

## Comparison of different bioinert columns dedicated for oligonucleotide separations

Oligonucleotides have become more and more important in genetic testing, research, and forensics. To date a variety of oligonucleotide-based approaches have been developed for different pharmaceutical applications. This has required more robust and highly sensitive analytical methods.

Ion pair reversed phase liquid chromatography remains as the gold standard. However conventionally used stainless steel for tubing and column hardware poses a special challenge for the analysis of oligonucleotides. Stainless steel provides mechanical resilience and compatibility with most solvents although many eluents such as methanol or acetonitrile can cause corrosion. The resulting positively charged surface can lead to metal leaching as well as

undesired ionic interactions with the analytes. Due to their electron rich backbone oligonucleotides can be irreversibly adsorbed. This non-specific adsorption has a negative influence on recovery and peak shape. The effect is even more critical when working at low to neutral pH, as metals are more electropositive under these conditions. To overcome this problem HPLC systems and columns can be passivated with strong acids or pre-conditioned with a similar sample. However, these procedures are time consuming and a recurring task. Furthermore, a change of sample can lead to non-specific adsorption again. A much more robust and simple solution is to use a fully bioinert system and bioinert column hardware.



The column body represents more than 70% of the surface that the analytes be in touch with [1]. Consequently, a bioinert 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. Together with the inert and robust YMC-Triart stationary phases with multi-stage endcapping, the bioinert coated YMC-Accura hardware provides the ideal solution for the analysis of oligonucleotides.

## Oligonucleotide separation

In this technical note two phosphorothioate RNAs were analysed using different C18 columns:

- **YMC-Accura Triart Bio C18** – a widepore phase (300 Å) in bioinert coated hardware
- **XBridge Premier Oligonucleotide BEH C18** – a bioinert coated column with standard pore (130 Å) dedicated for oligonucleotide separations
- **the corresponding stainless steel column XBridge Oligonucleotide BEH C18**
- **bioZen Oligo** – a core-shell phase with a 100 Å pore dedicated for oligonucleotides in titanium lined hardware with titanium frits.

Table 1: RNA sequences.

5'-U*C*A*U*C*A*C*A*C*U*G*A*A*U*A*C*C*A*A*U-3'	(20mer)
5'-G*U*C*A*U*C*A*C*A*C*U*G*A*A*U*A*C*C*A*A*U-3'	(21mer)

\*=Phosphorothioated

The RNAs were analysed at an elevated temperature of 65 °C. Furthermore, two different buffer concentrations were examined:

- 15 mM triethylamine (TEA) in combination with 400 mM 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), which is the optimum buffer according to previous studies [3]
- 4 mM TEA combined with 100 mM HFIP, which is better suited for detection with mass spectrometry (MS).

Further details are listed in Table 2.

Table 2: Chromatographic conditions.

Columns:	YMC-Accura Triart Bio C18 (1.9 µm, 30 nm) 50 x 2.1 mm ID XBridge Premier Oligonucleotide BEH C18 (2.5 µm, 13 nm) 50 x 2.1 mm ID XBridge Oligonucleotide BEH C18 (2.5 µm, 13 nm) 50 x 2.1 mm ID bioZen Oligo (1.7 µm, 10 nm) 50 x 2.1 mm ID
Part number:	TA30SP9-05Q1PTC
Eluent:	A) i) 15 mM TEA - 400 mM HFIP; ii) 4 mM TEA - 100 mM HFIP B methanol
Gradient:	i) 8–23%B (0–30 min) ii) 0–25%B (0–30 min)
Flow rate:	0.42 mL/min
Temperature:	65 °C
Injection:	1.0 µL
Detection:	UV at 260 nm
Sample:	Phosphorothioate RNA (All PS RNA)

Figure 1 shows the separation of the two RNAs analysed with all four columns designed for oligonucleotides using conditions i). While YMC-Accura Triart Bio C18 provides sharp peaks and good resolution with baseline separated peaks, the other columns provide very poor peak shapes. The peaks of the XBridge Premier Oligonucleotide BEH C18

and the XBridge Oligonucleotide BEH C18 columns are not even partly separated. The bioZen Oligo column gives only one very broad peak. Remarkably, aside from the decreased recovery of XBridge Oligonucleotide BEH C18, the peak shape is slightly better compared to the corresponding bioinert coated column.

