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Note d'application

Properties of the Gen-Pak™ FAX Column and Its Utility for Anion Exchange Analysis of Large Molecule Biologics

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Abstract

With a burgeoning pipeline of protein- and nucleic acid-based therapies, there is a need for improved ion-exchange methods that can confirm the concentration, integrity and relative abundance of components found within drug substances and drug products.

It has been found that a weak anion-exchanger, known as the Gen-Pak FAX Column, exhibits comparatively low retentivity and higher kinetic efficiency for various protein and oligonucleotide separations. Comparative testing with alternative strong anion exchangers underscores the uniqueness of the Gen-Pak FAX Column chemistry. Stationary phase swelling has been found to be less significant with a Gen-Pak FAX Column such that high flow rates and low ionic strength mobile phases can be more readily applied. In addition, the unique elution behavior of the Gen-Pak FAX Column has made it possible to develop novel separation conditions and thereby achieve unseen selectivites for the separation of oligonucleotide impurities.

Benefits

- Improved peak capacity for small oligonucleotides and an example neutral *pI* protein

- Lower column pressure that facilitates higher flow rate and higher throughput analyses
- Low retentivity and unique selectivity that can be used to achieve new separations

Introduction

As a result of their intrinsically charged nature, biopolymers (*i.e.* peptides, proteins, oligonucleotides, and large nucleic acids) are ideal substrates for ion-exchange (IEX) separations. Sequence, post-translational modifications, molecule length, and the presence of various conformers and isoforms can correlate with differences in the overall net charge. Efficient fractionation of the biomolecules can thus be achieved through their electrostatic interactions with a charged stationary phase. Due to its usually mild, non-denaturing elution condition, IEX is often applied as part of a manufacturing process to purify various types of biologics.

Because IEX has the ability to finely resolve differences in surface charge, it has also become an ideal analytical tool for monitoring charge variants of therapeutic proteins and impurities of therapeutic oligonucleotides.[1,2,3,4,5]

Most commercially available, therapeutic monoclonal antibodies (mAbs) have isoelectric points (p*I* values) greater than 8. Cation-exchangers (CEX) are thus regularly applied for mAb charge variant analysis.[6] However, IgG4 sub-class mAbs, like natalizumab, tend to have lower p*I*s, which makes them potentially suitable for analysis by anion-exchange (AEX) chromatography.[7] It is therefore relevant to assess the capability of an anion exchanger for protein separations. With the emergence of CRISPR technology, it has become necessary to develop new analytical techniques for Cas nucleases and ribonucleoprotein complexes. AEX might help with these protein materials, as well.

Just as importantly, AEX columns might be of help to oligonucleotide and nucleic acid analyses. These types of biomolecules are negatively charged (pK_{a1} = 2.14) due to the phosphate groups in their sugar-phosphate backbone. AEX has already been applied for the purification and analysis of synthetic DNA, RNA, natural products, and plasmids.[8,9,10] With each nucleotide added to a growing chain, a nucleic acid takes on an extra negative charge, which directly contributes to increased AEX retention. In theory, AEX should therefore provide selectivity for oligonucleotides if differing lengths.[11] Despite the promise of this selectivity, some analytical scale AEX methods lack robustness, as a result of poor analyte recovery and high carryover effects. We recently proposed new method considerations to improve AEX separations.[5] One of the main considerations is the use of weak AEX stationary phases instead of strong exchangers. The use of a weak exchanger might result in higher

recovery due to the inherently weaker binding strength between the solute and the stationary phase functionalities at the loading of the sample, especially when and where mobile phase pH can be optimally tuned. [5] In our investigations, we have found a correlation between the retentivity of the stationary phase and the recovery and carryover of large nucleic acids.

With this application note, we have chosen to review the properties of the Gen-Pak FAX Column because it has recently shown promise during modern method development experiments. While the Gen-Pak™ FAX Column was first introduced by Waters in the early 90s, it is based on 2.5 µm non-porous polymer particle technology that can still be advantageously applied today.

Herein, we discuss three example separations achieved with the Gen-Pak FAX Column and its application to the analysis of biopolymers including (1) an IgG4 mAb, (2) a 15 to 35 mer oligonucleotide dT ladder and (3) a model mixture of antisense oligonucleotides.

