High-Throughput Determination of Enantiopurity by Microplate Circular Dichroism

Samantha L. Pilicer, Justin M. Dragna, Adam Garland, Christopher J. Welch, Eric V. Anslyn,* and Christian Wolf*

ABSTRACT: Methods for the rapid determination of enantiomeric excess (ee) in asymmetric synthetic methodology development are increasingly in demand as high-throughput experimentation protocols in academia and industry are adopted. Optical approaches have been reported, many of which rely on the use of chemical derivatization or molecular assemblies, resulting in UV/vis, fluorescence, or circular dichroism (CD) signals that report the ee values. While UV/vis and fluorescence approaches benefit from readily available 96- and 384-well plate readers, until recently, no CD plate readers existed. Herein, we report the utility of using the EKKO CD plate reader to analyze a chlorocoumarin amine derivatization methodology for the ee determination of a diverse set of chiral amines with an error margin within ±7%. Linear calibration curves of ee versus CD responses for each amine were obtained, the minimum detectable and quantifiable ee values were calculated, the technique was applied to an asymmetric hydrogenation, and various interferents expected to be present in crude samples are explored. The technique described herein is found to be suitable for high-throughput experimentation that requires a parallel and rapid ee determination step.

INTRODUCTION

Rapid and accurate measurement of enantiopurity is a fundamental requirement in the fields of asymmetric synthesis and catalysis and for many ancillary studies of chiral pharmaceuticals, agrochemicals, and specialty chemicals.1 While chiral high-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) have been the gold standard for these tasks for decades,2 the use of chiroptical spectroscopy has been gaining renewed attention3 owing to a potential advantage in sample throughput. To date, many groups have demonstrated that chiroptical analysis rivals chromatography by reducing cost, waste production, and assay development efforts.4 A variety of methods for optical determination of enantiopurity have been reported, typically employing circular dichroism (CD), UV, or fluorescence spectroscopy.5 The sensing approaches range from the observation of inherent chiroptical signals of the molecules of interest6 to the use of probes that serve as reporters for chiral compounds that display little or no usable CD signal. Some chiroptical sensors are designed to undergo conformational changes upon covalent or noncovalent analyte binding that result in an induced CD signal,7 while others are assembled in a series of chemical reactions upon exposure to the analyte.8

The interest in the use of CD for enantiopurity determination motivated us to explore the reagents, instrumentation, and experimental protocols that would be required for practical high-throughput enantioselective analysis. A number of researchers have reported sensor designs and assay conditions that enable reliable, reproducible, and accurate ee values for different compound types, suggesting that a manageable collection of chiroptical sensors and assay protocols might afford a general approach for enantiopurity determination.9 While fluorescence and UV measurements have been conducted with plate readers for decades, gathering high-throughput chiroptical data in microwell plates has until recently meant interfacing a liquid handling autosampler with a CD spectrophotometer containing a flow cell, an approach that affords little or no speed advantage over conventional autosampler-limited chromatographic approaches.10

When developing an analytical method for chiral HPLC or SFC separation of enantiomers, multiple columns and elution conditions are often tested by trial and error before sufficient separation is achieved. Chiral amines, which are examined in this study, are a challenging class of substrates for chiral chromatography, where chemical derivatization is often used to improve the signal intensity and enantiomer resolution. By contrast, the development of chiroptical methods may be achieved with a single, generally applicable, small-molecule derivation of a diverse set of chiral amines with an error margin within ±7%. Linear calibration curves of ee versus CD responses for each amine were obtained, the minimum detectable and quantifiable ee values were calculated, the technique was applied to an asymmetric hydrogenation, and various interferents expected to be present in crude samples are explored. The technique described herein is found to be suitable for high-throughput experimentation that requires a parallel and rapid ee determination step.

