Sepax Technologies, Inc.

Quantitative Analysis of Tween 20 in Formulated MAb Samples Using Sepax Mixed-Mode SAX Column Chromatography

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APPLICATION NOTE

INTRODUCTION

Detergents are commonly used in the formulations for biological samples to improve the drug stability and solubility and to prevent denaturation and aggregation.[1,2] Of the detergents being used, the non-ionic, amphiphilic surfactants such as polysorbates 20 and 80 (Tween 20 and 80) as well as poloxamer 188 (Pluronic F68) are the most common ones due to their low toxicity and high biocompatibility. At concentrations above the critical micellular concentration, the monomeric surfactant molecules arrange into aggregates called micelles through hydrophobic interactions.[3] Therefore, surfactant quantification, is crucial to ensure a consistent buffer composition and is an important critical quality attribute. However, these surfactants are heterogeneous mixtures of amphiphilic molecules consisting of hydrophilic head and hydrophobic tail, making their quantification challenging.[1]

MIXED-MODE CHROMATOGRAPHY

Chromatographic methods for Tween 20 quantification ideally should provide excellent separation between the surfactant and other sample components, high throughput, minimal sample preparation required, as well as good sensitivity and precision. A step gradient is required to obtain a single peak due to the heterogeneity of surfactants like Tween 20. However, a step gradient on a traditional reverse phase column may not provide an adequate separation between the surfactant and other sample components and may have protein carryover issue.[4] Mixed-mode chromatography using hydrophobic interactions to retain neutral surfactants while anionic components elute in the dead volume due to the anionic resin is a solution to the difficulties associated with Tween 20 quantification. Challenges with mixed-mode chromatography methods can arise from lot to lot reproducibility. There are also reports of carryover issues with commercial mixed mode columns for non-ionic surfactant quantification. Therefore, Sepax developed the mixed-mode Monomix H2P-SAX resin with its unique chemistry that allows 100% aqueous buffer to be used for the high-throughput separation of Tween 20 and monoclonal antibodies (mAbs).

SEPAX MONOMIX H2P-SAX COLUMN

Method development using the Sepax Monomix H2P-SAX column (40 µm) for Tween 20 quantification is substantially easier than other vendors. The more hydrophilic nature of the Sepax H2P-SAX resin allows for better peak shape, a higher retained peak area, and less flow through when starting under 100% aqueous conditions, where protein samples are eluted in non-denatured form. Carry over was not noted despite starting under aqueous conditions. In contrast, competitor methods recommend starting the run with an optimized amount of organic buffer to obtain the best peak shape. The peak area and retention time collected using the Monomix H2P-SAX columns (40 µm) were also highly reproducible when examining three lots of resin at concentrations above and below the CMC. The excellent reproducibility, superb separation between non-ionic surfactants and mAbs, and ability to start under aqueous conditions makes the Monomix H2P-SAX column an attractive tool for nonionic surfactant quantification.

METHODS AND MATERIALS

Sample

Tween 20 (~1228 g/mol) was purchased from Millipore Sigma (St. Louis MO). NIST mAb, humanized IgG1κ was obtained from the National Institute of Standards and Technology (Gaithersburg MD). All samples were prepared with water from a Milli-Q water purification system. Samples of Tween 20 were prepared though serial dilution to 0.0004%, 0.0008%, 0.0016%, 0.0031%, 0.0063%, 0.0125%, and 0.025%. Samples of 1 mg/mL NIST mAb and 0.02% Tween 20, 1 mg/mL NIST mAb and 0.04% Tween 20, and 1 mg/mL NIST mAb and 0.08% Tween 20 were also prepared.

System and Detection

Analysis was conducted on an Agilent 1260 Infinity HPLC connected to an Agilent 1200 Series evaporative light scattering detector (ELSD) (Santa Clara CA). An ELSD produces a nonlinear response with increasing concentration. An external standard curve was generated by plotting the log of the concentration of the Tween 20 sample against the log of the peak area.

HPLC Column

Three Sepax Monomix H2P-SAX, 40 µm, 2.1 x 20 mm columns (Part Number: 282640990-2102) were used in this study along with a competitor's column (2.1 x 20 mm) for quantification. Three different resin lots were used.