Experimental

LC Analysis of Natalizumab and the Oligonucleotide Ladder

Sample preparation

Natalizumab (6 mg/mL) was obtained as an expired dose of Tysabri™ (natalizumab) (Biogen, Cambridge, MA), diluted to 1 mg/mL in water and injected without further preparation. An oligo dT ladder was prepared by reconstituting the contents of a vial of MassPREP™ Oligonucleotide Standard (p/n: 186004135 < https://www.waters.com/nextgen/glocal/shop/standards--reagents/186004135-massprep-oligonucleotide-standard.html>) in 100 µL water.

Mobile phase preparation

For the analysis of natalizumab and of oligo dT ladder, Tris-(hydroxymethyl)aminomethane (TRIS), and sodium chloride (NaCl) were purchased from Sigma- Aldrich (Buchs, Switzerland). Tris buffer was prepared as 20 mM solution, and its pH was adjusted to ~ 8.0. This 20 mM Tris buffer was used as mobile phase A. For mobile phase B, NaCl was dissolved (up to 600 mM) in 20 mM Tris buffer.

LC Conditions

LC system:	ACQUITY™ UPLC™ H-Class Bio System with Quaternary Solvent Manager [Equivalent to an ACQUITY Premier System with QSM FTN instrument configured with a High pH Kit]
Detection:	ACQUITY TUV Detector (Titanium Flow Cell, 5 mm, 1500 nL)
Wavelength:	260 nm or 280 nm
Data acquisition:	Empower™ Pro 3 Software Feature Release 3
Column:	Gen-Pak FAX Column (WAT015490); 4.6 x 100 mm Note: Other column types were acquired in a 4.6 x 50 mm configuration. The Gen-Pak FAX Column stationary phase was packed in a matching 4.6 x 50 mm configuration for comparison testing.
Column temperature:	30 °C
Sample temperature:	6 °C
Sample manager washes:	18.2 MΩ water
Seal wash:	10 % HPLC grade Methanol / 90 % 18.2 MΩ water (v/v)
Injection volume:	1 µL
Flow rate:	For natalizumab and oligo dT ladder separation: 0.72 mL/min
Mobile phase:	For natalizumab and oligo dT ladder separation:

	20 mM Tris pH 8.0 buffer. Salt gradient (NaCl) information is provided in the figure captions.
Syringe draw rate:	30 µL/min
Needle placement:	1.0 mm
TUV sampling rate:	2 Hz
Filter time constant:	none

Results and Discussion

General Considerations

Analytical anion exchange is best performed with non-porous resins. Despite their more limited binding capacity, non-porous particles are preferred for high-resolution analyses due to the low diffusivity of the biomolecules and strong nature of electrostatic interactions. A non-porous particle helps minimize mass transfer related band broadening. The biggest determinant of an AEX stationary phase's resolution capability is often its grafting (or the composition of surface that is applied to incorporate the charge bearing ligands).

As mentioned before, Waters first introduced the oligonucleotide specific GenPak FAX Column in the early 90s. We have been finding it to be more than fit for purpose for many of today's newest methods.

Here, in some past research work, our team compared different resins in a 4.6 x 50 mm column format. The Gen-Pak FAX Column stationary phase was tested alongside two other commercially available SAX resins, one based on a 4 µm non-porous particle and the other a 3 µm non-porous particle. Comparative measurements were performed by maintaining the same intrinsic gradient steepness and studying the quality of the separations obtained with the different AEX columns.

Analysis of IgG4 mAb (Natalizumab)

The columns were first tested for their ability to separate a protein sample. Natalizumab was selected because it

is a neutral p*I* mAb amenable to an AEX mode separation.

Within this work, each stationary phase was investigated for its resolving power and retentivity properties. Charge variant chromatograms obtained with a Tris pH 8 mobile phase system are displayed in Figure 1. The first observation to make is that the two strong AEX columns were significantly more retentive than the weak exchanger Gen-Pak FAX Column.

