



Advantages of Circular Dichroism (CD) Detection for the Determination of Fractionation Timing in Preparative Supercritical Fluid Chromatography (Prep-SFC) for Chiral Separations

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Summary

In chiral prep-SFC, samples are often overloaded making it difficult to determine when to start and end fractionation using UV chromatographic data.

A CD detector can differentiate enantiomers and generate positive/negative responses arising from eluting chromatographic peaks. JASCO's model X-LC 3195CD CD detector can simultaneously generate CD, UV and g-factor signals. The g-factor is a signal defined as CD/UV whose level is independent of the analyte concentration but dependent on enantiopurity. Therefore, the enantiopurity of g-factor-triggered fractions collected can be more accurately controlled, increasing collection efficiency and purity of the collected enantiomers

Introduction

Chiral separation is one of the best suited applications for SFC. The first chiral separation by SFC was demonstrated in 1986 by Hara et. al. of Tokyo University of Pharmacy and Life Sciences in collaboration with JASCO¹. In the 1980's, open-tubular capillary column SFC gained general attention but packed column SFC attracted only several research groups, including JASCO. In 1988, JASCO demonstrated fractionation of lemon-peel oils by preparative SFC², and also in 1995 JASCO succeeded in chiral separation by preparative SFC with stack injections, proving it was a very efficient and suitable method for fractionation of chiral compounds³. In the 1990's, interest in capillary SFC quickly disappeared due to limited development in instrumentation whereas packed column SFC including preparative SFC gained a lot of interest.

Definition of Circular Dichroism (CD). The relationship between absorption and concentration is expressed by the following equation.

$$\text{Absorbance (AU)} = \varepsilon c l \quad \text{----- (eq. 1)}$$

where ε = molecular absorption
 c = concentration (mol/L)
 l = optical path length

Chromophores near a chiral center in enantiomers exhibit different molecular absorptions to right- and left-handed circularly polarized lights. The different amounts of absorption by enantiomers cause a CD signal as illustrated in Figure 1
 This can be expressed by the following equation.

$$\begin{aligned} \text{CD} &= \text{AU}_L - \text{AU}_R = \Delta \text{AU} \quad \text{----- (eq. 2)} \\ &= \varepsilon_L c l - \varepsilon_R c l \\ &= (\varepsilon_L - \varepsilon_R) c l \\ &= \Delta \varepsilon c l \end{aligned}$$

where $\Delta \varepsilon$ is the difference in molecular absorption. Note that ε_L and ε_R are dependent on wavelength.

Definition of g-factor. The g-factor is defined as the CD signal divided by the AU signal at the same wavelength. Using equations 1 and 2, it is expressed as;

$$\begin{aligned} \text{g-factor} &= \text{CD} / \text{AU} = \Delta \text{AU} / \text{AU} \\ &= \frac{\varepsilon_L c l - \varepsilon_R c l}{\varepsilon c l} = \frac{\Delta \varepsilon}{\varepsilon} \quad \text{----- (eq. 3)} \end{aligned}$$

Equation 3 shows the g-factor does not include concentration c and path length l terms but includes only ε and $\Delta \varepsilon$, proving that the g-factor is independent of concentration and optical path length and dependent only on enantiopurity.

Flavanones. Flavanoids are a group of metabolites produced by plants that often exhibit biological activity. It is categorized into various subclasses including flavones, flavonols, flavanones, isoflavanones, anthocyanidins, and catechins. Flavanones have been defined as citrus flavanoids due to their unique presence in citrus fruits. Flavanones present a unique structural feature known as chirality, which distinguishes them from all other classes of flavonoids⁴. It has been recognized that stereospecific pomological disposition of racemic flavanones is important, and in 1986, Okamoto et al. demonstrated chiral separation of unsubstituted flavanone (2,3-Dihydroflavone) by HPLC on a cellulose triphenylcarbamate derivative based stationary phase⁵.

In this paper, we demonstrate a highly efficient fractionation method for flavanone by Prep-SFC using a CD detector.

Experimental

Instruments: Figure 2 shows the JASCO Preparative SFC system, with an X-LC 3195CD CD Detector, which was used for the experiment.



Figure 2. Preparative SFC System

Figure 3 shows the hydraulic diagram of the system. Components are listed in the figure legend. The SF-NAV software managed the total system control. The JASCO J-820 CD Spectrometer was used to measure the CD spectra of flavanone.

