Reducing adsorption on metal surfaces

The analysis of certain sensitive molecules is challenging when using a standard reversed phase liquid chromatography (RP) or hydrophilic interaction liquid chromatography (HILIC) method. For sensitive compounds such as certain peptides and proteins as well as oligonucleotides, glycans and several small coordinating compounds often peak tailing, loss of recovery and/or retention time shifts are observed.

This behaviour is due to undesired interactions of the analytes with metal surfaces present in the system including the column hardware. This non-specific adsorption on metallic surfaces predominantly occurs with acidic molecules containing e.g., phosphate or multiple carboxylate moieties.

This adsorption and the associated peak deformations strongly depend on the analyte and chromatographic conditions such as pH and ionic strength of the mobile phase. Increasing the ionic strength can lead to decreased adsorption while a low to neutral pH usually increases the adsorption, because the metal surface is more electropositive under these conditions. Furthermore, the adsorption is increased with the number of these moieties. The example in figure 1 demonstrates this phenomenon. Adenosine triphosphate (ATP) containing three phosphate groups shows much higher adsorption as it does not even elute from the column compared to adenosine di- (ADP) or monophosphate (AMP). In addition ADP also still shows a more deformed peak shape compared to AMP.

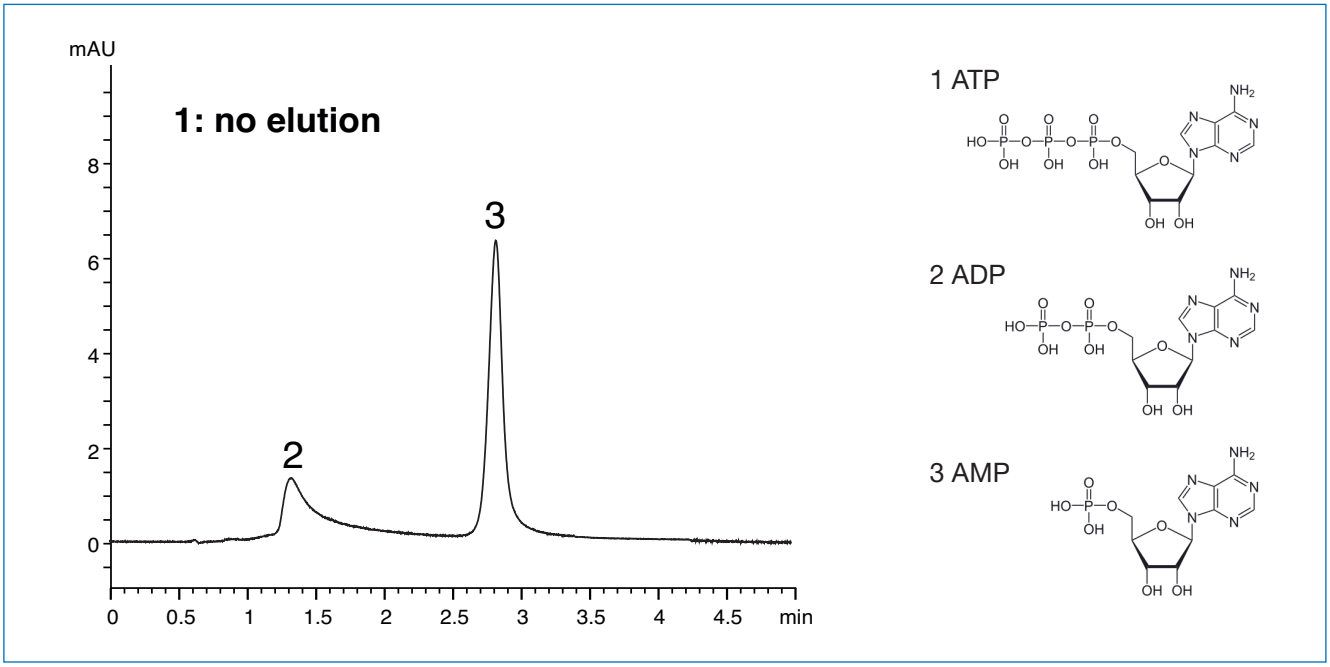

Figure 1: Analysis of ATP, ADP and AMP with a standard RP setup.