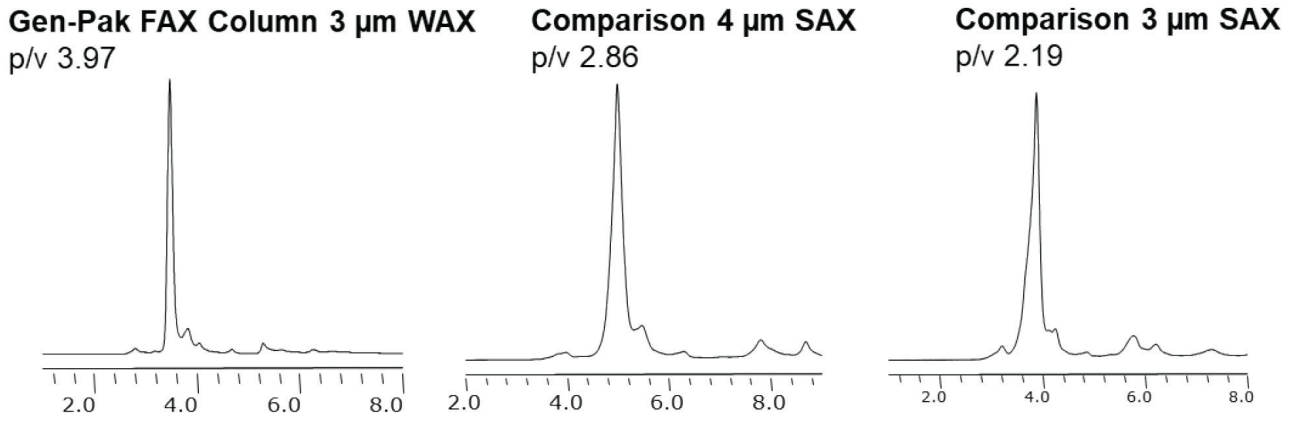

Figure 1. UV Chromatograms (280 nm) obtained for a pI ~ 7.8 monoclonal antibody (natalizumab) to demonstrate the applicability of three different AEX stationary phase to the separation of acidic and neutral pI proteins. Salt gradient separations were performed at 30 ˚C with a 0.72 mL/min flow rate, 20 mM Tris pH 8 buffer mobile phase, and a 10 min gradient running from 0 to 200 mM NaCl.

This lower retentivity can be advantageous or disadvantageous depending on the needs of an assay. We often find that IEX methods are largely still developed using empirical observations (trial and error). As such, it is valuable to have multiple tools (both weak and strong AEX columns) in the toolbox.

Figure 1 shows that the sharpest main isoform peak and the highest resolution (***i.e.*** between the main peak and the variant in the right shoulder) were observed with the Gen-Pak FAX Column. The peak to valley ratio was p/v = 3.97 on the weak exchanger and 2.86 and 2.19 on the strong exchangers.

An interesting method parameter in IEX is the pressure generated by the column. In IEX of large molecules, pressure might impact the separation efficiency and also the selectivity and overall retention.[13,14] In most modes of liquid chromatography, the porosity of the stationary phase is assumed to be constant and independent of the

mobile phase composition. However, the swelling of ion exchange stationary phases in the absence of salt (or sufficient ionic strength) is well-known and recently a so-called wetting/dewetting process was also discovered, proving that column porosity depends on the applied chromatographic conditions.[15,16,17,18] The swelling of IEX phases typically manifests as increased pressure (decreased column permeability) and as a change in apparent column porosity. Therefore, it has been widely recommended to use ion exchange columns with mobile phases containing at least 0.02 M buffer concentrations.[19]

The maximum backpressures produced by each column was documented and is reported in Figure 2. The two SAX columns exhibit high and very similar back pressures. Higher pressures are almost always observed when a mobile phase with a low ionic strength is passing through an IEX column. The permeability of the grafting is one of the primary determinants of this phenomenon. Different grafting compositions and thicknesses correlate with different pressure effects.[13] Note that the Gen-Pak FAX Column shows significantly higher permeability and correspondingly lower pressure. Accordingly, an analyst can potentially use a Gen-Pak FAX Column with lower ionic strength mobile phase without as much concern over swelling and pressurization. An analyst might also be able to more readily tune a high flow rate, high throughput method using the Gen-Pak FAX Column stationary phase.

