An Executive Summary

Achieving Greater Success with Chiral LC and SFC Analysis and Purification

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How the use of new stationary phases and packing technology leads to improved recognition, separation efficiency, and resolution in the analysis of chiral compounds.

Introduction

The accurate and efficient chiral separation of enantiomeric compounds is very important in fields such as pharmaceuticals, pesticides, and synthesis development. The striking differences in the chemical and physiological properties between enantiomers make these separations a key requirement in the development of many drugs. Often, only one enantiomer exhibits the desired therapeutic benefit, while the other may have an antagonistic effect—or even worse, a toxic side effect. A striking example of this behavior is the drug, thalidomide. Whereas R-thalidomide is an efficient sedative, S-thalidomide is a teratogen that caused severe birth defects in thousands of children in many countries. Since 1992, the US Food and Drug Administration (FDA) has required a complete, separate evaluation of the effects of all compounds that have the potential for chirality.

Chiral separation is also very important when it comes to raw materials used during the development of chemical syntheses. In the field of peptide chemistry, amino acids are critical raw materials whose chiral purity must be assessed to develop reliable synthetic pathways to new compounds. In addition, it is highly desirable to evaluate potential enantiomeric excesses after each synthesis step is. And to ensure unwanted chiral conversions do not occur in the body, the final compound's stability must be determined.

Chiral separations can be performed in several ways, including chiral derivatization and chromatographic techniques using either a chiral mobile phase additive or a chiral stationary phase. This summary discusses the benefits of chiral stationary phases and presents strategies for developing successful methods for chiral separations.

Chiral Stationary Phases

Chiral stationary phases have been in use since the early 1970s, when ligand exchange and protein-based phases were developed for high-performance liquid chromatography (HPLC).

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Cyclodextrin-based and Pirkle-type phases were some of the first next-generation options to be introduced, and were soon followed by polysaccharide-based chiral stationary phases such as the Lux® portfolio of chiral LC/SFC columns. These phases were developed in the mid-1980s and greatly enhanced the capabilities of analytical and preparative chromatographic methods for chiral compounds (1). Macrocyclic glycopeptide-based and cinchona alkaloid-based phases were then developed in the early-to-mid 1990s.

Even with all these options, polysaccharide CSPs have accounted for more than 90% of all chiral separations done by chromatographic methods since their introduction. The most common polysaccharides used are amylose and cellulose, which can be attached to the stationary phase as a coating, as seen in the earlier generations of these materials (**Figure 1**). They can also be immobilized by using a strong chemical bond, as in the Lux i-Amylose-1 and Lux i-Cellulose-5 CSPs. The immobilization process results in chiral phases with increased solvent robustness, allowing for the use of stronger solvents in the mobile phase or in the cleaning steps, without degradation of the chiral stationary phase. Their performance remains essentially unchanged, even after undergoing 200 column-volumes of various strong solvents.