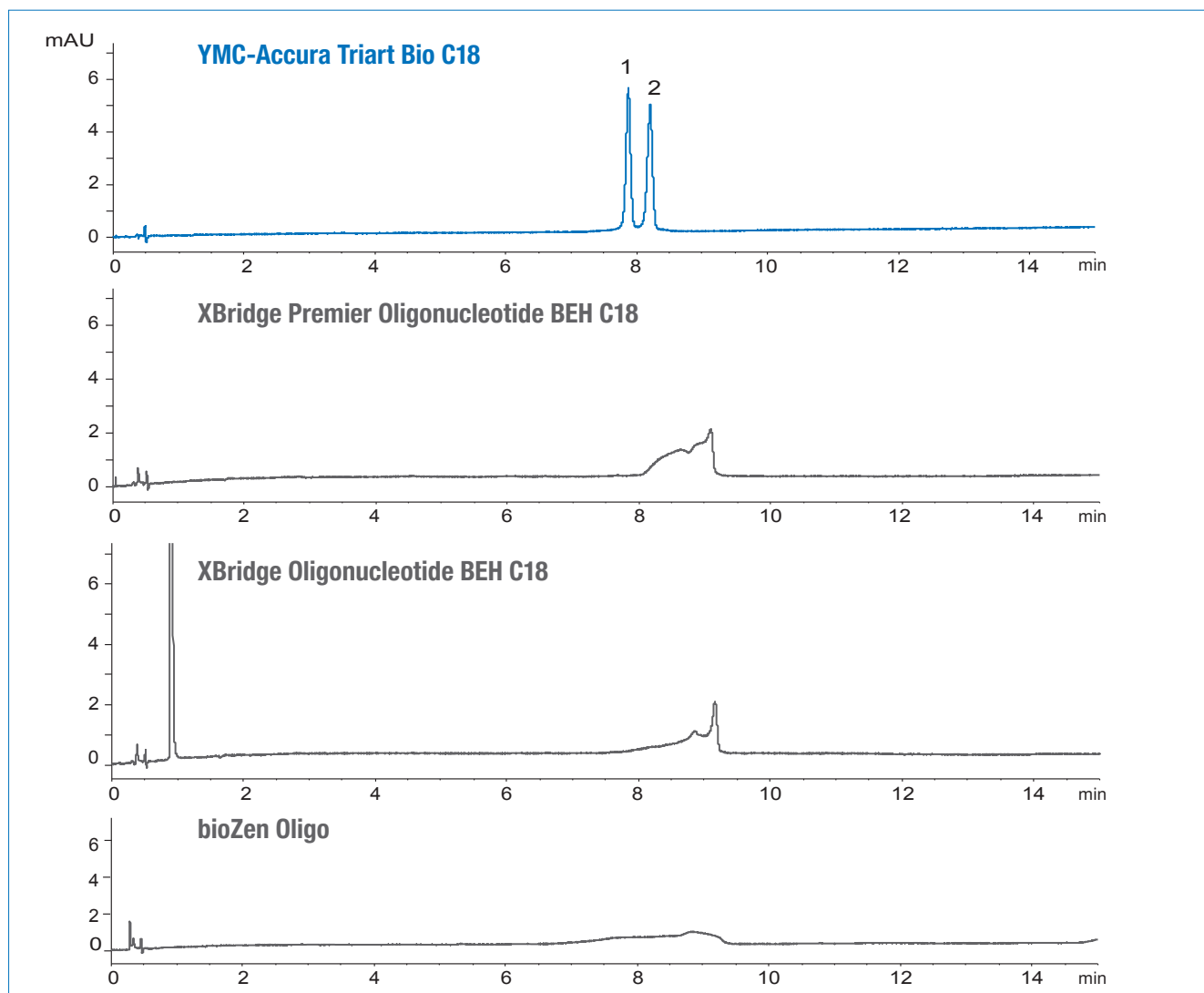


Figure 1: Separation of two PS RNAs (20 and 21mer) with 4 different C18 columns.

## Column reproducibility

To determine the reproducibility of the columns 30 consecutive injections were performed. Figure 2 shows that by using YMC-Accura Triart Bio C18 the peak shape, resolution and recovery remain consistent. Opposed to

this XBridge Premier Oligonucleotide BEH C18 shows slightly improved peak shapes while the corresponding stainless-steel column additionally shows higher retention. bioZen Oligo was not considered further for this test.

