the present method has high sensitivity. Its sensitivity can be evaluated from two values: the lowest enantiomeric excess (ee, which is defined as ee (%) = [(L-enantiomer − D-enantiomer)/(L-enantiomer + D-enantiomer)]) that can be determined at the lowest concentration of a sample. It should be noted that these two terms are interdependent of each other; namely, the limit of detection (LOD) on ee can be improved by increasing sample concentration or vice versa. In an attempt to estimate the sensitivity of the method, we performed measurements on samples of 2.0 mM or 178 μg/mL alanine with different ee’s in 4.0 mM α-CD in water. Results obtained are listed in Table 2. It is evident from the table that the method is not only effective but also very sensitive. It can accurately determine samples with concentrations as low as micrograms having an ee value as high as −90.00%(or +97.00%) and as low as 1.5%. Furthermore, even at an ee as low as 1.5% the relative error was only 0.66%

### CONCLUSIONS

Collectively, the results presented clearly demonstrate that enantiomeric compositions of a variety of amino acids with different shapes, sizes, and functional groups can be accurately and sensitively determined by the NIR spectrometric technique. Such analysis is possible because both enantiomeric forms of an amino acid are differentiated through their diastereomeric interactions with an added carbohydrate. The fact that the method works well with all three CDs and that sucrose is as effective as all three CDs clearly demonstrates that it is not necessary to have inclusion complex formation in order to produce effective diastereomeric interactions. Rather a simple adsorption of the analyte onto a carbohydrate is sufficient. Since inclusion complex formation is not a requisite, this method is not limited to the amino acid studies here but is rather universal and sensitive as it can, in principle, be used to determine enantiomeric compositions for all types of compounds with only microgram concentration and enantiomeric excess as low as 1.5% in water or in a mixture of water and organic solvent. Furthermore, it does not rely on the use of rather expensive carbohydrates such as cyclodextrins but is equally as effective even with a simple and inexpensive carbohydrate as sucrose.

### ACKNOWLEDGMENT

The authors are grateful to Cargill Corp. and Camo Corp., respectively, for their generous donation of cyclodextrins and the Unscrambler program version 8.0 used in this work.

### SUPPORTING INFORMATION AVAILABLE

Compositions of solutions used for calibration, predicted enantiomeric compositions of solutions of Phe, Ala, Leu, Isoleu, Val, and Cys in α-, β-, and γ-CD and of Ala in different concentrations of sucrose and α-CD in Tables S1–9. This material is available free of charge via the Internet at http://pubs.acs.org.

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