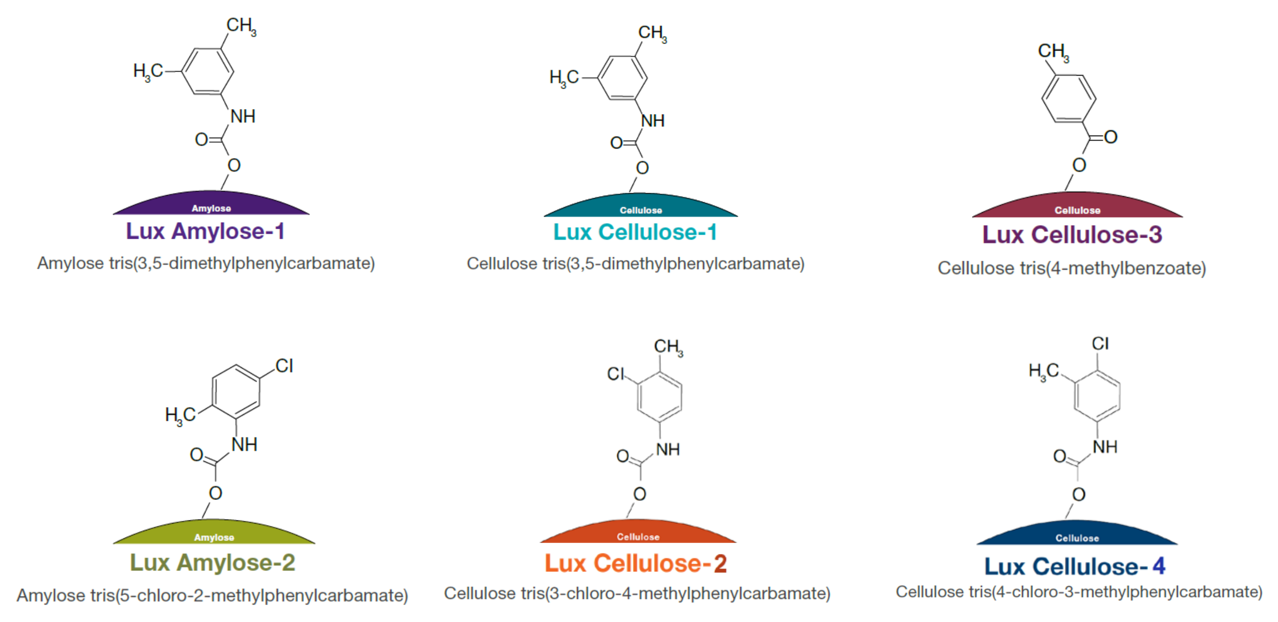
Alongside their broad enantioselectivity, polysaccharide CSPs can be used in a range of separation modes such as reversed-phase HPLC (RP-HPLC), normal-phase HPLC (NP-HPLC), polar organic, and supercritical fluid chromatography (SFC). Within the Lux family of polysaccharide CSPs, all eight phases are scalable from analytical to preparative chromatography because of high-quality manufacturing processes, packing technologies, and patented hardware systems such as, Axia™. This system uses an internal piston in the column, which is locked in place to help achieve very high packing density that improves resolution by as much as 30% (**Figure 2**). It also reduces column-to-column variation and increases column loadability.

The wide variety of chiral selectors available on polysaccharide supports makes it highly likely that one or more of them can separate any chiral compound. One issue, however, is that it is difficult to predict which selector will work for the chiral compound of interest. Unless a published method is already available, a thorough evaluation of all available CSPs is usually needed. What follows are several strategies for quickly developing successful methods using a variety of tools available from Phenomenex.

Recent advancement in immobilized chiral stationary phases. Phenomenex recently introduced the immobilized Lux i-Amylose-1 and Lux i-Cellulose-5, which both have unique selectivities intended to increase the chance of success in chiral screening. In addition, they are stable with strong solvents like ethyl acetate and tetrahydrofuran (THF), offer optimized surface chemistry, have improved immobilization technology, and are packed in analytical, semi-prep and preparative Axia-packed columns.

This combination of ruggedness and unique chiral selectivity provides multiple avenues to positively affect chiral success rates during screening across multiple industries. An example of the importance of additional chiral selectivity is shown in **Figure 3**. By adding Lux i-Cellulose-5 to this screen of one mobile phase and multiple CSPs, one could separate acebutolol, which could not be done under these conditions with the other CSPs.

Figure 1: Lux® polysaccharide-based CSPs (coated).



Lux Amylose-1
Amylose tris(3,5-dimethylphenylcarbamate)

Lux Cellulose-1
Cellulose tris(3,5-dimethylphenylcarbamate)