Received: June 12, 2020
Published: July 24, 2020
sensor and relatively little optimization of solvent and other sensing parameters, which can also be carried out in parallel if desired. However, the generation of a calibration curve that correlates the measured CD signal to the sample ee values is required. We note that this can be conveniently performed together with the sample preparation when using a CD plate reader, a direction pioneered by the Anslyn and Kahr laboratories.10 The advances of robust optical probes and user-friendly high-throughput CD sensing technology are most likely to accelerate reaction discovery and optimization efforts where large numbers of new catalysts and other parameters such as solvent, additives, or temperature need to be screened.

In order to investigate the performance of CD microplate spectroscopy for assessment of enantiopurity, we obtained an EKKO CD microplate spectrophotometer. This instrument executes fast circular dichroism measurements using vertical optics to accommodate a series of analytes in either 96- or 384-well microplates. Using this instrument, we set out to study a variety of chiral amines using a 4-chlorocoumarin reagent recently developed by Wolf and co-workers.11 This reagent reacts rapidly with a variety of compounds, including amines, which results in characteristic UV changes and a chiroptical signal at wavelengths greater than 300 nm, where interference from other chiral components is typically low. Herein, we report the first comprehensive assessment, method validation, and a real-time workflow protocol using this instrument. We decided to evaluate (a) the general utility of this plate reader for quantitative chiral amine analysis, (b) the tolerance for potentially interfering compounds, which is important in real-world applications, and (c) the feasibility of using a kit approach to chiroptical ee determination by performing the assay in microwell plates containing the preconcentrated 4-chlorocoumarin reagent. Altogether, this study showcases practical aspects relating to the speed, performance, robustness, and convenience of microplate measurements of enantiopurity by CD.

■ RESULTS AND DISCUSSION

As reported previously,11 the 4-chlorocoumarin A reacts quickly with primary and secondary amines, yet it is stable under normal storage conditions (Figure 1). To increase the ease of use, we found that evaporation of predeﬁned quantities of A in the wells of a microplate provides a loaded “kit”, which could be stored at least for several weeks at room temperature, or several months in the freezer, and then used as needed (Figure 1). Preparation of the reaction plates was carried out

Figure 2. Structures of chiral amines successfully tested with the 4-chlorocoumarin CD assay. Only one enantiomer is shown.
using a Genevac to concentrate the sensor in the 300 μL 96-well reaction plates (glass-coated polypropylene). Addition of 5.0 mM amine samples and stoichiometric amounts of triethylamine in acetonitrile to the individual wells of the reagent-loaded microplates leads to rapid dissolution of triethylamine in acetonitrile to the individual wells of the well reaction plates (glass-coated polypropylene). Addition of the amine to the reaction mixtures and transfer to the quartz plate were obtained, although the exact location of the CD signals at 395 nm and the sample ee’s were observed. The substrate derivatization is quantitative within 20 min and is typically performed in the presence of an equivalent of a base such as triethylamine. By comparison, the effect of Et3N and DBU on the induced CD signals showed only minor differences and no deviation from the linear increase in the measured chiroptical response of the coumarin probe to the substrate ee. We therefore decided to use triethylamine in all other studies.

Importantly, the method outlined in Figure 1 is generally applicable and the plate reader performed reliably with all analytes 1–21 including 3-hydroxypyrrroline, 20, which does not have the amino group directly attached to the chirality center. In general, similar CD spectra were observed after dilution of the 5.0 mM reaction mixture to 0.3 or 0.6 mM and transfer to the quartz plate were obtained, although the exact location of the CD maximum varied slightly between analytes. The CD amplitudes of the coumarin derivatives of 1, 7, 8, 10, 15, and 18 vary substantially, but we were pleased to find that accurate ee determination is possible even at relatively small ellipticities (see below). Again, plotting of the CD amplitudes measured at 395 nm versus the sample ee values of these coumarin measurement is sufficient for ee analysis, as we show below. We collected single-point measurements between 375 and 410 nm. While it is possible to obtain the continuous spectra, this would unnecessarily reduce sample throughput. We therefore decided to measure the CD sensor outputs every 5 nm in ACN, which proved sufficient for the identification of the CD curve and its maximum. In a similar fashion, the concentration of the amine can be obtained by measuring UV absorbances at a few selected wavelengths for comparison with a calibration curve. Representative CD spectra obtained by derivatization of the aliphatic and aromatic amines 2 and 6 of varying enantiomeric composition at 5.0 mM and subsequent dilution to 0.3 mM for the CD analysis are shown in Figure 3. The calibration curves for the 4-chlorocoumarin-derived products 2A and 6A were acquired and linear correlations between the CD signals at 395 nm and the sample ee’s were observed. The substrate derivatization is quantitative within 20 min and is typically performed in the presence of an equivalent of a base such as triethylamine. Comparison of the effects of Et3N and DBU on the induced CD signals showed only minor differences and no deviation from the linear increase in the measured chiroptical response of the coumarin probe to the substrate ee. We therefore decided to use triethylamine in all other studies.

