Chromatography Today Help Desk

Trouble with chiral separations

Introduction

The concept of a mirror image not being superimposable on certain shapes is a fundamental concept for understanding chirality. It is also a fundamental consideration when designing or choosing a suitable column for the separation or purification of a pair of enantiomers. Understanding how individual molecules interact through a three-point interaction, will ultimately ensure that the required selectivity is achieved in the separation. The required specificity of the separation is highly dependent on the two modes of retention for each of the chiral components. This has resulted in a multitude of new columns and also a wealth of research that investigates, at a molecular modelling level, the different interactions that each of the enantiomers have with the stationary phase.

This very highly selective nature of the stationary phase can also result in issues, as small changes to the surface can impact the selectivity of the column selected to separate the two components. This article will discuss how this very specificity can be a cause of problems when trying to perform quantitative analysis of an enantiomeric sample. It will look at highlighting the issue of additive memory effect, and how this impacts on the quantitative analysis. Finally, the article will then look to propose a range of solutions to address these challenges.

Problem statement

Analytical scientists are typically posed two broad questions.

1. What is in the sample?

2. How much is present of each component in the sample?

These questions are not trivial to resolve, and quantification of enantiomers presents even greater challenges, potentially unique challenges, that are not necessarily seen by a broader range of analytes. These challenges are primarily associated with the choice of column and how to obtain a separation. It is evident from the multitude of chiral stationary phases that are routinely employed in enantiomeric separation compared to the ubiquitous C18 stationary phase used for non-chiral based separations that there is not a single chiral column that separation scientists prefer.

In many separations a gradient is used to allow very differently retained components to be eluted in a reasonable amount of time, and to ensure that the peak width is reasonably consistent across the range of the separation, making quantification easier. The use of gradients, however, does result in an increase in the overall cycle time to analyse a sample, as part of the run is spent re-equilibrating the system ready for the next injection. For chiral separations, it is much more common to run the separation in an isocratic mode, as the compounds will have exactly the same chemical retention, and as a consequence there is a greater reliance on the nature of the stationary phase to separate rather than changes in a mobile phase.

The use of isocratic mobile phase compositions, although reducing or eliminating the re-equilibration time, does mean that there may not be a full elution of everything that has been injected onto the column. The issues associated with samples analysed using chromatography coupled to mass spectrometry due to very late eluting compounds [1] has been widely discussed previously. However, the help desk has not discussed the phenomena where not all all of the sample components are eluted from the column in a single cycle; which is associated with sample components actually changing the nature of the stationary phase. Components of the sample that are irreversibly adsorbed onto the surface of the stationary phase, can have an impact on the retention mechanism for all or part of the column resulting in the additive memory effect [2,3,4]. This can occur in all forms of separation if the column is not effectively reset after each injection, however it is much more prevalent in isocratic separations, and in particular separations which are very sensitive to small changes in the nature of the stationary phase.

As with all separations, chromatographers demand that the separation is robust from column to column. There are many examples that exist of separation scientists developing new methods on a column, only to find that this separation no longer performs to the same specifications when a new column is purchased [5]. This is often attributed to a lack of robust QC procedures within column and stationary phase manufacturing, which is very unfair on an industry that has dramatically improved the quality control criteria and tests performed on columns and stationary phases. For reversed phase columns the column manufacturers will routinely check batches of materials using a variant of a Tanaka test [6,7] and not just monitor the retention time and peak width of an aromatic hydrocarbon. For chiral columns the level of quality checks is even more robust, due to the nature of the separation.

However, in many cases it can be the method that causes the issue. The Help Desk has discussed this on a few occasions based on the premise that the assay is inherently unstable [5]. In this article a slightly different scenario will be discussed which is highly relevant to chiral separations. The use of additives and the possibility of an additive memory effect will be discussed and how this can be avoided by using appropriate solvents and wash systems to reduce the adsorption of an additive to the chiral stationary phase.

Setting the scene

There are a range of chiral stationary phases on the market and these can be very broadly broken down into six generic classifications [8,9].

- Pirkle phases
- Polysaccharide/Carbohydrate based phases where the primary mechanism for the formation of an analyte – Chiral Stationary Phase (CSP) complex is through attractive interactions, but where inclusion complexes also play an important role