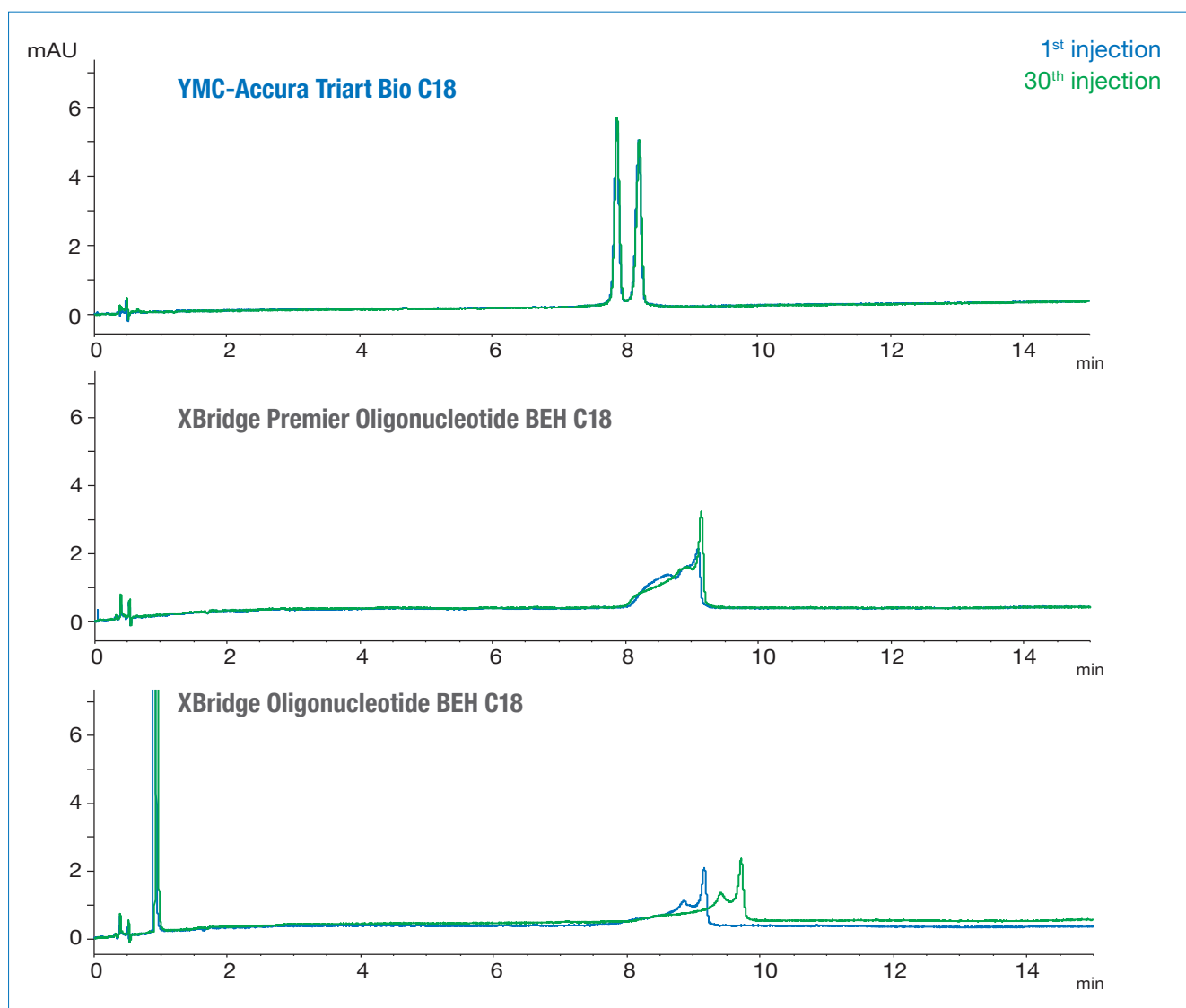


Figure 2: Comparison of 1<sup>st</sup> and 30<sup>th</sup> injection.

## MS compatibility

In addition, a buffer with lower concentration, 4 mM TEA-100 mM HFIP, was tested to obtain MS compatibility. Lower buffer concentrations usually led to decreased resolution in previous studies [3]. In comparison to the optimum buffer i) YMC-Accura Triart Bio C18 still provides good peak

shapes and resolution using the MS compatible buffer ii) (see Figure 3). XBridge Premier Oligonucleotide BEH C18 provides a slightly better peak shape and resolution and bioZen Oligo again shows one broad peak. The best results are again provided by YMC-Accura Triart Bio C18.