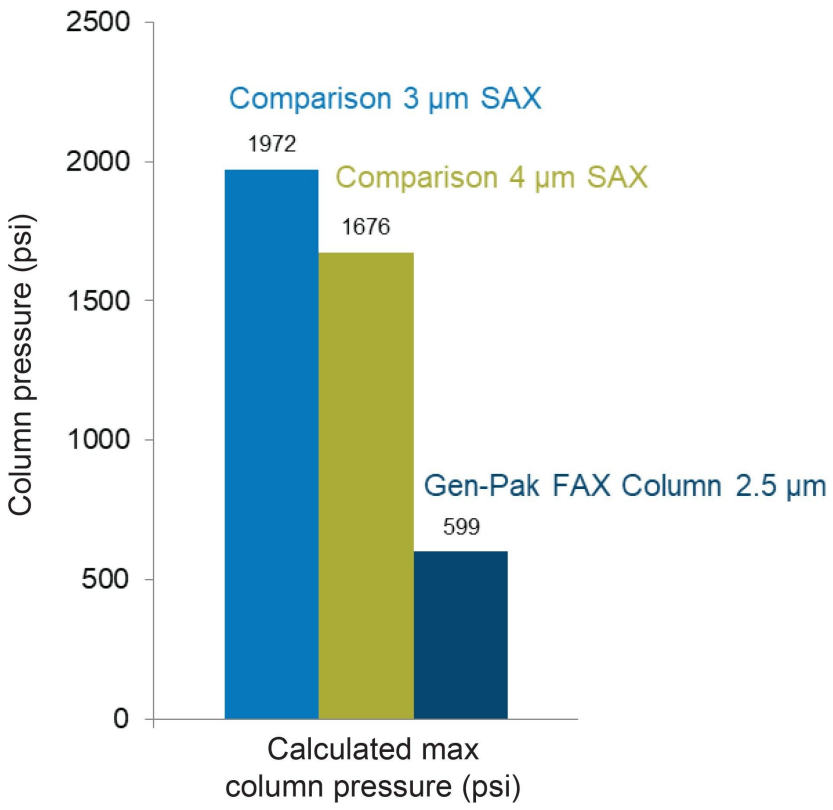

Figure 2. Maximum column pressures observed for three 4.6 x 50 mm columns packed with different AEX stationary phases during a salt gradient separation performed at 30 ˚C with a 0.72 mL/min flow rate, 20 mM Tris pH 8 buffer mobile phase, and a 10 minute gradient running from 0 to 200 mM NaCl.

Separation of 15 to 35-mer Oligonucleotide dT Ladder

Similar experiments were performed to compare a set of critical pairs within a 15 to 35-mer oligonucleotide dT ladder. Effective peak capacities were calculated for each set of peak profiles using the first and last retention times as the separation window. The Gen-Pak FAX Column particles were compared again to the two SAX stationary phases (Figure 3). Note that the Gen-Pak FAX Column again showed lower retentivity, and comparable or even higher peak capacity (*Pc*) values for these small oligos. A *Pc*=98 was obtained for the Gen-Pak FAX Column, while *Pc*=97 and 75 were obtained for the two SAX columns. The operating pressure was yet again much lower on the Gen-Pak Fax Column compared to the SAX phases (nearly a factor of three lower).

