How to improve the analysis of hydrophobic compounds

If one asks chromatographers what are the biggest challenges in RP-LC, very hydrophobic compounds will definitely be one of the answers. What makes them so difficult for reversed phase techniques? First, these substances usually have extended elution times on C18 columns, which results in higher amounts of organic solvents and valuable lab time being required. Also they may elute only partially or not at all which challenges the chromatographer's patience even more. Apart from chromatographic results, finding the appropriate sample solvent is also something that has to be strongly considered.

In summary, hydrophobic analytes demand a careful method development investigating nearly every accessible setting in LC. In this expert tip, the most important issues with hydrophobic substances are discussed and solutions are provided on how to overcome them.

- **Suitable stationary phases**
- **Consideration for mobile phases**
- **Sample solvents**
- **Carryover**
- **Structurally similar analytes**

1. Choose the right stationary phase

High retention times

Due to their high hydrophobicity these compounds elute late on C18 columns, which consumes time and solvents. Decreasing the stationary phase's hydrophobicity will remedy the situation. Choosing shorter alky ligands compared to C18 leads to less hydrophobic interactions between the analyte and the stationary phase. This results in shorter retention times without sacrificing the selectivity. In Figure 1 the comparison between a YMC-Triart C18 and a C8 column using the same chromatographic conditions is shown. The elution order stays the same, but the retention times decrease significantly, for example by 40% for triphenylene (peak 9).

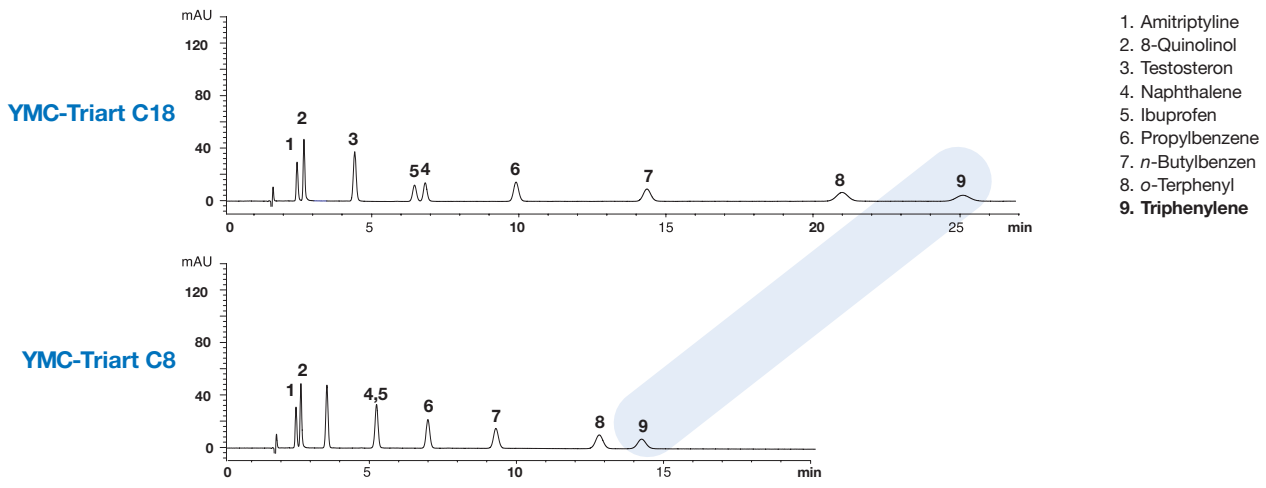

Figure 1: Comparison of the retention times using YMC-Triart C18 and C8 columns.

Chromatographic conditions

Columns:	YMC-Triart C18 (5 µm, 12 nm) 150 x 3 mm ID YMC-Triart C8 (5 µm, 12 nm) 150 x 3 mm ID
Part Nos.:	TA12S05-1503PTH TO12S05-1503PTH
Eluent:	20 mM H_3PO_4-KH_2PO_4 (pH 3.1)/methanol (25/75)
Flow rate:	0.425 mL/min
Temperature:	40 °C
Detection:	UV at 265 nm

Carryover or no elution

In addition to high retention times it is also possible that hydrophobic analytes elute only partially or even worse: they don't elute at all, even when using 100% organic solvent. This is the case if the hydrophobic interactions between the analyte and the stationary phase are very strong and the mobile phase's elution strength is not high enough. To minimise this, analyses can be run using increased temperatures, but reducing the hydrophobicity of the stationary phase may also be needed. As an example, reduced peak areas due to carryover were observed for the analysis of hydrophobic amyloid β peptides using a YMC-Triart C18 column. By choosing YMC-Triart Bio C4 with a shorter alkyl ligand, carryover was diminished and retention times reduced.