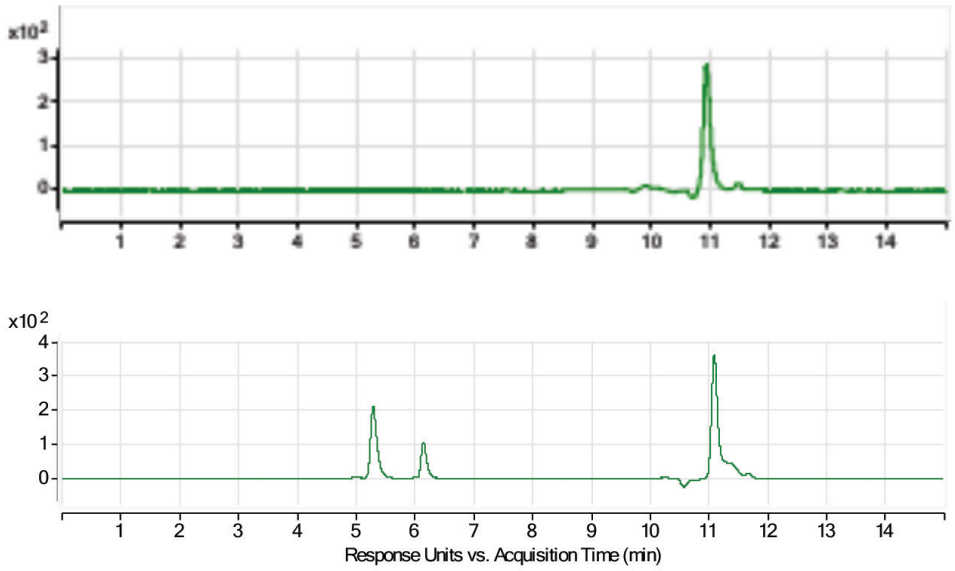

Figure 1: Irreversible adsorption occurring on a prototype SEC column, compared to final column. The three main peaks relate to Fab, Fc fragments of an IgG protein and the solvent peak. It is very evident that the peaks are not eluting due to adsorption issues.

- Cyclodextrins and crown ethers where the analyte forms an inclusion complex
- Chiral ligand exchange chromatography where the analyte forms part of a metal diastereo-isomeric complex
- Protein based phases

The nature of the retention mechanism of any component is complex with many of these columns due to the complexity of the stationary phase, which will typically have at least three modes of retention and have a very specific topography to ensure a suitable retention and separation. Small changes to the nature of the stationary phase can be very impactful on the separation when resolving chiral components [10]. An example of such sensitivity can be seen where changing the length of the linker group to a Pirkle style CSP [10], resulted in a substantially improved separation for the enantiomers under investigation. The explanation for this relates to greater access to a potential binding site, at the bottom of the CSP, which is sterically blocked with a short chain linker, but becomes accessible when a longer chain linker group is used.

It is often assumed, incorrectly, that everything that is injected onto a HPLC column will eventually elute from the column in the first injection. There are many examples in the world of protein analysis where it is not uncommon for the column to require several injections to stabilise the stationary phase and separation. Figure 1 shows an example of an irreversible adsorption occurring on a prototype SEC column. It can be clearly seen that on the first injection there are no peaks eluting which correspond to the F_c and F_{ab} fragments of the IgG protein that was injected on the system. The subsequent injections show the compound eventually eluting, and by the third injection the compounds are fully eluting from the column. In this scenario the analyte is changing the nature of the stationary phase making it more inert when the proteins initially adsorb; after the surface has been modified there is no further adsorption of the proteins. For size exclusion the use of an isocratic mobile phase is common, and the retention mechanism [11] is based on the available volume within the column that is accessible to the molecule of interest. Size exclusion separations are not based on any form of adsorption occurring, and so in this case it is very evident that there is an issue with the initial inertness of the column, which is rectified by a few conditioning injections. One of the reasons for choosing this example is that the mobile phase is not highly elutropic, and so adsorption can be irreversible, which will obviously change the nature of the stationary phase.

In the example of the protein separations it is very evident what is happening in the separation, as no peak elution occurred. In order to identify the impact this effect may have on a chiral separation, Ye [12], looked at the effect of adding an ethanesulfonic acid (ESA) modifier to the mobile phase for the separation of a series of amino acid esters. Table 1 gives the summary of the findings from Ye's work

Table 1: The effect of the column history, specifically the use of ethanesulfonic acid, on the separation factor for a series of chiral separations. In all cases a ChiralPak AD column was used with ethanol-hexane mobile phase (12).

Test probe	ESA used on column prior to analysis	Separation Factor
Leucine, isobutyl ester	No	1.16
	Yes	1.67
Leucine, methyl ester	No	1.17
	Yes	1.53
Phenylalanine, methyl ester	No	1.12
	Yes	1.85
4-Cl-Phenylalanine, methyl ester	No	1.17
	Yes	2.95
4-Cl-Phenylalanine, ethyl ester	No	1.09
	Yes	4.43