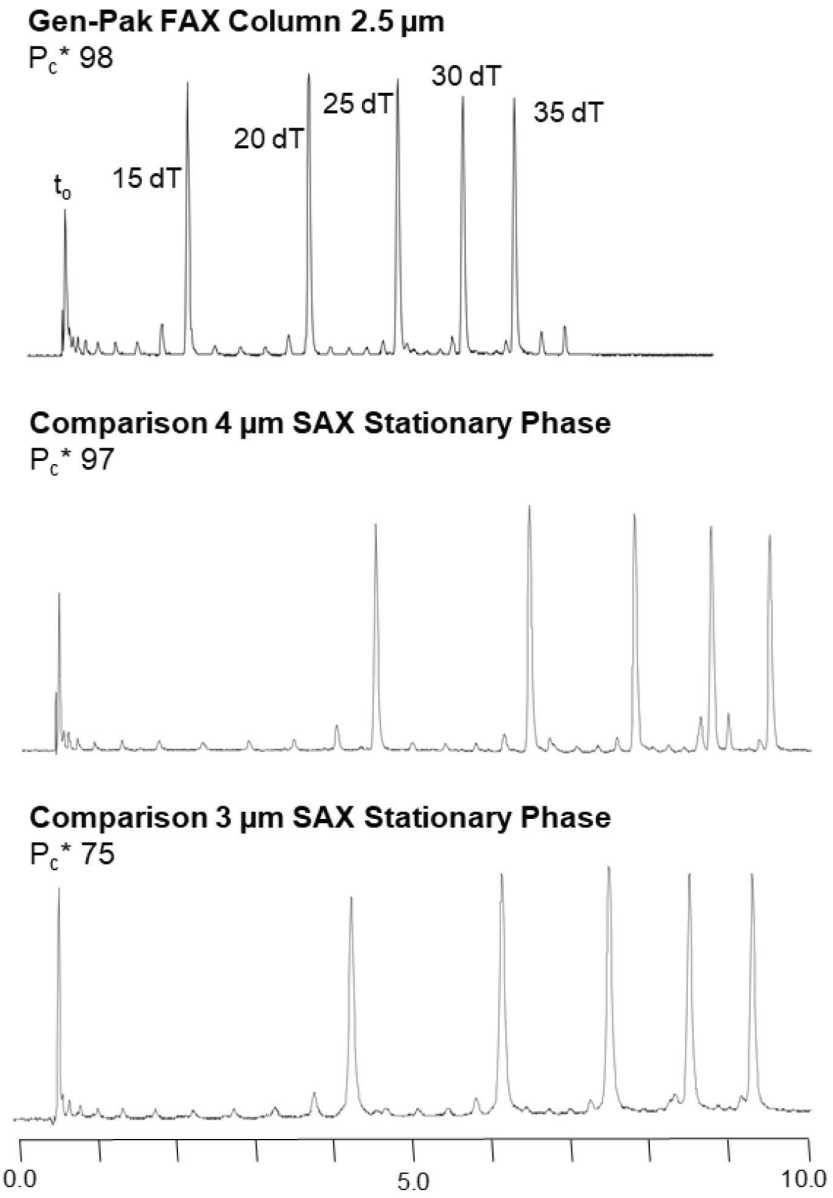

Figure 3. UV Chromatograms (260 nm) obtained for an oligo dT ladder using various 4.6 x 50 mm AEX columns. Salt gradient separations were performed at 30 ˚C with a 0.72 mL/min flow rate, 20 mM Tris pH 8 buffer mobile phase, and a 10 minute gradient running from 300 to 600 mM NaCl.

Separation of an Antisense Oligonucleotide Model Mixture

Recently, researchers Stilianos Roussis and Claus Rentel published a new method for antisense oligonucleotides (ASO) impurity analysis.[12] They were looking for a separation that would give a new type of selectivity and facilitate the detection of deaminated impurities. Of the columns tested, it was a Gen-Pak FAX Column that was seen to be amenable to a specially devised mobile phase condition. It was found that the best eluent for achieving a unique oligonucleotide selectivity was one comprised of 75% methanol. This novel condition limited the amount of eluent salt concentration because of solubility issues. Sodium bromide and guanidine were both used in the eluent at 250 mM concentrations. The lower retentivity of the Gen-Pak FAX Column stationary phase facilitated the development of this particular method. The authors of this work concluded that the most significant beneficial effect of the weak AEX column (Gen-Pak FAX Column) was its compatibility to afford elution with a high-organic, low-salt mobile phase. With these novel AEX conditions, it was possible to avoid extraneous hydrophobic, polar/hydrophilic and H-bonding interactions and maximize a single, ionic/electrostatic mechanism of separation. The authors observed improved separation performance for phosphorothioate (PS) oligonucleotide impurities based mainly on ionic/electrostatic differences.

The reported separations were also said to benefit from pH gradient effects corresponding to the electrostatic potential of the stationary phase and analyte ionization. An extraordinary degree of separation was achieved by this new WAX method in comparison to SAX chromatography. For the first time, the extent of deamination of phosporothioate oligonucleotides was directly determined by liquid chromatography.

An example of the new selectivity for an ASO impurity analysis is shown in Figure 4. Please note, that such separation is not achievable with ion-pair reverse phase (IP-RP) chromatography. Therefore, both weak AEX and IP-RP modes can be considered for comprehensive and complementary analysis of an oligonucleotide.