![Figure 3](https://dx.doi.org/10.1021/acs.joc.0c01395)

Figure 3. Top: chiroptical sensing of 2 and 6 at 100.0, 80.0, 40.0, 0.0, −40.0, −80.0, 100.0% ee with 4-chlorocoumarin A at 5.0 mM in the presence of 10 equiv of triethylamine in acetonitrile. After 20 min, samples were diluted to 300.0 μM with the same solvent and transferred to the quartz plate for CD analysis. Bottom: plotting of the CD intensities measured at 395 nm versus % ee.
derivatives reveals linear correlations (Figure 4 and Supporting Information).

With linear regression equations in hand, 24 samples of 1, 2, 4, 13, or 19 with randomly chosen enantiomeric composition were prepared, derivatized, and analyzed as described above at a single wavelength (395 or 405 nm, Table 1). The results show that the error margin is within ±7%, which is typically acceptable for high-throughput screening applications. Standard errors were obtained from regression analysis and used to determine the minimum ee quantifiable (MEQ), as defined by eq 1. In each case, the standard error is below the MEQ, indicating that the calibration curve is reliable for the analyses. The MEQ is generally below 5%, which underscores the good sensitivity of the CD analysis.

\[
\text{MEQ} = \frac{(\text{ee cal std. error}) \times 5}{2} 
\]

Having established the general analyte scope, accuracy, and speed of chirality sensing, we decided to examine the possibility of asymmetric reaction analysis with multiplate CD spectroscopy (Scheme 1). For this purpose, we chose to follow a literature protocol that produces the secondary amine 19 via hydrogenation of imine 22 using a catalyst formed from a commercially available Ir dimer and the phosphinooxazoline ligand 23. After 16 h, the crude product mixture was filtered through a cotton plug and a small 5.0 mM aliquot was applied to the 4-chlorocoumarin sensing assay, as described above. CD analysis at 405 nm.

Table 1. Determination of Enantiomeric Excess of Samples of Random Enantiomeric Composition and MEQ Analysis Using CD Intensities at 395 or 405 nm Measured with the Plate Reader after Reaction with the 4-Chlorocoumarin Reagent A 