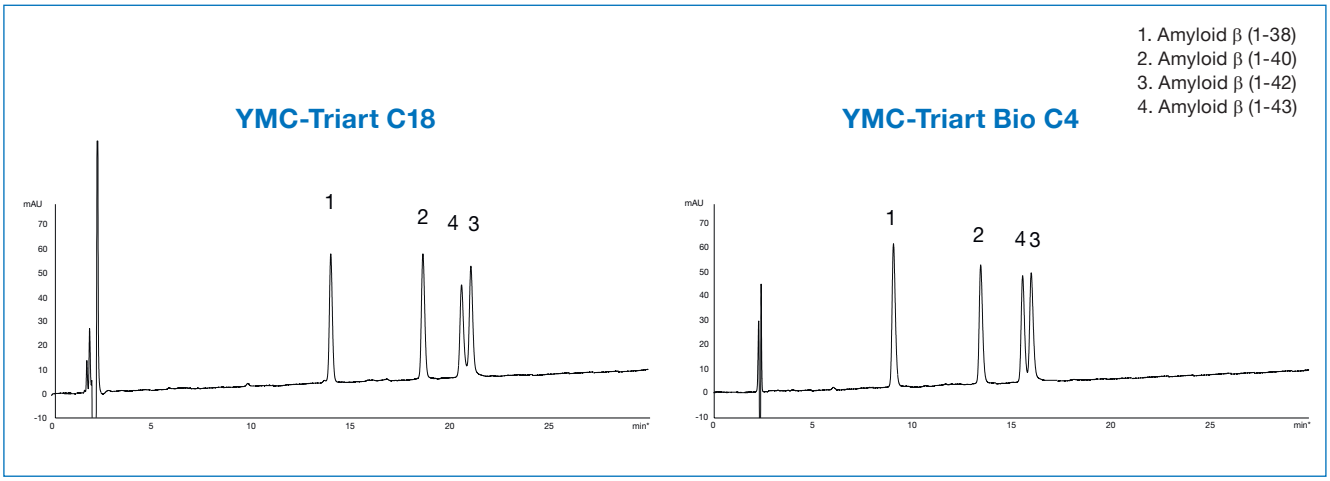

Figure 2: Separation of amyloid β peptides using YMC-Triart C18 and YMC-Triart Bio C4 columns.

Chromatographic conditions

Columns:	YMC-Triart C18 (5 µm, 12 nm) 150 x 3 mm ID YMC-Triart Bio C4 (5 µm, 30 nm) 150 x 3 mm ID
Part Nos.:	TA12S05-1503PTH TB30S05-1503PTH
Eluent:	A) water/TFA (100/0.1) B) acetonitrile/TFA (100/0.1)
Gradient:	25–40%B (0–30 min), 90%B (30–40 min), 25%B (40–55 min)
Flow rate:	0.4 mL/min
Temperature:	70 °C
Detection:	UV at 220 nm
Injection:	4 µL (each 0.1 mg/mL)

Structurally similar compounds

If structurally similar compounds have to be separated the selectivity of the stationary phase is crucial. Using conventional C18 columns often is not sufficient to achieve sufficient resolution. Figure 3 shows the separation of the hydrophobic vitamins D_2 and D_3 using a conventional C18 (YMC-Triart C18) column and a high carbon load C18 column (YMC-Triart C18 ExRS). The resolution increases considerably using a YMC-Triart C18 ExRS column since it has a great selectivity for hydrophobic and structurally related substances.