Chromatographic conditions

Column:	YMC-Triart C18 (3 µm, 12 nm) 50 x 2.1 mm ID
Part No:	TA12S03-05Q1PT (standard hardware)
Eluent:	5 mM $HCOONH_4$
Flow rate:	0.21 mL/min
Temperature:	25 °C
Detection:	UV at 265 nm
Injection:	1 µL (10 µg/mL)

This makes the analysis of sensitive compounds extremely challenging. Different measures are found to optimise the separation of these substances:

- Passivation of the system and column
- Additives to the mobile phase
- Sample pre-conditioning of the system and column
- (Bio)inert system and column

Passivation of the system and column

There are several approaches to passivate stainless steel systems. Citric, nitric or phosphoric acid as well as etidronic acid can be used for this treatment [1]. During the passivation procedure a chromium oxide (Cr_2O_3) layer is formed while iron is dissolved at the metal surface. This prevents ionic interactions with metal surfaces. A possible passivation procedure is the following [2]:

1. Flush the system with 0.5 % phosphoric acid in acetonitrile/water (90/10) overnight (16 h) at room temperature
2. To clean the system, flush it with ultrapure water for 1 h.

One has to make sure that the stationary phase is stable at such low pH before performing this procedure.
Another way to passivate the system is a treatment with an EDTA-solution. For example, Fleitz et al. flushed their system with 40 mM EDTA-Na_2 solution for 24 h and could decrease the adsorption of multiply phosphorylated peptides effectively for 3 months [3].

However, the treatment's durability is limited and must be repeated regularly. In some cases, adsorption increases after a few hours of run time. Presumably, the adsorption of multivalent ions such as phosphate, citrate or etidronate is in competition with the adsorption of sensitive ionic analytes such as multiple phosphate or carboxylate containing compounds.

Additives to the mobile phase

In case the column is not stable to a passivation process, selected additives which mask the active sites can mitigate adsorption. The addition of citric or phosphoric acid, EDTA or medronic acid to the mobile phase or sample diluent effectively minimises sample adsorption [1].
An effective reduction of adsorption can be achieved by adding 1 ppm citric acid to the mobile phase [4].

A second approach is to add e.g., citrate to the sample diluent. For phosphorylated peptides a 50 mM citrate solution as sample solvent could effectively decrease the adsorption [5].
Nevertheless, these additives have an adverse effect on detection with mass spectrometry (MS) since they are not volatile or lead to ion suppression.

Sample pre-conditioning of the system and column

Another effective treatment to prevent ionic interactions with metal surfaces is to condition the system including the column with the target or a similar substance. In this case, the metal surface is saturated with substance so that further adsorption is prevented. While this is effective for some analytes it is not a permanent measure. The effect can be reduced due to sample change e.g. longer oligonucleotides interact more strongly with the metal surface.

Furthermore, cleaning with a basic wash leads to a sample desorption, so that the pre-conditioning must be repeated after such cleaning processes.

Sample pre-conditioning is depended on the sample itself, the system and the column. Lardeux et al. observed gradually increased peak areas with a plateau reached after 15 injections of an oligonucleotide sample [6].

