Separation Report No. 074

Separation of Monoclonal Antibodies Using TSKgel HPLC Columns

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1. Introduction

In recent years, advances in biotechnology have resulted in increased use of monoclonal antibodies (MAbs) as the main constituent of therapeutic drugs and as diagnostic agents. Because of this increased commercial application, it is important for the bioanalytical chemist to have access to the tools for rapid and simple purification of MAbs. There are many published reports about MAb purification by high-performance liquid chromatography (HPLC) and FPLC (fast protein liquid chromatography); a select group of references is included at the end of this Separation Report. This document describes the analysis and isolation of MAbs by HPLC using TSKgel columns. Several application examples are discussed.

2. Separation Mode and Purification Method

Table 1 shows the HPLC separation modes used in the analysis and isolation of MAbs, together with their main features. Ion exchange (IEC), hydrophobic interaction (HIC) and affinity (AFC) have large sample capacity and high selectivity; as such, they are often used in MAb purification. IEC is said to be used in almost 75% of the MAb purifications, often following ammonium sulfate fractionation (HIC). Though gel filtration chromatography (GFC) is inferior with respect to resolution or sample load, it is often used as the final purification method and as a purity check for MAb preparations since this method constitutes a simple step for the removal of dimers and higher molecular weight aggregates.

Table-1 Features of HPLC separation modes for monoclonal antibody purification

Separation mode	Separation mechanism	TSK-GEL	Feature
Gel filtration (size exclusion) chromatography (GFC, SEC)	Molecular size	TSKgel G2000SW$_{XL}$ TSKgel G3000SW$_{XL}$ TSKgel G4000SW$_{XL}$	Though resolution and sample load are inferior to other separation modes, it allows simple removal of dimers and aggregates, causes of MAb antigenicty. It can also be used in final purification or as a purity check. Separation of Fab and IgM (TSKgel G4000SW$_{XL}$) is also possible.
Ion exchange chromatography (IEC)	Ionic interaction	TSKgel DEAE-5PW TSKgel SP-5PW TSKgel CM-5PW	Resolution is high and the sample load is greater. Anion exchange plays an important role for the separation of MAb sub-classes and impurities. Cation exchange allows simple purification by step-wise elution because many of the impurities are not retained on the column.
		TSKgel DEAE-NPR TSKgel SP-NPR	The non-porous resin (NPR) column can be used for rapid analysis and fractionation of trace amounts of MAb (μg to ng); purity check of MAb fractions is possible instead of electrophoresis.
Hydrophobic interaction chromatography (HIC)	Hydrophobicity	TSKgel Ether-5PW TSKgel Phenyl-5PW TSKgel Butyl-NPR	Resolution is high and the sample load is good. TSKgel Ether-5PW, particularly, has high recovery of hydrophobic MAbs. HIC allows simple purification by step-wise elution. TSKgel Butyl-NPR is ideal for micro-fractionation.
Affinity chromatography (AFC)	Bioaffinity	TSKgel Chelate-5PW TSKgel Tresyl-5PW	Immobilized metal chelate affinity chromatography (IMAC). Activated affinity support. Ligands such as antigen are immobilized to absorb and purify MAb.

In general, it is said that using a single HPLC mode (with the exception of GFC) provides a MAb purity of about 95%. Thus, high purity preparations of MAb for use in diagnostic applications can be obtained using a single HPLC column. However, a purity of 99% and higher is required when MAbs are used for medicinal purposes. This level of purity can be achieved using a single Protein A affinity step, which is based on antibody-antigen interaction. A minimum of two different separation modes are required when less specific HPLC separation modes are applied, such as IEC-HIC or IEC-GFC.

Furthermore, recent demands for preparation of high purity MAb solutions (approx. 99.9% purity or higher) require three different separation modes, for instance, HIC-IEC-GFC or AFC-IEC-GFC. In addition, it is necessary to select the proper elution method, whether linear elution, gradient elution or step-wise gradient elution, depending on the purity required, the time allowed for purification and the cost of each process of preparing the MAb preparation. Table 2 lists the literature in which various TSKgel products were used for MAb purification along with a brief summary about each mode. While most methods consist of IEC and GFC, reports are included based on HIC and AFC as well.