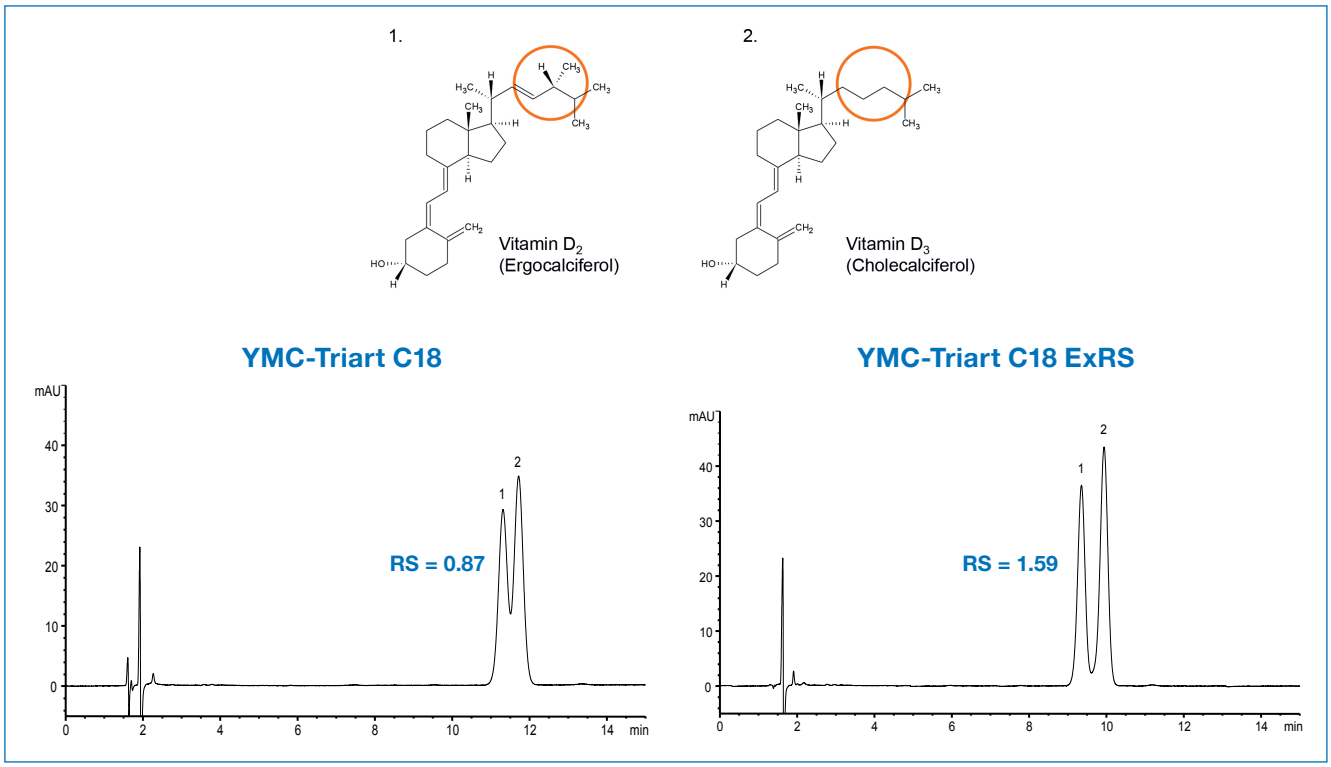

Figure 3: Separation of vitamin D_2 and D_3 using YMC-Triart C18 and YMC-Triart C18 ExRS columns.

Chromatographic conditions

Columns:	YMC-Triart C18 (5 µm, 12 nm) 150 x 3 mm ID YMC-Triart C18 ExRS (5 µm, 8 nm) 150 x 3 mm ID
Part Nos.:	TA12S05-1503PTH TAR08S05-1503PTH
Eluent:	THF/acetonitrile (10/90)
Flow rate:	0.425 mL/min
Temperature:	30 °C
Detection:	UV at 265 nm
Injection:	4.25 µL (10 µg/mL)

2. Consider the mobile phase

Another option is to make the mobile phase more attractive for the non-polar analytes and decrease the polarity. For example, instead of using water/methanol mixtures, tetrahydrofuran (THF) and acetonitrile can be used as eluents as used in the vitamin application shown above in Figure 3. Both solvents provide a lower polarity. However, it is very important to consider the miscibility of the solvents. Figure 4 shows the miscibility of commonly used solvents.