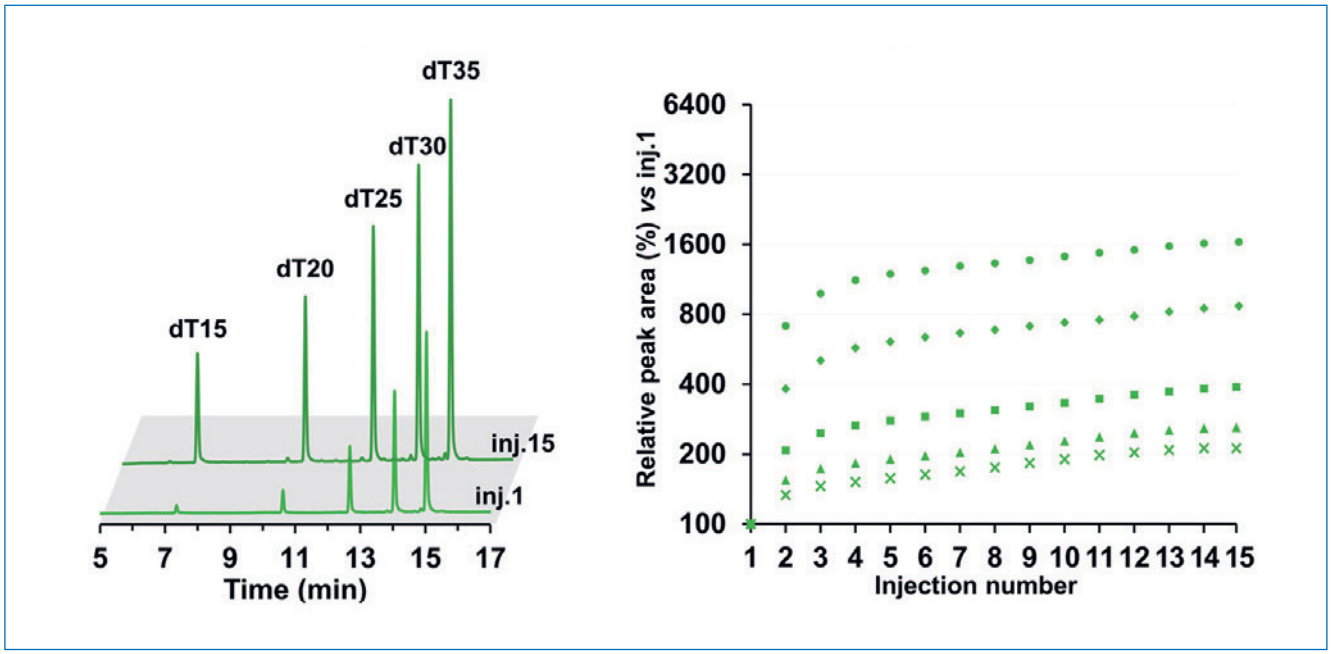

Figure 2: Pre-conditioning with an oligonucleotide mixture [6].

Chromatographic conditions

Column:	YMC-Triart C18 (1.9 µm) 150 x 2.1 mm ID
Part No.:	TA12SP9-15Q1PT
Eluent:	A) 15 mM TEA – 400 mM HFIP* (pH 7.9)
	B) 15 mM TEA – 400 mM HFIP*/methanol (50/50)
Gradient:	30–50%B (0–20 min)
Flow rate:	0.4 mL/min
Temperature:	70 °C
Detection:	UV at 260 nm
Injection:	2 µL
Sample:	Deoxythymidine oligonucleotide mixture dT15–35 (2 µM)

**1,1,1,3,3,3-hexafluoro-2-propanol*

(Bio)inert system and column

As all the above measures are temporary, new (bio)inert systems and column hardware have been developed. The column body represents more than 70% of the surface that the analytes is in contact with [6]. Consequently, a (bio)inert column body and frits will provide a distinct improvement in performance. Different bioinert concepts are available: a bioinert coating of the stainless steel column body and frits, PEEK-lined stainless steel columns in combination with PEEK frits, and columns made of titanium. A bioinert coating and a PEEK-lining are preferable to titanium because, with the latter, metal erosion can still occur, resulting in a contamination of the silica bed. The eluted titanium is able to cause an ionic bonding with free residual silanol groups causing adsorption of the analytes onto the stationary phase. This effect is particularly pronounced for stationary phases without or with insufficient endcapping [2]. Therefore, YMC provides the bioinert YMC-Accura Triart series of columns which have a bioinert coating on the column body and frits as well as the PEEK-lined YMC-Triart metal-free columns.

Figure 3 shows the results Lardeux et al. received using a YMC-Triart C18 metal-free column. The column provided high recovery and sharp peaks from the first injection. Furthermore, a comparison of the peak areas reveals that the recovery is higher using a bioinert column even after pre-conditioning the standard stainless steel column [6].