Table-2 Key literature featuring TSKgel columns for the separation of monoclonal antibodies

Literature No.	Column	Sample	Antibody type	Outline
1	TSKgel G3000SW	Ascites, cultured supernatant	IgG_1, F(ab)2	Review article on MAb against class I, MHC antigen
2	TSKgel G3000SW	Ascites	IgG_1, F(ab')2	Separation of IgG_1 and F(ab')2
3	TSKgel G3000SW	Ascites	IgG_1, IgG_{2b}, IgG_3	Final purification of MAb purified by IEC Recovery is 85% and higher
4	TSKgel DEAE-5PW TSKgel G3000SW	Ascites, cultured supernatant	IgG_1, IgG_{2a} to the total of 8 species	1 step IEC purification of MAb by sophisticated eluent Recovery is 95%
5	TSKgel G3000SW	Ascites	IgG_1, IgG_{2a}, IgG_{2b}	Purity check on MAb purified by IEC
6	TSKgel DEAE-5PW	Ascites	IgG_1, IgG_{2a}, IgG_{2b}	1 step IEC purification of MAb by sophisticated eluent Recovery is 92% and higher
7	TSKgel Phenyl-5PW	Ascites, cultured supernatant	IgG	Examination of separation by IEC and HIC IEC and HIC were found to provide good separation
8	Toyopearl DEAE-650M Toyopearl CM-650M	Cultured supernatant	IgG_{2b}	Rapid separation of sample; simple removal of phenol red indicator. Recovery of activity is 90% and higher
9	TSKgel SP-5PW TSKgel G4000SW	Ascites	IgG_1, IgM	Simple purification by IEC, automatic control by IEC-GFC connection. Recovery of activity is 80% and higher
10	TSKgel SP-5PW	Cultured supernatant	IgG	Step-wise gradient elution of pretreated sample (100mL) Purity 99%
11	TSKgel Chelate-5PW	Ascites	IgG	Application of IMAC, purity increased 10-fold in single step
12	TSKgel DEAE-5PW TSKgel G3000SW	Ascites	IgG_1, IgG_{2a}, IgG_{2b}, IgM	MAb purification on preparative columns using a sophisticated eluent. Recovery is 89% and higher
13	TSKgel DEAE-5PW TSKgel G3000SW	Ascites	IgG_1	Comparative examination of purification by IEC and AFC on preparative columns. Purity 99.2% and recovery of activity 72%
14	TSKgel DEAE-5PW	Ascites	IgM (2 species)	Purification of IgM to high purity in 1 step using simultaneous gradient of salt and pH
15	TSKgel Ether-5PW TSKgel $G3000SW_{XL}$ TSKgel DEAE-NPR	Ascites	IgG_1	Examination of MAb separation conditions by HIC, simple purification in step-wise elution

3. Applications

3.1 Gel Filtration Chromatography (GFC, SEC)

GFC is inferior to other chromatographic modes for the separation of MAbs in terms of resolution and sample load. IgG is often expressed in mouse ascites fluid in the presence of large amounts of albumin. When injecting crude samples into a GFC column, it is often not possible to obtain satisfactory resolution between MAb (IgG) and albumin. Thus GFC is more commonly used for separating partially purified samples rather than for direct MAb purification from crude ascites samples.

Figure 1 shows an example of MAb (IgG_1) separation by GFC from mouse ascites fluid. Although it was found that separation of IgG and albumin could be improved somewhat by changing the eluent pH and salt species (pH 5.0, 0.1mol/L Na_2SO_4), the resolution would not be sufficient for most applications. For a detailed examination of this separation, see Separation Report No. 62.

To isolate IgM, TSKgel G4000SW_{XL} is best suited since the larger pore size enables separation of larger proteins.

3.2 Ion-Exchange Chromatography (IEC)

Ion Exchange Chromatography steps either consist of an anion exchange packing material with DEAE (diethylaminoethyl) functional groups or a cation exchange packing material with SP (sulfopropyl) or CM (carboxymethyl) functional groups. In general, DEAE anion exchangers are used as the last step in MAb purifications. DEAE is also very suitable for the separation of IgG subclasses. In both cases a linear gradient method is applied. Best separations are obtained at pH 8 or higher. Figures 2 and 3 show the separation of MAb (IgG_1) from mouse ascites fluid and cell culture supernatant on TSKgel DEAE-5PW. In the case of mouse ascites fluid, shown in Figure 2, IgG_1 elutes in about 15 minutes, well separated from the impurities that elute before or after IgG_1 such as transferrin (about 11 minutes) and albumin (about 22 minutes). However, when injecting cell culture supernatant, as shown in Figure 3, MAb could not be detected because it was present at trace levels.