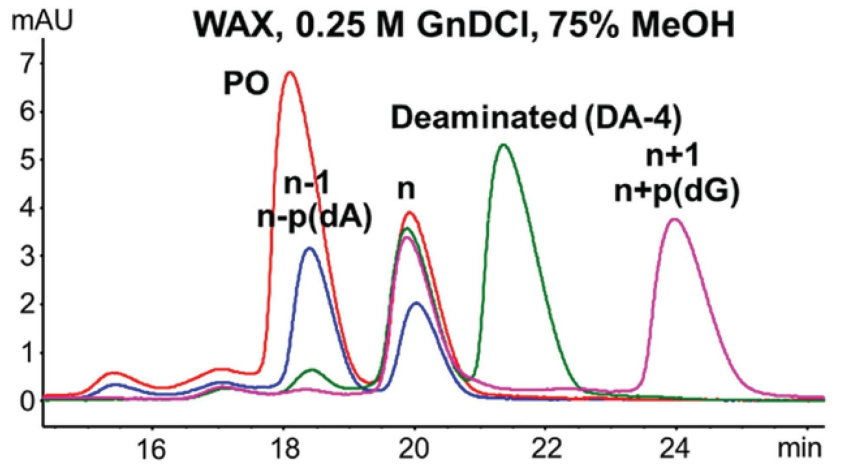

Figure 4. UV Chromatograms obtained for model oligonucleotide mixtures as obtained with a Gen-Pak FAX Column. Eluent: 5 mM Na_3PO_4, 250 mM NaBr, 250 mM GnDCl in 75% methanol. With permission from Ref.[12]

Conclusion

This brief review document and application note presents a few select examples in which the advantages of the weak anion-exchange Gen-Pak FAX Column are effectively applied. For various protein and oligonucleotide/nucleic acid separations, the Gen-Pak FAX Column shows lower retentivity compared to strong AEX columns. Such low retentivity may result in several benefits, especially when mobile phase pH can be optimally tuned.

Due to the inherently weaker interactions occurring between the solutes and the stationary phase, the separation efficiency is significantly improved. Higher peak capacity was observed for natalizumab and a 15 to 35-mer oligonucleotide dT ladder with the Gen-Pak FAX Column compared to other SAX columns. In other work, the low retentivity of this column has been found to be beneficial for large nucleic acid analysis (i.e. intact mRNA) where there is seen to be an improvement in solute recovery and method robustness.[5]

The permeability of the Gen-Pak FAX Column was found to be favorable too. As it shows significantly higher permeability, an analyst can potentially use a Gen-Pak FAX Column with lower ionic strength mobile phase

without as much concern about swelling and pressurization. This high permeability also enables one to work at high flow rates such that high throughput methods can be developed.

Finally, the lower retentivity of the Gen-Pak FAX Column has inspired the development of a novel type of AEX method wherein a high-organic (up to 75% MeOH) eluent composition is combined with NaBr and guanidine to achieve new selectivites. Researchers have proposed that these conditions minimize non-specific interactions and maximize an electrostatic interaction-based separation mechanism.

References

1. S. Fekete, A. Beck, J.L. Veuthey, D. Guillarme, Ion-exchange Chromatography for The Characterization of Biopharmaceuticals, *J. Pharm. Biomed. Anal*. 113 (2015) 43–55, http://dx.doi.org/10.1016/j.jpba.2015.02.037 <http://dx.doi.org/10.1016/j.jpba.2015.02.037>
2. H. Yang, S.M. Koza, W. Chen, Plasmid Isoform Separation and Quantification by Anion-exchange Chromatography (AEX), Waters Application Note, 720007207, March 2021.
3. A. Kanavarioti, HPLC Methods for Purity Evaluation of Man-Made Single-Stranded RNAs, Scientific Reports 9, 2019, 1–13, https://doi.org/10.1038/s41598-018-37642-z <https://doi.org/10.1038/s41598-018-37642-z> .
4. S. Fekete, H. Yang, K. Wyndham, M. Lauber, Salt gradient and ion-pair mediated anion exchange of intact messenger ribonucleic acids, *J. Chromatogr*. Open 2 (2022) 100031, https://doi.org/10.1016/j.jcoa.2022.100031 <https://doi.org/10.1016/j.jcoa.2022.100031>
5. S. Fekete, M. Lauber, Salt Plug Injection Methods for Improved Anion Exchange Analyses of Large Nucleic Acids, Waters Application Note, 720007991, July 2023.
6. A. Goyon, M. Excoffier, M.C. Janin-Bussat, B. Bobaly, S. Fekete, D. Guillarme, A. Beck, Determination of isoelectric points and relative charge variants of 23 therapeutic monoclonal antibodies, *J. Chromatogr*. B 1065–1066 (2017) 119–128, http://dx.doi.org/10.1016/j.jchromb.2017.09.033 <http://dx.doi.org/10.1016/j.jchromb.2017.09.033>