Method

The running conditions are illustrated in Tables 1 and 2.

Table 1: HPLC and ELSD Run Conditions	
Mobile Phase A	2% formic acid in water
Mobile Phase B	2% formic acid in isopropyl alcohol (IPA)
Flow Rate	1 mL/min
Column Temperature	25 °C
ELSD Temperature	80 °C
ELSD Pressure	3.5 bar
ELSD Gain	6

Table 2: Monomix H2P-SAX Method	
Time	Mobile Phase B
0.0	0
2.5	0
2.51	100
4.5	100
4.51	0
7.0	0

RESULTS AND DISCUSSION

One of the most crucial method development parameters for a step gradient is the starting buffer composition. In methods for non-ionic surfactant quantification, starting with too much organic will result in poor retention of the surfactant retaining on the column resulting in a larger flow though peak at the dead volume. In contrast, equilibrating without organic could result in samples sticking to the column. Therefore, most commercial columns for surfactant quantification require the end user to optimize the starting conditions for each sample. The Monomix H2P-SAX column performed best when starting under aqueous conditions, as shown in Figure 1. A slight shoulder was noted when utilizing 100% mobile phase A as the starting condition, which is likely due to the more hydrophilic components of the heterogenous Tween 20 sample.

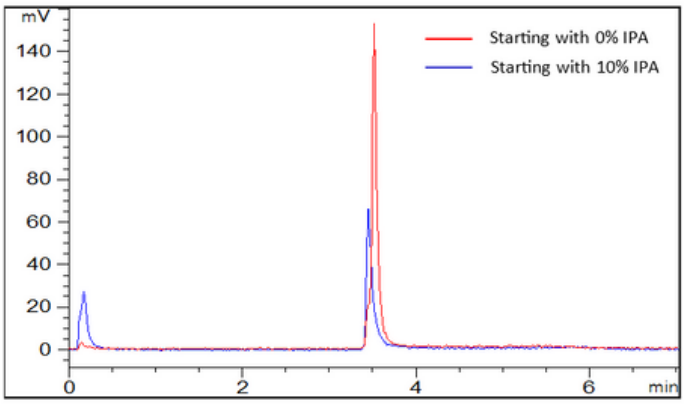

Figure 1. Chromatograms associated with injecting 50 µL of 0.0063% Tween 20 onto a Monomix H2P-SAX column. Red trace: Starting with the recommended starting conditions of 0% mobile phase B; Blue trace: starting with 10% mobile phase B; Gradient: a step gradient at 2.51 min to 100 mobile phase.

When injecting 50 µL of 1 mg/mL NIST mAb and 0.02% Tween 20, the Monomix H2P-SAX column also provides a good separation and peak shape for the mAb and surfactant when starting the run with 100% mobile phase A as noted in Figure 2.

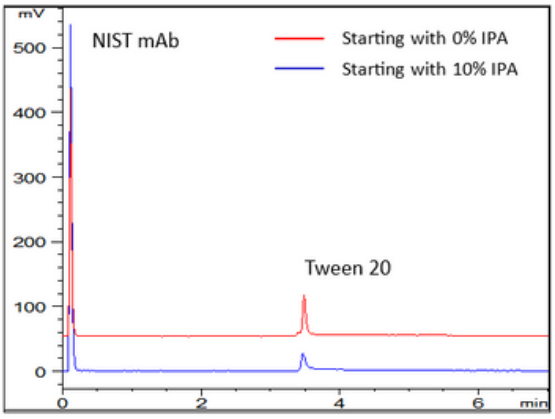

Figure 2. Overlay of chromatograms developed from injecting 50 µL of 1 mg/mL NIST mAb and 0.02% Tween 20 using the recommended method for the Monomix H2P-SAX column. Red Trace starting at 0% mobile phase B; Blue Trace: starting with 10% organic buffer.

The anionic characteristics of the Monomix H2P-SAX resin does not allow the NIST mAb to retain on the column while the nonionic Tween 20 was retained until introducing organic buffer. The unique characteristics of the Monomix H2P-SAX resin suggests that starting condition optimization is not required. A reproducible signal was obtained on the Monomix H2P-SAX column across three injections (see Figure 3).