<table>
<thead>
<tr>
<th>entry</th>
<th>analyte</th>
<th>sample (% ee)</th>
<th>CD (% ee)</th>
<th>absolute error (% ee)</th>
<th>standard error</th>
<th>MEQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>86.0 (R)</td>
<td>83.5(^a)</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>56.0 (R)</td>
<td>53.4(^d)</td>
<td>2.6</td>
<td></td>
<td>86</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>28.0 (R)</td>
<td>23.2(^c)</td>
<td>4.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>46.0 (S)</td>
<td>45.9(^b)</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>72.0 (S)</td>
<td>73.0(^b)</td>
<td>1.0</td>
<td>2.7</td>
<td>3.5</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>28.0 (R)</td>
<td>24.4(^d)</td>
<td>3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>46.0 (S)</td>
<td>42.1(^c)</td>
<td>3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>72.0 (S)</td>
<td>70.3(^b)</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>92.0 (S)</td>
<td>92.7(^c)</td>
<td>0.7</td>
<td>2.8</td>
<td>4.4</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>86.0 (R)</td>
<td>87.4(^d)</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td>56.0 (R)</td>
<td>56.6(^d)</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>28.0 (R)</td>
<td>28.4(^d)</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>4</td>
<td>46.0 (S)</td>
<td>44.4(^d)</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>4</td>
<td>72.0 (S)</td>
<td>72.5(^b)</td>
<td>0.5</td>
<td>1.2</td>
<td>1.5</td>
</tr>
<tr>
<td>15</td>
<td>1(^f)</td>
<td>86.0 (R)</td>
<td>89.3(^d)</td>
<td>3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1(^f)</td>
<td>56.0 (R)</td>
<td>60.9(^d)</td>
<td>4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1(^f)</td>
<td>28.0 (R)</td>
<td>35.0(^d)</td>
<td>6.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>1(^f)</td>
<td>46.0 (S)</td>
<td>46.5(^d)</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>1(^f)</td>
<td>72.0 (S)</td>
<td>76.7(^d)</td>
<td>4.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1(^f)</td>
<td>92.0 (S)</td>
<td>91.3(^d)</td>
<td>0.7</td>
<td>4.2</td>
<td>4.3</td>
</tr>
<tr>
<td>21</td>
<td>19</td>
<td>86.0 (R)</td>
<td>84.4(^d)</td>
<td>1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>19</td>
<td>56.0 (R)</td>
<td>58.5(^d)</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>19</td>
<td>28.0 (R)</td>
<td>32.9(^d)</td>
<td>4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>19</td>
<td>72.0 (S)</td>
<td>70.9(^d)</td>
<td>1.1</td>
<td>2.9</td>
<td>3.6</td>
</tr>
</tbody>
</table>

\(^a\)Solutions were prepared as described above and CD analysis was conducted at 395 nm. \(^b\)Measurements at 0.6 mM (1 equiv of Et,N) in ACN. \(^c\)0.3 mM (10 equiv of Et,N). \(^d\)0.6 mM (10 equiv of Et,N). \(^e\)0.3 mM (2 equiv of Et,N). \(^f\)CD analysis at 405 nm.
The general robustness of chromatographic methods in particular with respect to impurities that may be separated from the targeted enantiomers during the separation process is an attractive feature and an ultimate test for optical methods. The effects of up to 10 equiv of a wide variety of possible solvents/interferents on the reaction between 4-chlorocoumarin A and amine 2 and the subsequent CD assay protocol were studied (Figure 5 and Supporting Information). We found that typically encountered solvents such as tetrahydrofuran, methanol, chloroform, acetone, water, ethyl acetate, toluene, dimethylformamide, and dimethylsulfoxide, and even equimolar amounts of enantiopure Binol or [Rh(COD)/Cl]$_2$, are well tolerated and have only minor effects on the CD signal. The presence of excess of triphenylphosphine, a nucleophile that can be expected to compete with the amine for the reaction with A, resulted in a decrease in the CD signal. While this shows that interference with this particular sensing method is possible, it simply determines the application boundaries and provides valuable guidance for the user to avoid complications.

**CONCLUSIONS**

The studies reported herein clearly demonstrate the potential for chiroptical methods to become an important contributor in the high-throughput analysis of enantiopurity. Using an existing derivatization methodology, we found that the CD plate reader gave accurate and relatively free from interference ee values with the potential of screening 96 samples in under 4 min. While the method was applied solely to one optical assay and with only chiral amines, the workflow can be applied to the wealth of derivatization and supramolecular assembly techniques currently available to the synthetic methodology community. We can imagine parallel analysis of multiple chiral functional groups with other optical approaches to extend the utility of the method, thereby obviating the use of chiral HPLC techniques for an initial screen of ee values.