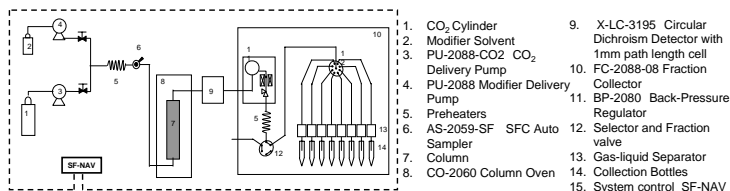


Figure 3. Hydraulic diagram of the Prep-SFC System.

Materials. An analytical chiral column (CHIRALPAK IA, 4.6 mm I.D. x 250 mm L, CHIRALPAK OD-H 4.6mm I.D. x 250mm L), semi-preparative column (CHIRALPAK AD-H, 10mm I.D. x 250mm L) and a preparative column (CHIRALPAK JA, 20 mm I.D. x 250 mmL) were purchased from Daicel Chemical Industries, Ltd., Osaka, Japan. Carbon dioxide (99.99%) was supplied by, Nippon Tansan, Co., Ltd., Tokyo, Japan. The methanol used as a modifier was purchased from the Kanto Chemical Co., Inc. (Cica-reagent >99.5%) and the Racemic flavanone (2,3-Dihydroflavone) was purchased from Wako Pure Chemicals (Purity >97.0%), Osaka, Japan. The chemical structure of flavanone and its CD spectra is shown in Figures 4 and 5 respectively. The CD spectra measured with the CD detector is also shown for comparison.

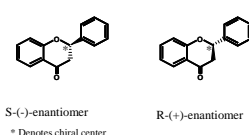


Figure 4. Structure of unsubstituted flavanone (2,3-Dihydroflavone).

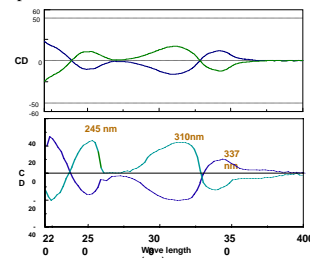


Figure 5. CD spectra of flavanone by J-820 CD spectrometer and X-LC 3195CD detector, respectively

Results and Discussion

UV- and g-factor triggering. In order to compare the enantiopurities of UV- and g-factor-triggered fractions, we separated flavanone on a semi-prep CHIRALPAK AD-H column (10 mm I.D. x 250 mm L.). Fractions were subjected to separation on an analytical CHIRALPAK OD-H column (4.6 mm I.D. x 250 mm L.) to measure enantiopurity of each fraction. Figure 6 shows the chromatogram of UV-triggered semi-preparative separation (left), on which fractionation time windows are indicated. As you can see, the separation of the peaks was poor. Therefore, collection of Fraction 1 was ended far before the peak valley to obtain a high enantiopurity. The right chromatogram shows the analytical separation for the determination of enantiopurity of fraction. Enantiopurities were calculated to be 99±1% for Fraction 1 and 80±1% for Fraction 2. Note that the elution order was reversed due to the different column used for the analytical separation.

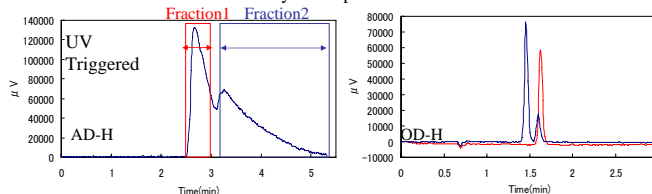


Figure 6. Chromatograms of UV-triggered fractionation by a semi-prep column (left) and measurement of enantiopurities of fractions (right). The semi-prep elution conditions are: column; CHIRALPAK AD-H (10mm ID x 250mmL); CO₂ flow rate = 15mL/min at -10°C; modifier, ethanol @ flow rate of 1.5mL/min; pressure = 20MPa; column temperature = 25°C; wavelength @ 310nm; sample, flavanone (12.5mg). The analytical elution conditions are: column; CHIRALPAK OD-H (4.6mm ID x 250mmL); CO₂ flow rate = 2mL/min at -10°C; modifier, ethanol @ flow rate of 0.2mL/min; pressure = 20MPa; column temperature = 25°C; wavelength @ 310nm.

Figure 7 shows the chromatogram of a g-factor-triggered semi-preparative separation (left), on which fractionation time windows are indicated. As indicated, fractions were taken when g-factors maintained levels where enantiopurities exhibited high levels. The right chromatogram was an analytical separation for the determination of enantiopurity of fraction. Enantiopurities were calculated to be 99±1% for Fraction 1 and 96±1% for Fraction 2.

The enantiopurity of UV-triggered Fraction 2 was 80%, whereas that of the g-factor-triggered Fraction 2 was 96%. This result suggests that the g-factor is a useful indicator for determining when to start and end fractionation in order to obtain high enantiopurity in prep-chiral separation.