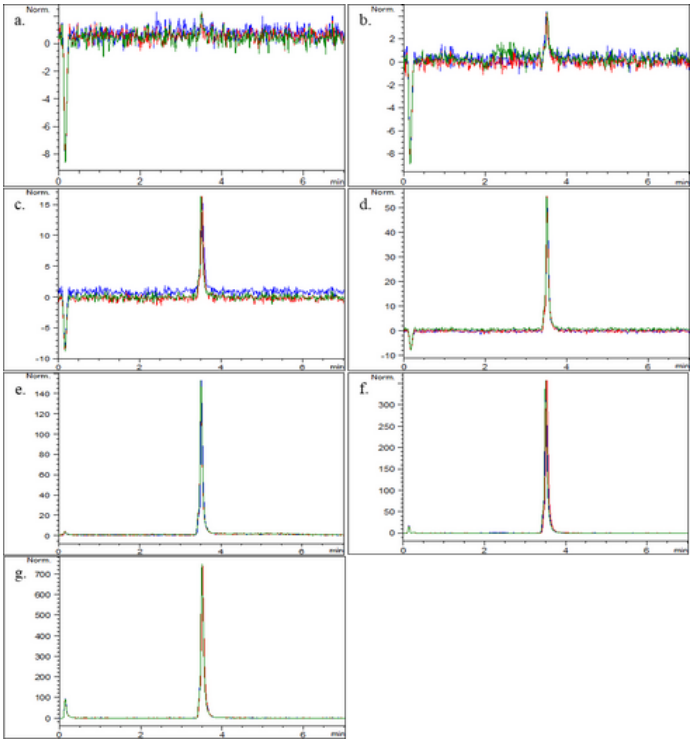

Figure 3. Repeatability study. Overlays of triplicate injections of 50 µL a. 0.0004%, b. 0.0008%, c. 0.0016, d. 0.0031, e. 0.0063%, f. 0.0125%, and g. 0.025% Tween 20 on the same Monomix H2P-SAX column. Data was background subtracted from an injection of water.

The peak area was reproducible across a range of Tween 20 concentrations above and below the CMC. The CMC for Tween 20 was reported as 0.006% according to the manufacturer.[7] The Tween 20 peak was not observed when injecting 50 µL of 0.0004% Tween 20, but a peak was detected when injecting 50 µL 0.0008% Tween 20 (see Figure 3b, average peak area 34.37 ± 2.64). Figure 4 illustrates a good lot-to-lot reproducibility by demonstrating a consistent retention time, peak area, and peak shape across three resin lots. A linear range of 0.0008-0.0125% Tween 20 was also obtained across all three resin beds, as shown in Figure 5.

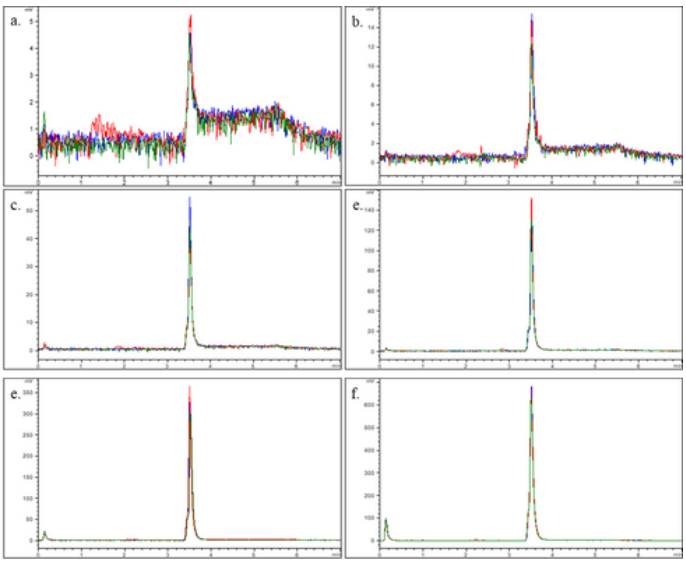

Figure 4. Lot-to-lot consistent study. Overlays of three injections of 50 µL of 0.0008% (a), 0.0016% (b), 0.0031% (c), 0.0063% (d), 0.0125% (e), and 0.0250% Tween 20 (f) performed on three different resin lots.