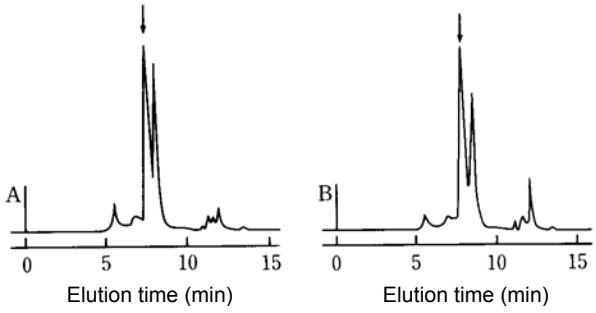

Figure 1 Separation of monoclonal antibody by GFC

Column:	TSKgel G3000SW_{XL}, 7.8mm ID × 30cm
Eluent:	A: 50mmol/L sodium phosphate buffer (pH 6.7) + 0.3mol/L NaCl B: 50mmol/L sodium phosphate buffer (pH 5.0) + 0.1mol/L Na_2SO_4
Flow rate:	1.0mL/min
Temperature:	25°C
Detection:	UV@280nm
Sample:	Anti-human proline hydroxylase (IgG_1), diluted solution of mouse ascites (50µl)

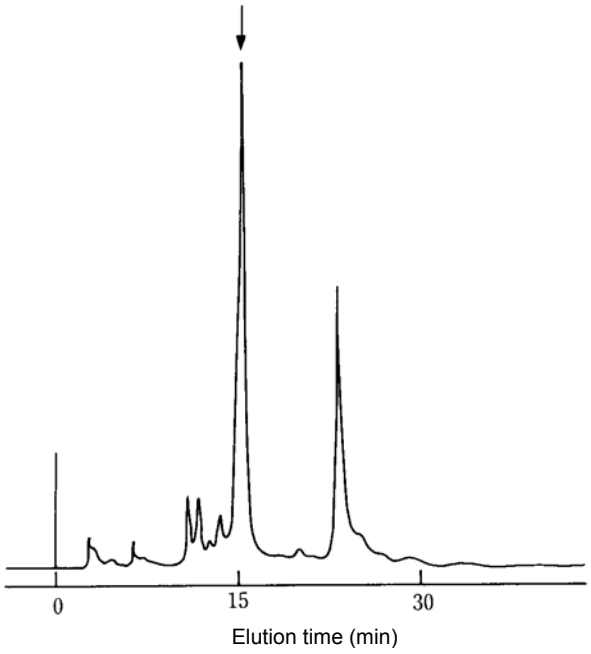

Figure 2 Separation of monoclonal antibody by IEC

Column:	TSKgel DEAE-5PW, 7.5mm ID × 7.5cm
Eluent:	A: 20mmol/L Tris-HCl (pH 8.5) B: A + 0.5mol/L NaCl A → B linear gradient (60 min.)
Flow rate:	1.0mL/min
Temperature:	25°C
Detection:	UV@280nm
Sample:	Anti-human albumin (IgG_1), diluted solution of mouse ascites (168µg in 40µl)

In contrast to anion exchange, with a cation exchanger such as SP or CM, sample throughput is higher because albumin and other abundant proteins pass through the column instead of being absorbed at the usual eluent pH. It is also possible to purify MAbs simply by step-wise gradient elution. Figure 4 shows a chromatogram in which MAb (IgG_1) is separated from cell culture supernatant on a TSKgel SP-5PW column. Clearly, the elution pattern and the selectivity differ as a function of the buffer in the eluent. In this case, better results are obtained with acetate rather than citrate buffer.

Though non-porous packing materials are not suitable to purify large quantities of monoclonal antibodies, they are the right column type to conduct a quick purity check of a MAb preparation since the high efficiency of NPR columns allow for fast separations of trace sample amounts.

Figure 5 demonstrates that a sample of mouse ascites fluid can be separated on a TSKgel DEAE-NPR column with similar resolution as that obtained on a TSKgel DEAE-5PW column (see Figure 2). Non-porous resin columns such as TSKgel DEAE-NPR are especially useful to check sample purity or to monitor expression levels, as they allow very short analysis times. Finally, although sample capacity is limited, columns packed with non-porous resin can be used to isolate nanogram amounts of MAb in a sample (see Separation Report No.65 for details).