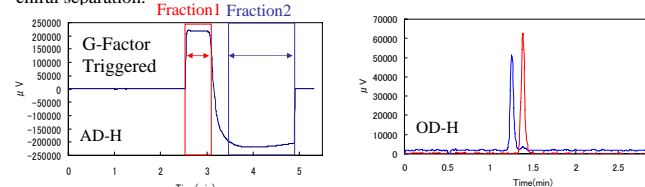


Figure 7. Chromatograms of the g-factor-triggered fractionation by a semi-prep column (left) and measurement of enantiopurities of fractions (right). Elution conditions are identical to those given in Figure 6 caption.

Loading capacity. In chiral prep-SFC separation, the loading capacity onto the column is a key factor in high productivity. Before performing preparative SFC, we evaluated the loading capacity of the chiral stationary phase (IA) by using an analytical scale column. Figure 8 shows chromatograms obtained by loading a CHIRALPAK IA (4.6 mm I.D. x 250 mm L.) with various sample amounts; 0.116, 0.232, 0.580 and 1.16 mg. These numbers can be expressed as (sample mass)/(unit mass of chiral stationary phase); 0.0464, 0.0928, 0.232, 0.464 mg/g, assuming that a 4.6 mm I.D. x 250 mm L. column contains 2.5 g of chiral stationary phase (CSP). As shown in chromatograms #1-3 sample loading up to 0.232 mg/g exhibits baseline separation, and fractionation can easily be triggered by the UV chromatogram signal. However, when the loading becomes 0.464 mg/g, peak resolution gets worse and it is difficult to determine when to start or end fractionation by the UV chromatogram.

The g-factor traces, top right, also clearly show that #1-3 have clear gaps between pairs of plateaus, however, #4 trace corresponding to chromatogram #4 does not have a gap, but the signal continuously changes. This means that in #4, there is a band between two peaks where enantiopurity is constantly changing. If we avoid collection of this band, it is possible to fractionate enantiomers with high purity, even though the loading is so high that the peak shapes deteriorate.

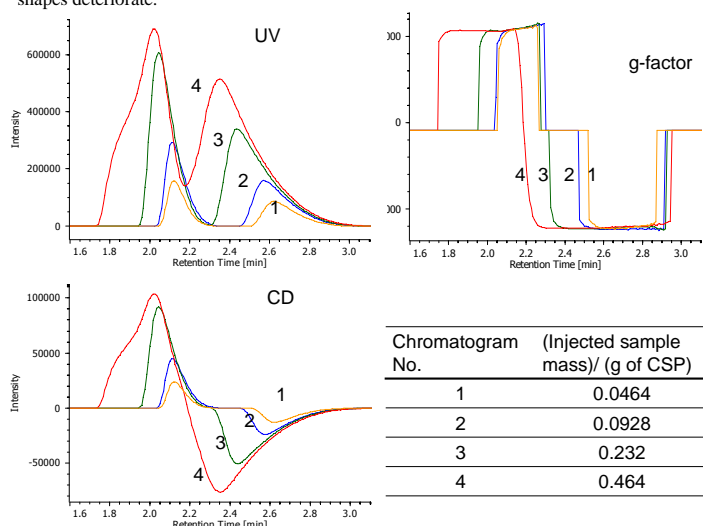


Figure 8. Overlaid Chromatograms of various sample loading. Elution conditions: column; CHIRALPAK IA, 4.6 mm I.D. x 250 mm; CO₂ flow rate @3.2mL/min; MeOH flow rate @0.8mL/min; Column temp @40°C; pressure @20MPa; wavelength: 310nm; Sample, flavanone.

Effect of loading on resolution and retention times. The left graph in Figure 9 shows the relationship between sample loading and resolution (Rs). The right graph shows a variation in retention time with sample loading. As clearly seen in these graphs, Rs deteriorates and retention times become shorter as the sample loading becomes high. This means that the equilibrium in the chromatographic system can no longer hold, i.e. overloading is occurring, and there is more sample solute population in the mobile phase than in the stationary phase, resulting in slipping of solute in the column.

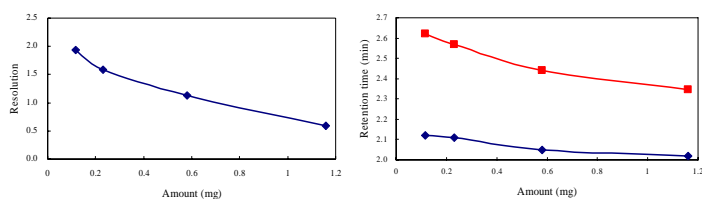


Figure 9. Relationship between sample loading and Rs (left), and variation of retention time with sample loading (right).