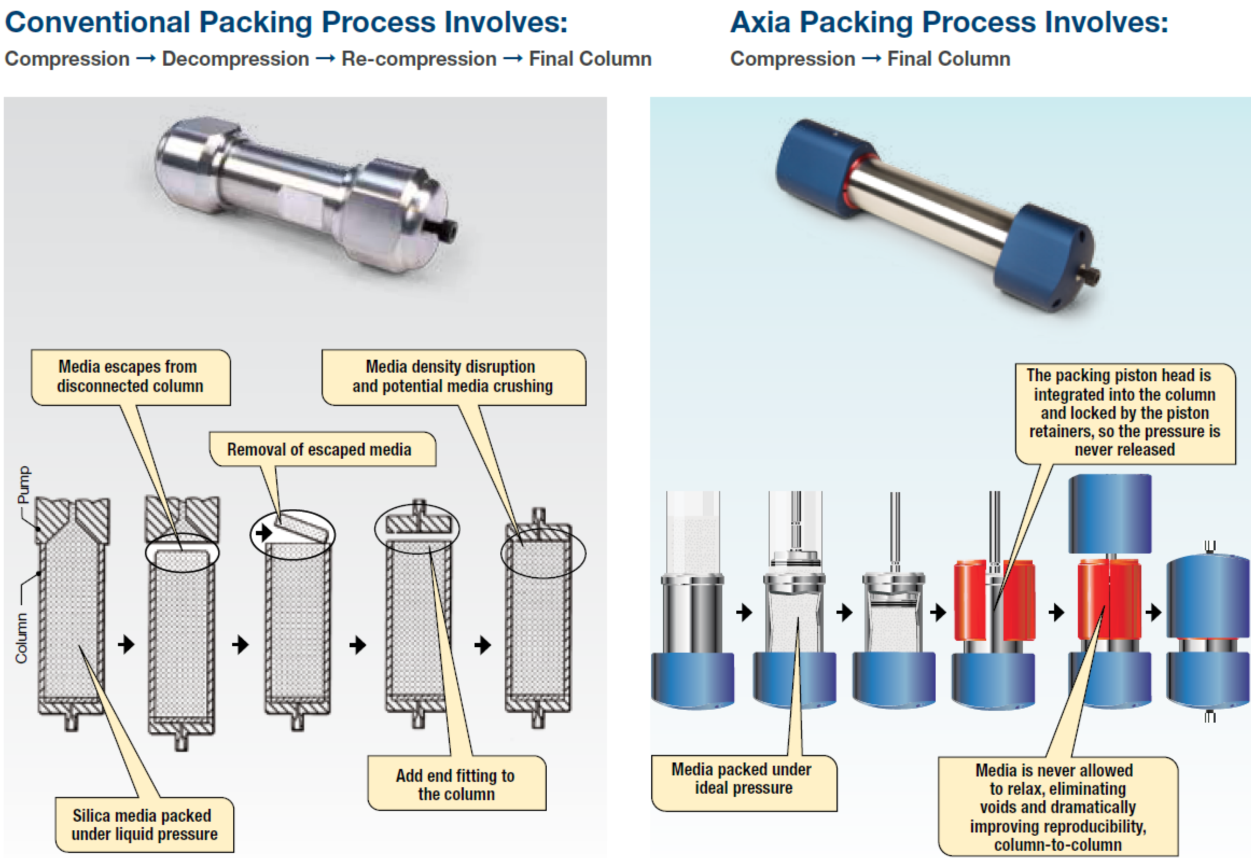
Lux Cellulose-3
Cellulose tris(4-methylbenzoate)

Lux Amylose-2
Amylose tris(5-chloro-2-methylphenylcarbamate)

Lux Cellulose-2
Cellulose tris(3-chloro-4-methylphenylcarbamate)

Lux Cellulose-4
Cellulose tris(4-chloro-3-methylphenylcarbamate)

Figure 2: Axia™ packing technology.



In a separate analysis of 56 pharmaceutical compounds, the use of six Lux CSPs with one SFC mobile phase yielded an 88% success rate of chiral resolution (**Figure 4**). This is a good example of the overall utility of these stationary phases and their ability to effectively resolve a range of enantiomers with differing functional groups.

More simplistically though, additional separation power for chiral LC can be achieved by adding a phase with broad enantioselectivity, like the Lux i-Amylose-1. For instance, **Figure 5** shows an example of metalaxyl enantiomeric separation.