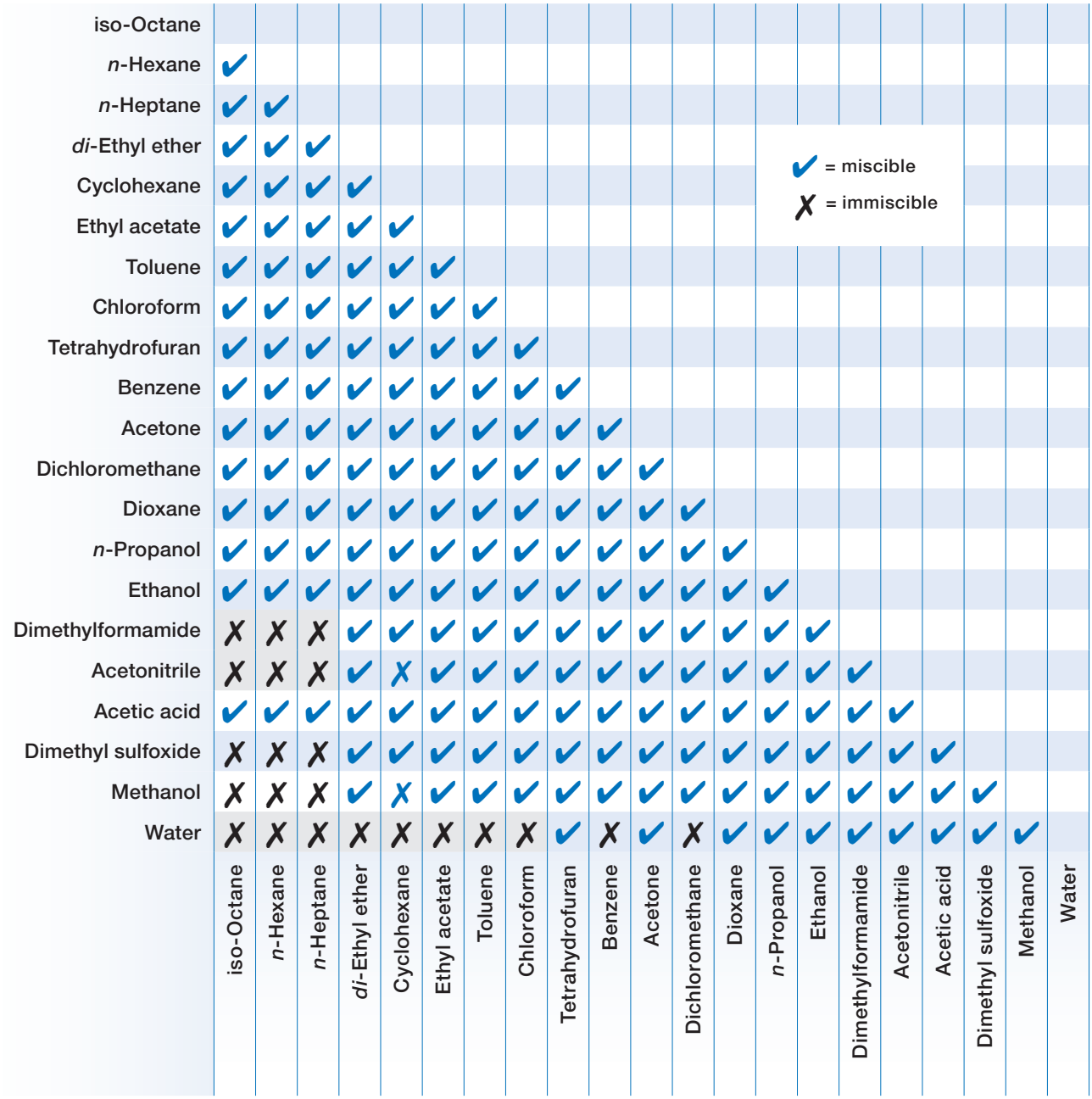

Figure 4: Miscibility of commonly used solvents.

3. Check the solubility

To dissolve very hydrophobic analytes, organic solvents are required as these compounds are nearly insoluble in water. However, organic solvents have high elution strengths in RP chromatography which may lead to deformed peak shapes. Diluting the sample can be one way to overcome this obstacle. However, if gradient conditions are used it is essential to make sure the sample is soluble over the entire gradient and not just for starting and/or finishing conditions. If the sample precipitates inside the column, it will block the column resulting in increased backpressure and decreased column performance.

If deformed peak shape occurs, reducing the injection volume can also be useful as the total amount of organic injected is reduced. However, the final injection volume depends of course on the analysis. As a rule of thumb, the injection volume should be 0.1% or less of total column volume, when injecting in solvents stronger than your initial mobile phase. Total column volumes of commonly used column dimensions are shown in Table 1.

Table 1: Total column volumes [mL] for several column dimensions.

Length/ID	1.0 mm	2.0 mm	3.0 mm	4.6 mm
50 mm	0.05	0.2	0.4	0.8
100 mm	0.08	0.3	0.7	1.7
150 mm	0.1	0.5	1.1	2.5
250 mm	0.2	0.8	1.8	4.2

In addition, the solubility differences for various solvents have to be considered. For example, lipids show a higher solubility in alcohol compared to acetonitrile. But using methanol as a sample solvent and acetonitrile as mobile phase will have a negative effect on the chromatography and therefore, the organic solvent in the sample should be the same as the one used in the mobile phase.

Conclusion

The analysis of hydrophobic substances can be challenging. During method development many things have to be considered in order to install a reliable and robust method. But addressing the issues mentioned above will help to establish a method the chromatographer is satisfied with. Therefore, for all future method development:

- ✔ **Choose the right stationary phase**
- ✔ **Consider the mobile phase and**
- ✔ **Check the solubility.**

Even if issues in your analysis are not obviously visible, keeping the underlying pitfalls connected to very hydrophobic compounds in mind will improve your routine work, speed up any troubleshooting and lead to more robust and reproducible methods. For example, implementing high organic column wash steps into your method can greatly improve column performance and life time.