Preparative chiral separation of flavanone. Figure 10 shows chromatograms of a preparative chiral separation of flavanone with a 30.2mg injection. This sample loading corresponds to 0.639 mg/g of CSP that is approximately 40% greater than the value we obtained from the loading capacity test with an analytical column. Still, the peak shape is much better than the one in the loading capacity test, and we can clearly see the gap between the two plateaus in the g-factor trace.

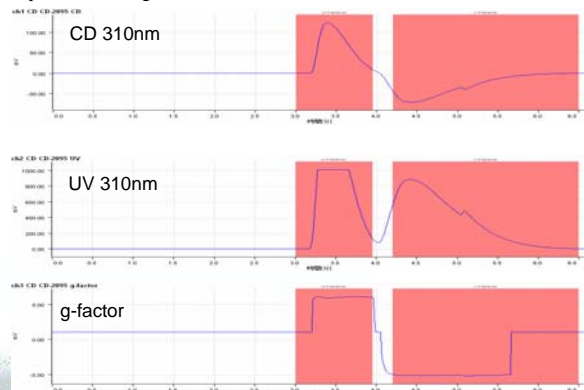


Figure 10. Chromatograms of preparative chiral separation of flavanone. Elution conditions are: column, CHIRALPAK IA (20 mm I.D. x 250 mm L.); CO₂ flow rate @34ml/min; Modifier, methanol flow rate @6mL/min; column temp @40°C, pressure @20MPa, wavelength UV and CD @310nm; sample, 30.2mg of flavanone

Stacked injection. Figure 11 shows preparative chromatograms of flavanone with stacked injections. Fractionation start of Fraction 1 was triggered at the UV peak's rising edge and the end was triggered at the g-factor's drop edge. The second fraction start timing was at the drop finishing edge of the g-factor signal and stop timing was at the vanishing UV peak.

The sample amount was 30.2 mg and the injection-to-injection interval was 3.8 min.

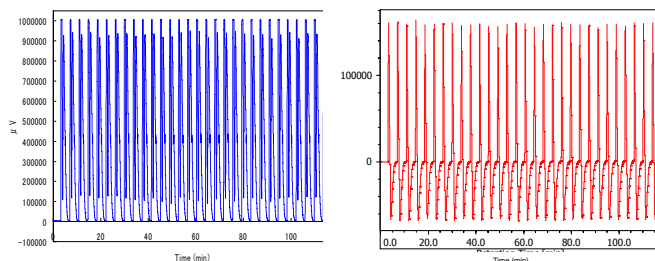


Figure 11. Chromatograms with stacked injections. The injection-to-injection interval is 3.8 min. Elution conditions are the same as described in Figure 10 caption.

Enantiopurity of each fraction. We performed analytical chiral separations of each fraction to determine the enantiopurity and recovery. Figure 12 shows the chromatograms obtained by the analytical separations. Optical purities of two fractions were higher than 99.9%. The error margin of $\pm 0.1\%$ was calculated by the signal-to-noise ratio of the chromatograms. The recovery was higher than 80%. Therefore, by running the system for 24 hours, 11.32 g of racemic flavanone can be separated and 4.5 g of each enantiomer will be recovered. The amounts are adequate for biological tests.

Furthermore, the band between Fractions 1 and 2 could be collected and re-chromatographed. By this arrangement the recovery could be significantly improved.

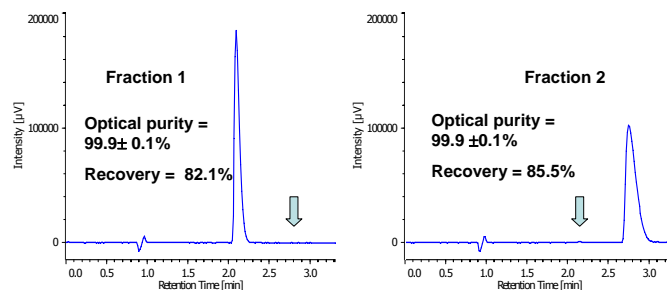


Figure 12. Enantiopurity and recovery of flavanone fractionation.

Conclusion

We obtained 99.9 \pm 0.1% pure enantiomers of flavanone. The recoveries for the nantiomers were 82.1% and 85.5%, respectively. Collection of the band between the fractions and re-chromatography of the collection could improve the recovery.

In summary;

- 1) The JASCO CD detector is a powerful tool for chiral fractionation.
- 2) The g-factor triggering enables high enantiopurity and recovery even though the peak separation is incomplete.
- 3) JASCO's SF-NAV software allows any combination of UV/g-factor triggering for fractionation, in addition to the system control.

References

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