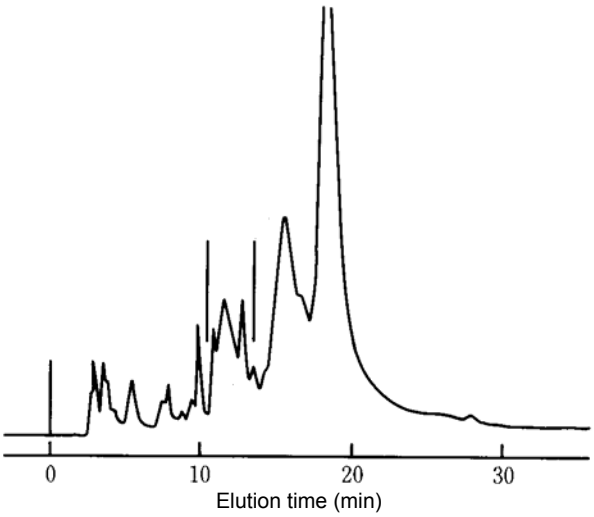

Figure 3 Separation of monoclonal antibody by IEC

Column:	TSKgel DEAE-5PW, 7.5mm ID × 7.5cm
Eluent:	A: 20mmol/L Tris-HCl (pH 8.8)
	B: A + 0.5mol/L NaCl
	A → B linear gradient (30 min.)
Flow rate:	1.0mL/min
Temperature:	25°C
Detection:	UV@280nm
Sample:	Anti-HLA-A, B, C (IgG_1),
	NS-1 culture supernatant

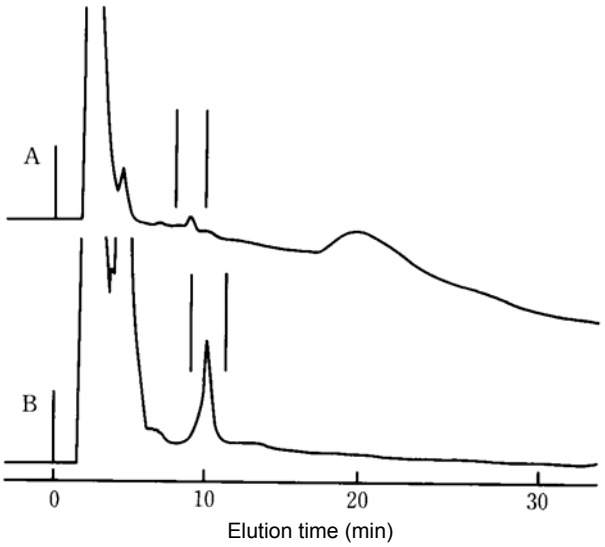

Figure 4 Separation of monoclonal antibody on IEC

Column:	TSKgel SP-5PW, 7.5mm ID × 7.5cm
Eluent:	A: a: 20mmol/L citrate buffer (pH 5.7)
	b: a + 0.5mol/L NaCl
	a → b linear gradient (30 min.)
	B: c: 20mmol/L acetate buffer (pH 5.7)
	d: c + 0.5mol/L NaCl
	c → d linear gradient (30 min.)
Flow rate:	1.0mL/min
Temperature:	25°C
Detection:	UV@280nm
Sample:	Anti-HLA-A, B, C (IgG_1),
	NS-1 culture supernatant (500µg in 500µl)

3.3 Hydrophobic Interaction Chromatography (HIC)

HIC is widely used as a method of protein purification and it is now often one of the steps in MAb purification. Traditionally, TSKgel Phenyl-5PW has mainly been used for the separation of proteins by HIC, while TSKgel Ether-5PW is better suited for the separation and recovery of such hydrophobic proteins as membrane proteins and proteins with molecular weights of 100,000Da or higher.

Figure 6 shows the separation of mouse ascites on TSKgel Phenyl-5PW and Ether-5PW columns, respectively. On the TSKgel Phenyl-5PW column the MAb (IgG_1) peak overlaps with the albumin peak at about 47 minutes. In contrast, on the TSKgel Ether-5PW column the MAb peak is well separated from the albumin peak, which elutes at about 13 minutes. In addition, MAb elutes faster from the TSKgel Ether-5PW column than TSKgel Phenyl-5PW when using the same separation conditions due to the difference in hydrophobicity of the packing materials. As shown in Figure 6, it is evident that in this study TSKgel Ether-5PW is the better choice for MAb separation by HIC.