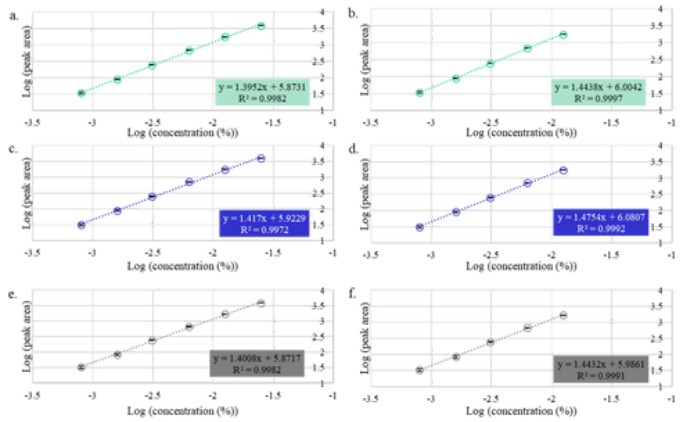

Figure 5. Standard curves associated with three lots of Monomix H2P-SAX resin with plots a-b, c-d, and e-f being from the same lot. Plots a, c, and e contain data from injections of 50 µL 0.0008-0.0250% Tween 20. Plots b, d, and f contain data from injections of 50 µL of 0.0008-0.0125% Tween 20.

Compared to competitor's column, the Monomix H2P-SAX column produces similar peak areas for the Tween 20 and flow through peak when injecting just Tween 20, as shown in Figure 6.

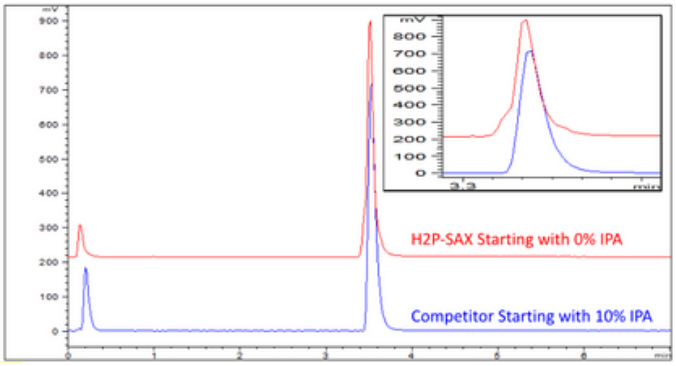

Figure 6. Overlaid chromatograms associated with the injection of 50 µL of 0.025% Tween 20 gathered with the Monomix H2P-SAX (red) and competitor column (blue). The Monomix H2P-SAX column utilized the recommended method while the competitor column started using 10% mobile phase B as recommended by the manufacturer.

The major difference comes from the starting conditions. Competitor columns for nonionic surfactant quantification recommend starting with at least 10% organic buffer to obtain the best peak shape (see Figure 7).

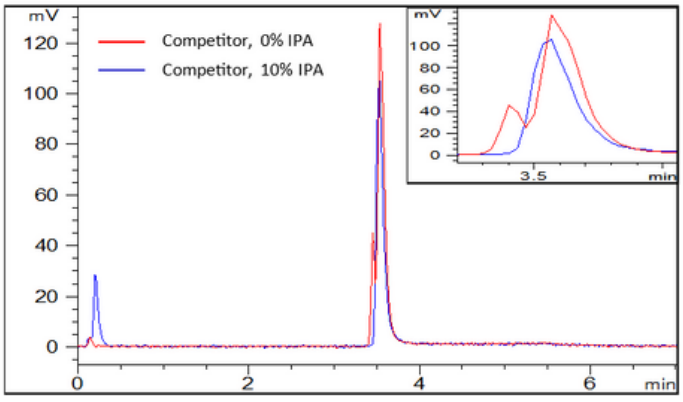

Figure 7. Chromatograms of 50 µL of 0.0063% Tween 20 onto the competitor's column when starting the method with 0% mobile phase B (red) and 10% mobile phase B (blue).

When starting the run with 100% mobile phase on the competitor's column, a split peak was observed, this peak was at the front of the peak and lines up with the small shoulder observed with the H2P-SAX column when run on the same condition. In contrast, the Monomix H2P-SAX column performed best when starting with 100% mobile phase A.