**EXPERIMENTAL SECTION**

**General.** All reagents and analytes were used as purchased. Chiral amines obtained as HCl salts were used as is and neutralized with equimolar triethylamine during the derivatization reactions. Acetonitrile was dried over 3 Å molecular sieves (heated to 170 °C for 24 h prior to use). All chiroptical analyses were performed using an EKKO CD microplate reader and a Hellma Suprasil quartz 96-well plate equipped with a quartz lid and silicon buffer mat inlay. Spectral scanning was conducted every 5 nm from 375−410 nm with 3.0 s integration time and 40 mdeg sensitivity. All stock solutions of the

![Scheme 1. Comparison of CD and Chiral HPLC ee Analysis of an Asymmetric Imine Hydrogenation Mixture](image)

![Figure 5. Effects of molecular interferents on the CD sensing output.](image)
reagent, additive/interferents, and chiral analytes were prepared using acetonitrile as the solvent.

**Chiroptical Analysis.** Solutions of 4-chloro-3-nitrocoumarin, A, (5.00–6.00 mM) were prepared and distributed into vials. Stock solutions of each enantiomer of the chiral analyte of interest were prepared (0.025 or 0.25 M) in acetonitrile for either 10.0 or 100.0 μL additions to the reagent (1:1 A/analyte ratio) allowing for the click reaction to occur at 5.00 mM. The reactions were conducted in the presence of 1.0–10.0 molar equiv of triethylamine or DBU. After 20 min, aliquots of the reaction solutions were diluted to 600.0 and/or 300.0 μL. Calibrations were constructed with the quartz 96-well plate and varied ee's (−100, −80, −40, 0, 40, 80, and 100%) of the analytes. EKKO parameters were set to scan every 5 nm at 375–300 nm, and varied ee of the reaction to occur at 5.00 mM. The reactions were conducted in the presence of 1.0–10.0 molar equiv of triethylamine or DBU. After 20 min, aliquots of the reaction solutions were diluted to 600.0 and/ or 300.0 μL. Calibrations were constructed with the quartz 96-well plate and varied ee's (−100, −80, −40, 0, 40, 80, and 100%) of the analytes. EKKO parameters were set to scan every 5 nm at 375–300 nm, and varied ee of the reaction to occur at 5.00 mM. The reactions were conducted in the presence of 1.0–10.0 molar equiv of triethylamine or DBU. After 20 min, aliquots of the reaction solutions were diluted to 600.0 and/or 300.0 μL. Calibrations were constructed with the quartz 96-well plate and varied ee's (−100, −80, −40, 0, 40, 80, and 100%) of the analytes.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/10.1021/acs.joc.0c01395.

Details of the CD sensing procedures, CD spectra, and method validation (PDF)

**AUTHOR INFORMATION**

**Corresponding Authors**

Eric V. Anslyn — Department of Chemistry, The University of Texas at Austin, Austin, Texas 78712, United States; Email: anslyn@utexas.edu

Christian Wolf — Department of Chemistry, Georgetown University, Washington, DC 20057, United States; orcid.org/0000-0002-4471-3753; Email: cw27@georgetown.edu

**Authors**

Samantha L. Pilicer — Department of Chemistry, Georgetown University, Washington, DC 20057, United States

Justin M. Dragna — Enantiosense, LLC, Austin, Texas 78723-4648, United States

Adam Garland — Water Less, LLC, Houston, Texas 77027, United States

Christopher J. Welch — Indiana Consortium for Analytical Science & Engineering (ICASE), Indianapolis, Indiana 46202, United States; orcid.org/0000-0002-8899-4470

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.joc.0c01395

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was gratefully supported by NSF SBIR (UTA18-001517), NSF (CHE-176413S), and NSF GOALI (CHE 1665040) grants, as well as the Welch Chair Regents Chair (EVA, 1-0046).

**REFERENCES**


(j) Wezenberg, S. J.; Salassa, G.; Escudero-Adán, E. C.; Benet...