Method Development

In chiral separations, the goal is to achieve a minimum resolution (R_s) of 1.5 between enantiomer peaks, with a selectivity (α) of at least 1.1. R_s is a direct function of three parameters: In addition to α, R_s is affected by the column efficiency (*N*) and the capacity factor (k), which is itself a function of the compound affinity for the stationary phase, for a given eluent. *N* is a limited factor, as increases in *N* result in increased back pressure. *k* is ineffective at values higher than 10. This leaves α as the key driver for resolution, and α depends primarily on the type of stationary phase chosen.

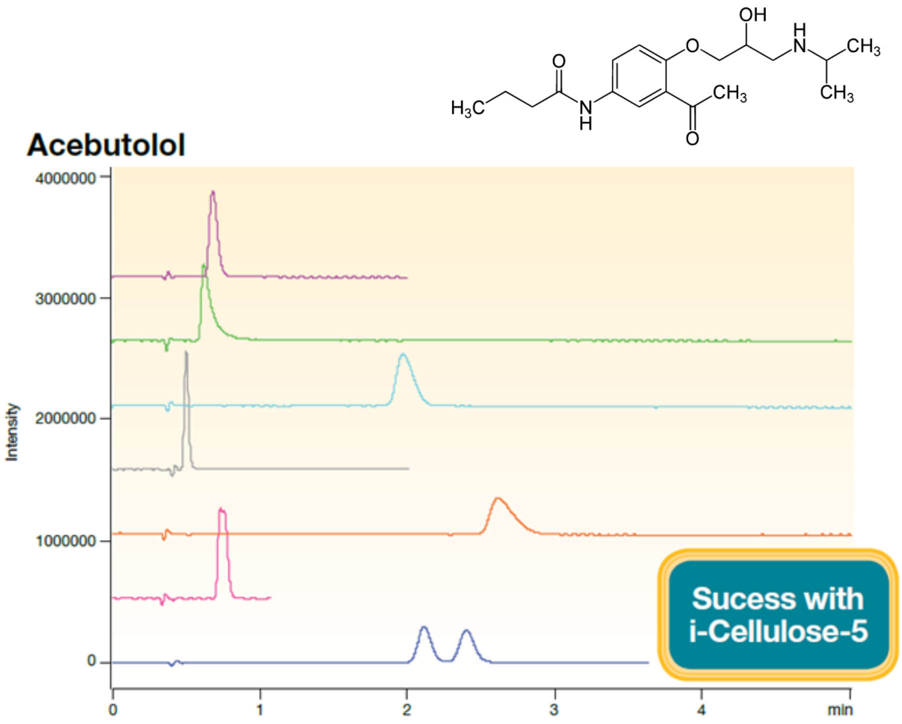
The first step in method development is to choose the mobile phase from among the four common separation modes available for polysaccharide based CSPs: normal phase, polar organic, reversed phase, and SFC. The next step is to screen representative CSPs using a column selector and then optimize the mobile phase conditions (**Figure 6**). The screening is best done under isocratic conditions, and if the desired R_s is not obtained, adjustments with ethanol and other modifiers should be tested. If that proves ineffective, then hexane–isopropyl alcohol (IPA) or pure organic phases are tried. Acetonitrile–IPA and methanol–IPA can also be useful in some cases.

Suggestions for SFC method development, which involve starting out with methanol and CO_2 as the mobile phase, with ammonium hydroxide as an additive, are summarized in **Figure 7**.

Case Studies

Fluorenylmethyloxycarbonyl chloride (FMOC)-isoleucine is a chiral amino acid composed of a mixture of D and L enantiomers. It can be effectively separated by four different cellulose-based CSPs (**Figure 8**) using a normal phase eluent such as n-hexane–ethanol–formic acid (80%, 20%,

Figure 3: A perfect complement to other Lux® phases.