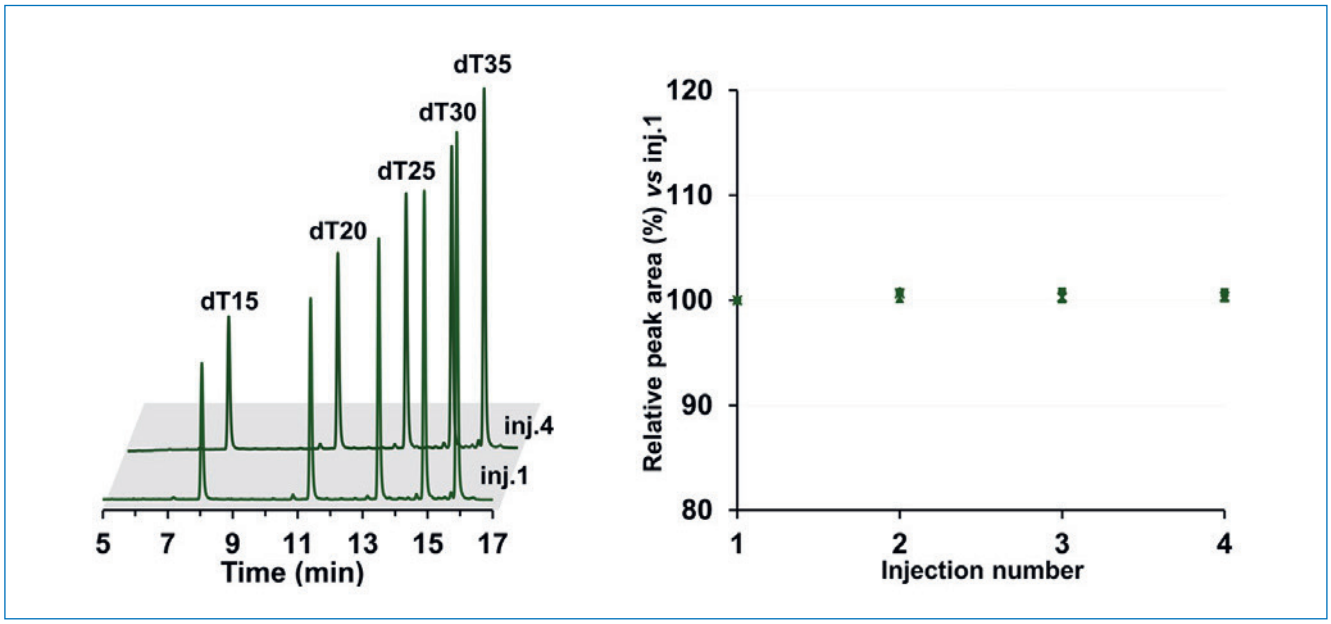

Figure 3: Consecutive injections of an oligonucleotide mixture using a YMC-Triart C18 metal-free column. No pre-conditioning is necessary [6].

Chromatographic conditions

Column:	YMC-Triart C18 metal-free (1.9 µm) 150 x 2.1 mm ID
Part No.:	TA12SP9-15Q1PTP
Eluent:	A) 15 mM TEA – 400 mM HFIP* (pH 7.9)
	B) 15 mM TEA – 400 mM HFIP*/methanol (50/50)
Gradient:	30–50%B (0–20 min)
Flow rate:	0.4 mL/min
Temperature:	70 °C
Detection:	UV at 260 nm
Injection:	2 µL
Sample:	Deoxythymidine oligonucleotide mixture dT15-35 (2 µM)

**1,1,1,3,3,3-hexafluoro-2-propanol*

Expert tip

The recently introduced coated YMC-Accura column hardware is less hydrophobic compared to PEEK-lined hardware and more robust. In contrast to PEEK-lined hardware no special connectors are necessary.

In Figure 4 the inertness of the YMC-Accura column hardware is demonstrated with consecutive injections of an RNA ladder in comparison with a regular stainless steel column.