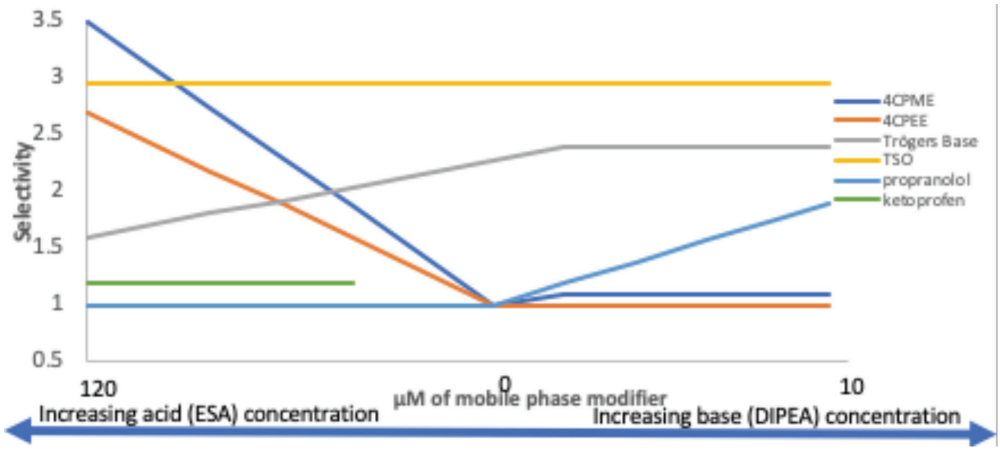

Figure 2: The effect of altering the mobile phase additive concentration from acid to basic on the selectivity for a series of enantiomers (4CPME, 4CPEE, Trögers Base, TSO, propranolol and ketoprofen).

where it can be seen that the ESA can have a pronounced effect on the separation and so suggests that it could be an ideal probe to evaluate the memory effects associated with mobile phase additives in chiral separations.

This work was taken further by Guiochon [13] who did a more systematic study of the impact of mobile phase additives on the retention of different enantiomer pairs on a column packed with amylose tris (3,5-dimethylphenylcarbamate), ChiralPak AD (Chiral Technologies, Daicel Group), stationary phase. The researchers used previously obtained data to construct a calibration curve for the resolution of a select group of compounds as a function of the mass of acid (ESA) or base N, N DiIsoPropylEthylAmine (DIPEA) loaded onto the column. The effective mass loading of either acid or base was from 100 µmol ESA to 100 µMol DIPEA. A range of compounds were chosen including 4 Chloro Phenylanaline Methyl Ester (4CPME), 4 Chloro Phenylanaline Ethyl Ester (4CPEE), Trögers base, TransStilbene Oxide (TSO), propranolol and ketoprofen. A schematic of the data collated is shown in Figure 2.

Once the calibration data had been collected the researchers took 5 different ChiralPak AD columns which had different mobile phase histories to determine the extent that either an acid or base had induced a memory effect on the column. The mobile phase used was 90/10 (v/v) hexane/ethanol, and the same mobile phase solution was used for all of the experiments. All samples (4CPEE, 4CPME, ketoprofen, propranolol, Tröger's base, and TSO) were made at a concentration of approximately 1 mg/mL in a solution of 90/10 (v/v) hexane/ethanol. Each column was kept at a temperature of 40°C when in use, and the flow rate was chosen to ensure that the void marker eluted at the same time from the different columns. All columns were equilibrated for at least twenty column volumes with the mobile phase prior to having samples injected. The injection sequence followed for each column was: 4CPEE, 4CPME, ketoprofen, propranolol, Tröger's base, and then TSO.

The data showed that the 4 chlorophenylanaline compounds could be used successfully as a test probe to indicate the mobile phase history of the column, with the other test compounds not being ideal. The authors concluded that the supposed steady state that the columns reached was more akin to a saddle point, Figure 3, as to a true stable point hinting that the history of the column could be very impactful on the current separation, and that trying to re-establish the original separation may require a substantial effort, if indeed it is possible at all.

Thus, there is clearly a potential issue with additive memory effects when using base or acidic modifiers for chiral separations. The use of normal phase isocratic separations can potentially increase the sensitivity of these types of separations [2], where small amounts of water present in the mobile phase can be impactful on the retention mechanism, increasing the persistence and

Figure 3: An example of a saddle point. It should be noted that this is not the most stable solution but does represent a part of parameter space where a steady state exists and to achieve the original steady state would require a large degree of perturbation.

stabilisation of the memory effect. It was noted that the persistence of the memory effect can last for several thousands of column volumes.

It is therefore evident that in developing a chiral separation that some care has to be taken to ensure the history of the column does not impact the robustness of the assay. The use of acidic or basic modifiers has been shown to impact the relative retention and this must in some manner be based on the modifier interfering with the binding between the CSP and the analyte. The fact that some separations have virtual stable saddle points means that re-equilibration may not be a viable solution and instead when developing a new chiral separation, use of an appropriate test to determine the degree of memory effect that is present may be advisable, or use a new column and record carefully the history of the column.

It is very evident that the history of the column, particularly in chiral separations can have a substantial impact on the observed resolution. It is common in purification studies to retain a column for the duration of the project for just such reasons. Tracking and recording the usage of a column is also critical to ensuring the effective performance of an assay. Simple measures like these can be very impactful and ensures

avoiding the pain of having to redevelop an assay. In these times of great uncertainty, having a robust test that can be applied uniformly applies not just to the world of separation science but perhaps gives direction for the even greater challenges that we face.

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