Sucess with i-Cellulose-5

Conditions for all columns:

Columns: Lux 3 µm Amylose-2
Lux 3 µm Amylose-1
Lux 3 µm Cellulose-4
Lux 3 µm Cellulose-3
Lux 3 µm Cellulose-2
Lux 3 µm Cellulose-1
Lux 3 µm i-Celluose-5

Dimensions: 150 x 3.0 mm

Mobile Phase: 80 % CO_2 / 20 % Methanol + 0.1 % isopropylamine and 0.1 % TFA

Flow Rate: 3 mL/min

Detection: UV @ 220 nm

Temperature: 30 °C

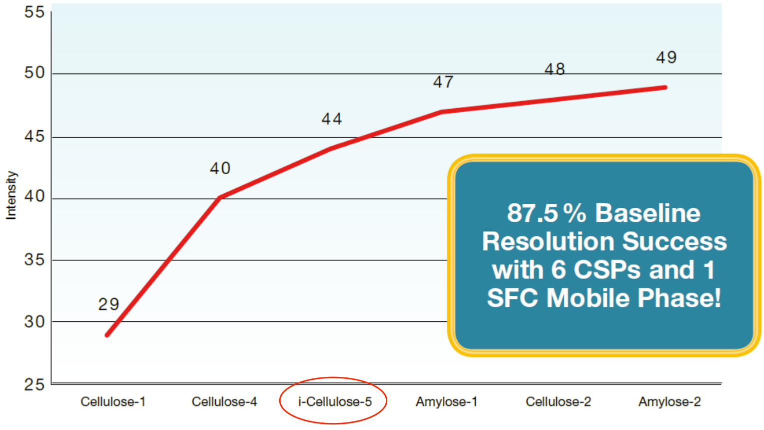
System: JASCO® 4000 Series Analytical SFC

Jasco is a registered trademark of Jasco Products Company.

Figure 4: Increase SFC screening success rate with Lux® i-Cellulose-5.

- 56 pharmaceutical compounds
- 1 mobile phase
- 6 Lux chiral phases

Cumulative baseline separation with Lux phases



87.5 % Baseline Resolution Success with 6 CSPs and 1 SFC Mobile Phase!

SFC Screen

Columns: Lux 5 µm Cellulose-1
Lux 5 µm Cellulose-4
Lux 5 µm i-Celluose-5
Lux 5 µm Amylose-1
Lux 5 µm Cellulose-2
Lux 5 µm Amylose-2

Dimensions: 250 x 4.6 mm

Conditions for all columns:

Mobile Phase: 80 % CO_2 / 20 % Methanol + 0.1 % Isopropylamine and 0.1 % TFA

Flow Rate: 3 mL/min

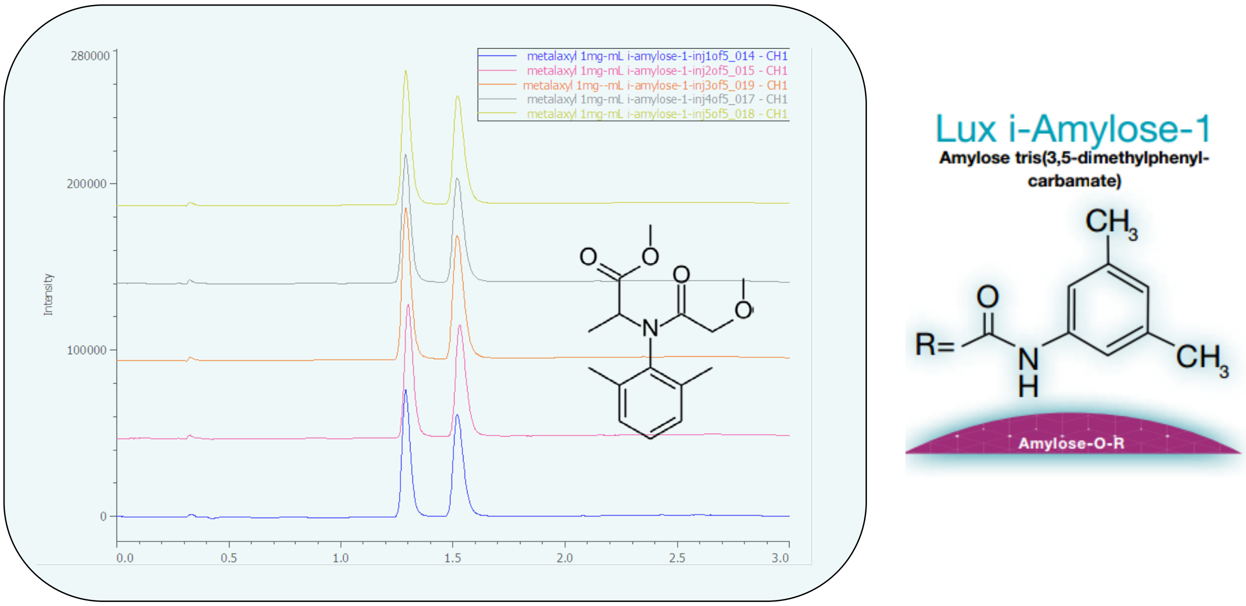
Detection: UV @ 220 nm

Temperature: 30 °C

System: JASCO® 4000 Series Analytical SFC

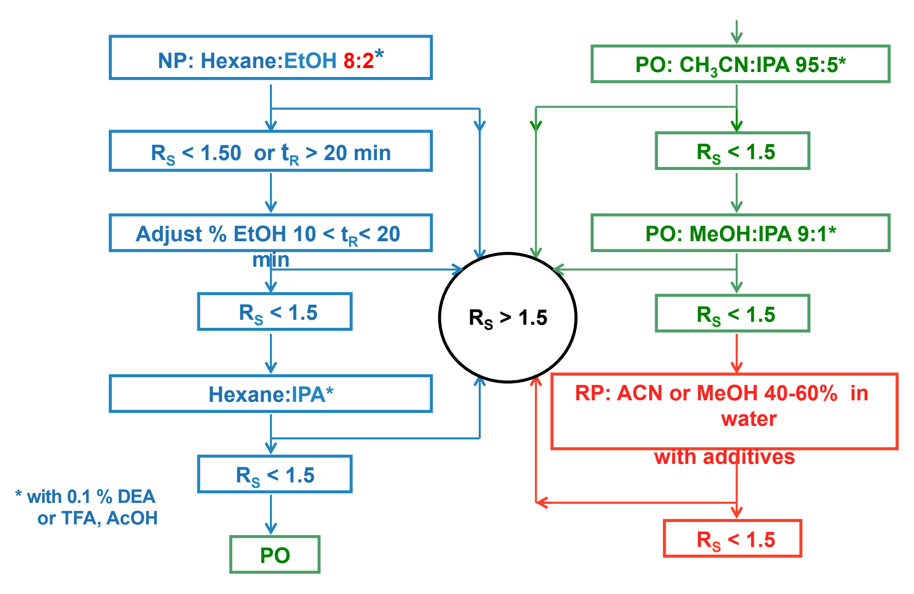
Jasco is a registered trademark of Jasco Products Company.

Figure 5: Industry: Environmental - Metalaxyl enantiomeric separation.



0.1%, respectively, by volume). Interestingly, the elution order can be reversed according to the chiral selector that's used or the mobile phase composition. Adjustment to the additive concentration can also be used to reverse the elution order: varying formic acid from 0.1% to 1% flips the elution order. The same effect can be obtained by varying the temperature from 5 °C to 50 °C.

Figure 6: Suggested screening protocol - HPLC.