Both columns were evaluated using mixed samples of NIST mAb and Tween 20, as shown in Figure 8, and both columns exhibited excellent separation between the NIST mAb and Tween 20 even with increasing concentrations of Tween 20. Similar peak areas were achieved with both columns as highlighted in Table 3. The Monomix H2P-SAX column was able to achieve these results despite equilibrating under aqueous conditions.

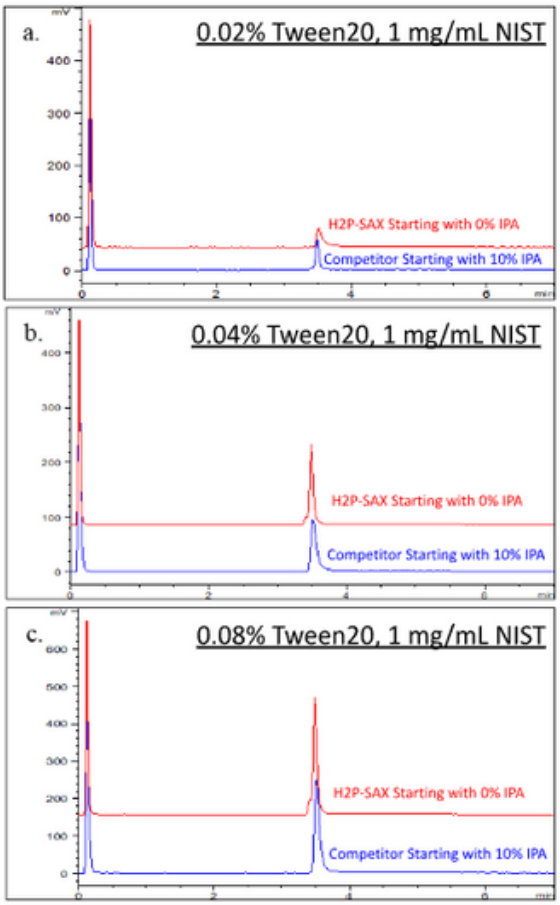

Figure 8. Chromatograms associated with injections of 5 µL of 0.02% Tween 20 and 1 mg/mL NIST (a), 0.04% Tween 20 and 1 mg/mL NIST (b), and 0.08% Tween 20 and 1 mg/mL NIST (c) on the Monomix H2P-SAX (red) and competitor column (blue)

Table 3. Peak Information from Different Sample Injections

Column	Starting Condition	Sample	Flow Though Peak Area	Tween 20 Peak Area	Plate Count	Tween 20 Tailing
Monomix H2P-SAX	100% Mobile Phase A	1 mg/mL NIST mAb, 0.02% Tween 20	742.6	235.2	8672	1.23
		1 mg/mL NIST mAb, 0.04% Tween 20	750.6	573.3	8618	0.97
		1 mg/mL NIST mAb, 0.08% Tween 20	997.6	1367.1	8288	0.86
Competitor	10% Mobile Phase B	1 mg/mL NIST mAb, 0.02% Tween 20	1065.1	236.6	6486	2.44
		1 mg/mL NIST mAb, 0.04% Tween 20	1080.0	615.8	7860	1.58
		1 mg/mL NIST mAb, 0.08% Tween 20	1249.3	1374.0	7658	1.44

CONCLUSIONS

The Sepax Monomix H2P-SAX column (40 µm) is an attractive, high throughput option for nonionic surfactant quantification. Here, the reproducibility of the Monomix H2P-SAX column (40 µm) was highlighted along with the linearity associated with the analysis of Tween 20. The convenience of the Monomix H2P-SAX column is apparent when it comes to method development. Competitor columns require optimization of the starting condition to ensure the best performance. In contrast, the more hydrophilic Monomix H2P-SAX resin performed best without organic in the starting conditions. By starting in aqueous conditions method development is not as intensive allowing user to save time and resources.

The Sepax Monomix H2P-SAX resin could potentially be used for other applications beyond Tween 20 quantification. The resin is compatible with mass spectrometry to characterize the different components of Tween 20 or monitor the degradation and oxidized components with a longer column and optimized, gradient method.

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