7. A. P. Liu, Y. Yan, S. Wang, N. Li, Coupling Anion Exchange Chromatography with Native Mass Spectrometry for Charge Heterogeneity Characterization of Monoclonal Antibodies, *Anal. Chem.* 94 (2022) 6355–6362. https://doi.org/10.1021/acs.analchem.2c00707 <https://doi.org/10.1021/acs.analchem.2c00707>

8. J.R. Thayer, *et al*., Identification of RNA Linkage Isomers by Anion Exchange Purification With Electrospray Ionization Mass Spectrometry of Automatically Desalted Phosphodiesterase-II Digests. *Anal. Biochem*. 399 (2010) 110–117. http://dx.doi.org/10.1016/j.ab.2009.11.009 <http://dx.doi.org/10.1016/j.ab.2009.11.009>

9. J.R. Thayer, R.M. McCormick, N. Avdalovic, High-Resolution Nucleic Acid Separations by High-Performance Liquid Chromatography, in Methods in Enzymology, 1996, Academic Press. 147–174. http://dx.doi.org/10.1016/s0076-6879(96)71009-1 <http://dx.doi.org/10.1016/s0076-6879(96)71009-1>

10. A. Eon-Duval, G. Burke, Purification of Pharmaceutical-Grade Plasmid Dna by Anion-Exchange Chromatography in an Rnase-Free Process. *J. Chromatogr.* B, 804 (2004) 327-335. http://dx.doi.org/10.1016/j.jchromb.2004.01.033 <http://dx.doi.org/10.1016/j.jchromb.2004.01.033>

11. K. Cook, J. Thayer,Advantages of Ion-Exchange Chromatography for Oligonucleotide Analysis. *Bioanalysis*, 2011. 3(10): p. 1109-1120. http://dx.doi.org/10.4155/bio.11.66 <http://dx.doi.org/10.4155/bio.11.66>

12. S. G. Roussis, C. Rentel, Separation of Phosphorothioate Oligonucleotide Impurities by Wax Hplc Under High Organic Content Elution Conditions, *Anal. Biochem*. 659 (2022) 114956, https://doi.org/10.1016/j.ab.2022.114956 <https://doi.org/10.1016/j.ab.2022.114956>

13. A. Murisier, M. Lauber, S.J. Shiner, D. Guillarme, S. Fekete, Practical Considerations on the Particle Size and Permeability of Ion-Exchange Columns Applied to Biopharmaceutical Separations, *J. Chromatogr*. A 1604 (2019) 460487, https://doi.org/10.1016/j.chroma.2019.460487 <https://doi.org/10.1016/j.chroma.2019.460487>

14. H. Lardeux, D. Guillarme, M. Imiołek, S. Fekete, M.A. Lauber, Pressure–Enhanced Liquid Chromatography as a Suitable Approach to Improve Selectivity for Large Molecule Separations, LCGC Supp., *Advances in LC Column Technology,* 41 (2023) 28–34. https://doi.org/10.56530/lcgc.na.ts6090k5 < https://doi.org/10.56530/lcgc.na.ts6090k5>

15. G.V. Samsonov, V.A. Pasechnik, Ion Exchange and the Swelling of Ion- Exchange Resins, Russian Chem. Rev. 38 (1969) 547–565, https://doi.org/10.1070/ RC1969v038n07ABEH001761

16. U.M. Ingle, A.M. Lali, Significance of Porosity and Pore Accessibility for the Se- Lection of Ion Exchange Adsorbents for Chromatographic Purification of Macro- Molecules, *Acta Chromatogr* 29 (2017) 5–24,

https://doi.org/10.1556/1326.2017.29.1.01 <https://doi.org/10.1556/1326.2017.29.1.01>

17. B. Gong, Y. Shen, X. Geng, Preparation of Strong Cation Exchange Packings Based on Monodisperse Poly(Glycidyl Methacrylate-Co-ethylenedimethacrylate) Particles and Their Application, *J. Liq. Chromatogr.* Relat. Technol. 26 (2003) 963–976, https://doi.org/10.1081/JLC-120018896 <https://doi.org/10.1081/JLC-120018896>

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