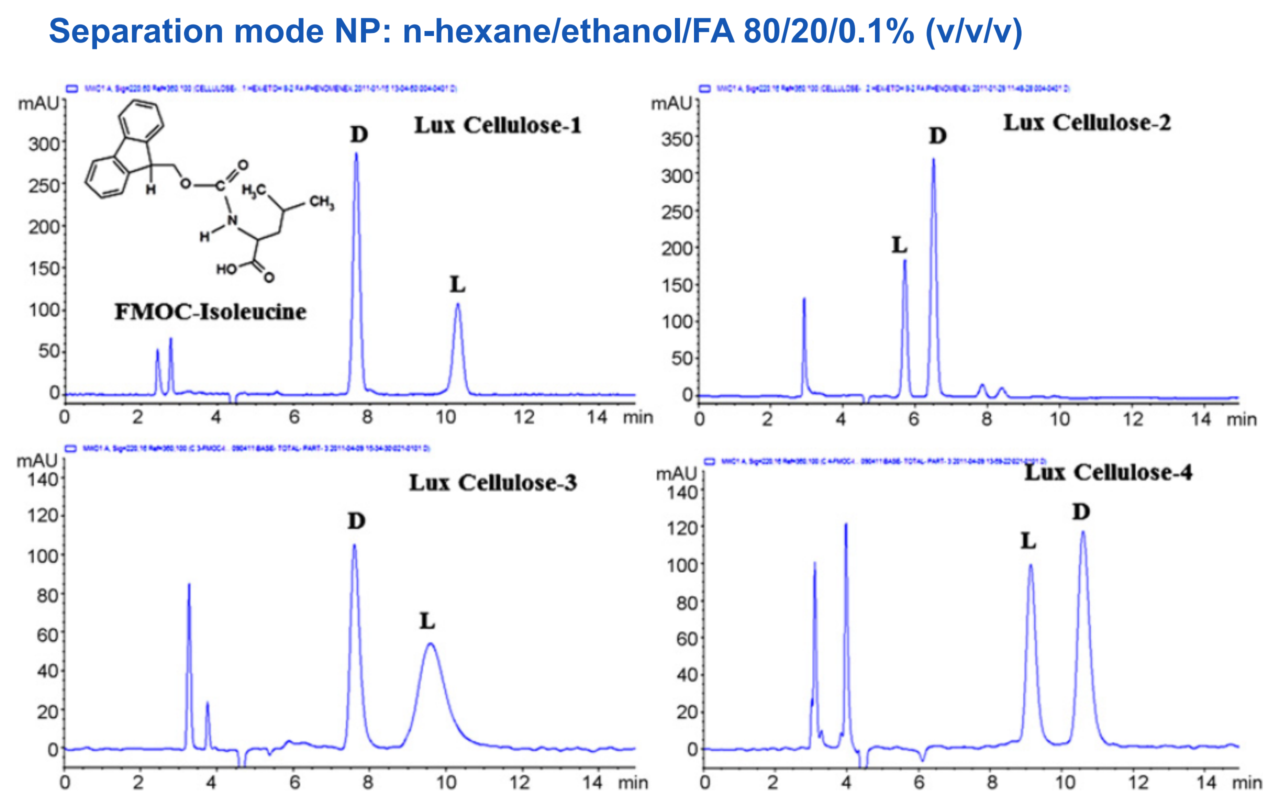
Preparative Purfication

Chiral purification by preparative liquid chromatography can reduce or eliminate the need for complex asymmetrical syntheses, which is very important in the pharmaceutical industry. A combination of immobilized polysaccharide-based CSPs and Axia™ packing technology can be used to quickly make large amounts of pure enantiomers, usually under isocratic conditions that are transferable to simulated moving bed (SMB) technology. Using this technology, chiral purification of the drug, warfarin, was possible at a flow rate of 20 mL/min using hexane–ethanol as the mobile phase and a cellulose-based CSP, at ambient temperature, with very high resolution (R_s=3.7).

Tools and Resources

Numerous pharmaceuticals, pesticides, and raw materials have already been successfully separated into their respective enantiomers, and the methods—over 1,700 of them—are available on the Phenomenex website. These include proton-pump inhibitors, beta blockers, anti-allergic agents, pain relievers, and antifungal drugs, among others. An online application name search tool allows researchers to browse the entire application library. Searches can be filtered by compound class, industry, technique, separation

Figure 8: Complementary selectivities.