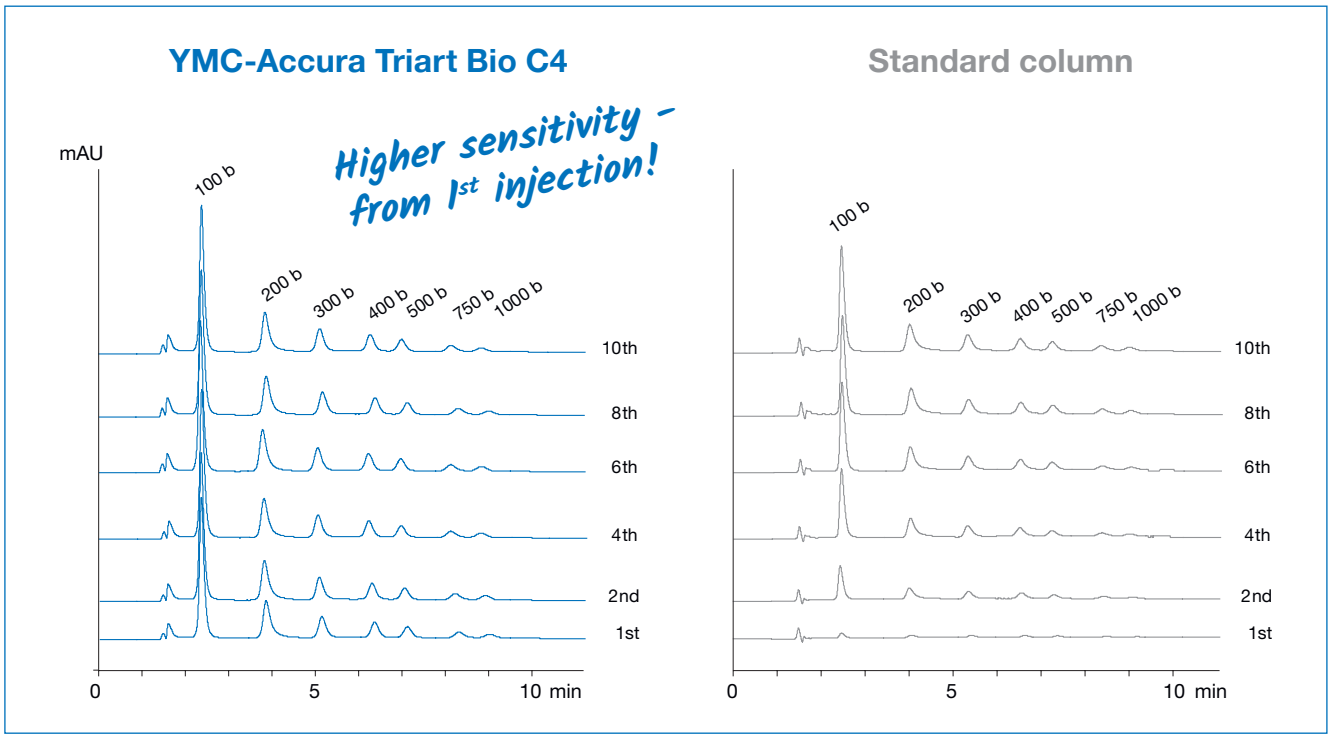

Figure 4: Analysis of an RNA ladder using the bioinert coated YMC-Accura Triart Bio C4 (left) and corresponding stainless steel column (right).

Chromatographic conditions

Column:	YMC-Accura Triart Bio C4 (1.9 µm, 30 nm) 100 x 2.1 mm ID
Part No.:	TB30SP9-10Q1PTC
Eluent:	A) 50 mM TEAA* (pH 7.0)/acetonitrile (95/5) B) 50 mM TEAA* (pH 7.0)/acetonitrile (50/50)
Gradient:	9–14%B (0–10 min), 80%B (10–15 min)
Flow rate:	0.2 mL/min
Temperature:	80 °C
Detection:	UV at 254 nm
Injection:	1 µL (0.25 mg/mL)
Sample:	100–1,000 bases (Century™-Plus RNA Markers)

* Triethylammonium acetate

References:

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