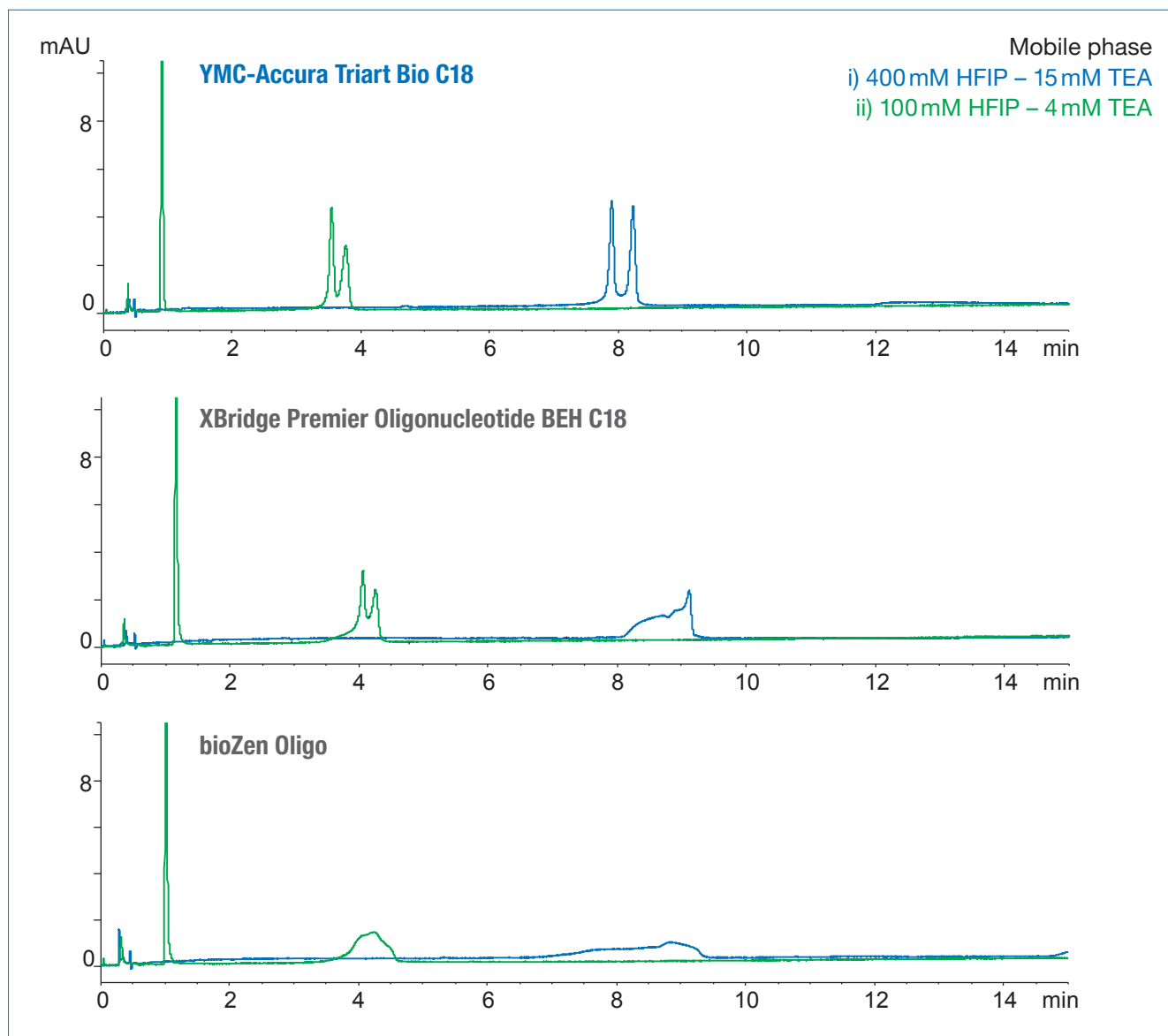


Figure 3: Analysis of two PS RNAs with 3 different C18 columns using a highly concentrated buffer i) (blue) and a buffer ii) (green) with lower concentration.

## Conclusion

For these short PS RNA oligonucleotides YMC-Accura Triart Bio C18 provides the optimum separation results compared to other columns specifically designed for oligonucleotide separations. In addition to the superior long-lasting bioinert column hardware, the larger pore of 300Å and general column selectivity might be the reason for these results. The results of XBridge Premier Oligonucleotide BEH C18 might be improved by buffer adjustments, but the column did not provide sufficient separation. The reason for the unexpected improved results using the lower buffer concentration

ii) is not fully understood. It is not likely that the smaller pore size is the cause as tests with the corresponding YMC standard pore size (120Å) gave the normally expected results (not shown). Nevertheless, a different pore design as well as differences in stationary phase selectivity could be decisive for this unique phenomenon. From a chromatographic point of view, YMC-Accura Triart Bio C18 provided the best peak shapes and resolution for both buffer concentrations.

Consequently, YMC-Accura Triart Bio C18 is an ideal tool for oligonucleotide analysis.

### References:

- [1] Gilar, M.; DeLano, M.; Gritti, F. *Mitigation of Analyte Loss on Metal Surfaces in Liquid Chromatography*. *J. Chromatogr. A* 2021, 1650, 462247. DOI: 10.1016/j.chroma.2021.462247
- [2] De Pra, M.; Greco, G.; Krajewski, M. P.; et al. *Effects of Titanium Contamination Caused by Iron-Free High Performance Liquid Chromatography Systems on Peak Shape and Retention of Drugs with Chelating Properties*. *J. Chromatogr. A* 2020, 1611, 460619. DOI: 10.1016/j.chroma.2019.460619
- [3] Daniel Eßer, Ken Tsutsui, Chiaki Matsumura, Noriko Shoji, Hiroki Kanezaki, *Analytical study of the Separation Behaviour of Oligonucleotides on IP-RPLC*, Poster by YMC presented at ISC 2022

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