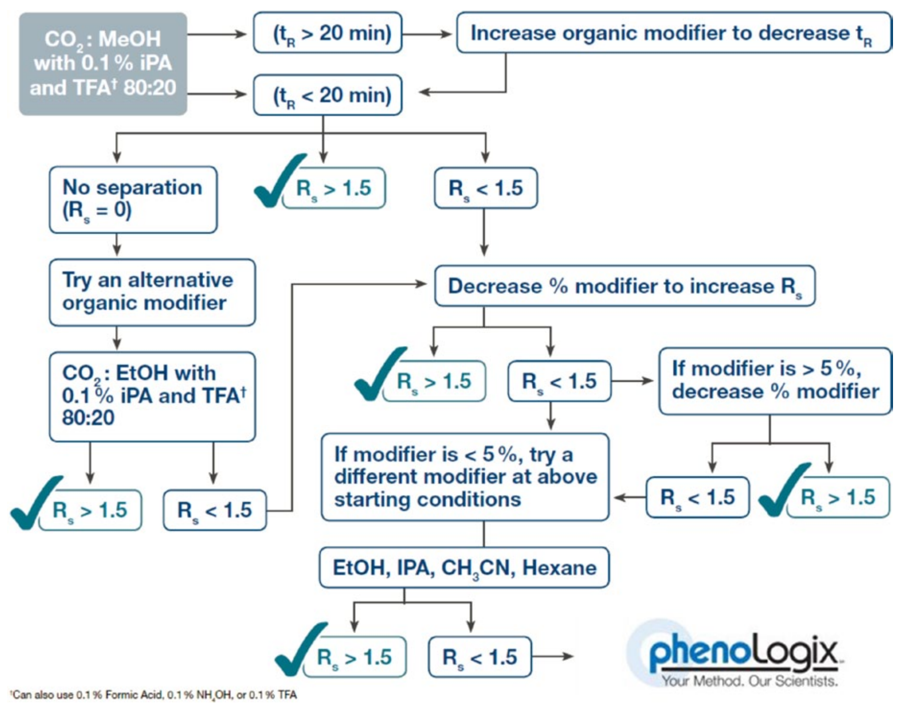
Chankvetadze B., Farkas T. *et al. J. Chromatogr. A* **2011** p 6554.

mode, and column phase, among other parameters. The results for each search include column details, running conditions, retention times, type of detector used, selectivity, and compound structure.

Another approach is to use the application structure search. Here, researchers can use tools similar to ChemDraw™ to draw structures and find matches to compounds already on the database. Structures can also be copied from other sources and pasted into the search window. The result of the search is a set of potential match compounds that either have the same structure or are very similar, with the relevant applications and methods available for their separation.

A third approach is to take advantage of the PhenoLogix™

Figure 7: SFC screening suggestion - SFC.



†Can also use 0.1 % Formic Acid, 0.1 % NH_4OH, or 0.1 % TFA

free chiral screening service by sending a sample of the compound of interest to their facility. A large number of Lux CSPs will be screened in the common separation modes (NP, RP, and PO), or even by SFC (upon request). Typical turnaround times are 10 days; confidentiality agreements are accepted if requested.

Summary

Polysaccharide based CSPs offer wide chiral recognition ability in a variety of HPLC and SFC separation modes using the full range of traditional mobile phases, with high selectivity. Recent advances in immobilization technology increases the solvent resistance and robustness of polysaccharide CSPs significantly, allowing for the use of stronger solvents in the mobile phase and in the cleaning of the columns. Improvements in preparative column packing technology involving internal pistons improve the loadability and efficiency of the columns, decreasing column-to-column variations and making them especially useful for SFC and purification methods. Several online application search tools are available to researchers for quickly finding available methods and conditions for chiral separations using polysaccharide based CSPs.

References

1. Chankvetadze B., *Journal of Chromatography*, A **2012** p 26 (Review)
2. De Klerck K.; Mangelings D.; Vander Heyden Y. *J. of Supercritical Fluids* **2013**, p 50-59