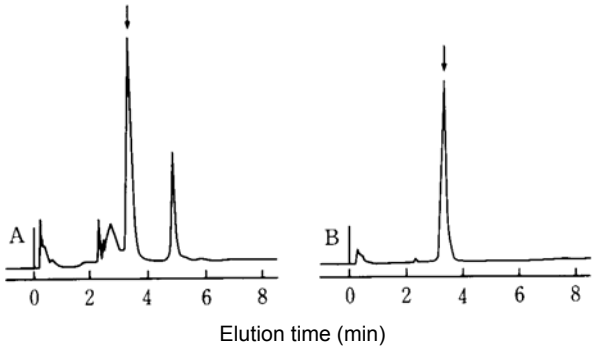

Figure 5 Purity check on monoclonal antibody fraction by IEC

Column: TSKgel DEAE-NPR, 4.6mm ID × 3.5cm
Eluent: a: 50mmol/L Tris-HCl (pH 8.5)
b: a + 0.5mol/L NaCl
a → b linear gradient (10 min.)
Flow rate: 1.5mL/min
Temperature: 25°C
Detection: UV@280nm
Sample: Anti-fowl 14K lectin (IgG_1)
A: Mouse ascites (× 4, 5μl)
B: TSKgel HA-1000, MAb fraction (100μl)

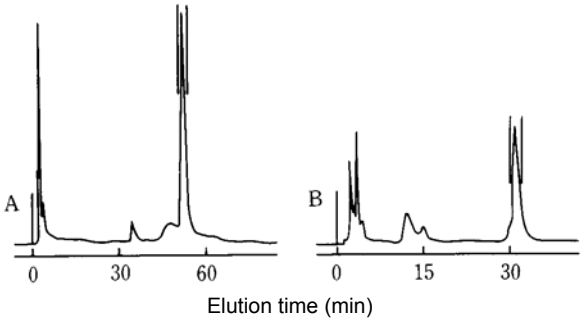

Figure 6 Separation of monoclonal antibody by HIC

Column: A: TSKgel Phenyl-5PW, 7.5mm ID × 7.5cm
B: TSKgel Ether-5PW, 7.5mm ID × 7.5cm
Eluent: a: 0.1mol/L sodium phosphate buffer (pH 7.0) + 1.5mol/L ammonium sulfate
b: 0.1mol/L sodium phosphate buffer (pH 7.0)
a → b linear gradient (60 min.)
Flow rate: 1.0mL/min
Temperature: 25°C
Detection: UV@280nm
Sample: Anti-fowl 14K lectin (IgG_1)
Mouse ascites (1.5mg in 100μl)

Figure 7 shows that a large sample volume can be injected on the TSKgel Ether-5PW column. 3mL of mouse ascites (2-fold dilution, 1.0mol/L ammonium sulfate) was directly injected on the column and eluted with a linear gradient or a step-wise gradient elution. A chromatogram nearly identical to Figure 6 was obtained for linear gradient elution (data not shown). With step-wise gradient elution, most of the impurities pass through the column, while only MAb is absorbed at the starting ammonium sulfate concentration of 1.0mol/L. The absorbed MAb was eluted with 0.5mol/L ammonium sulfate. Clearly, step-wise gradient elution provided a simple separation of this large sample volume.

Moreover, if one wishes to purify a large quantity of MAb, it is also possible to employ packing materials developed for low pressure liquid chromatography (Toyopearl resins) instead of using an HPLC column.

Toyopearl Ether-650M has a much larger particle size (~65μm) than TSKgel Ether-5PW (10μm) and is thus less efficient, although its selectivity is similar to that for TSKgel Ether-5PW. Please consult our website or request a copy of the Process Chromatography catalog for more information about the relationship between TSKgel columns and Toyopearl resins.

Similar to IEC, a non-porous resin column is available in the TSKgel line of HIC columns. Using TSKgel Butyl-NPR, MAb purity check or micro-purification can be rapidly achieved. Figure 8 shows the separation of mouse ascites on a TSKgel Butyl-NPR column. The separation was completed in about 3 minutes and it is clear that MAb is well separated from the impurities.

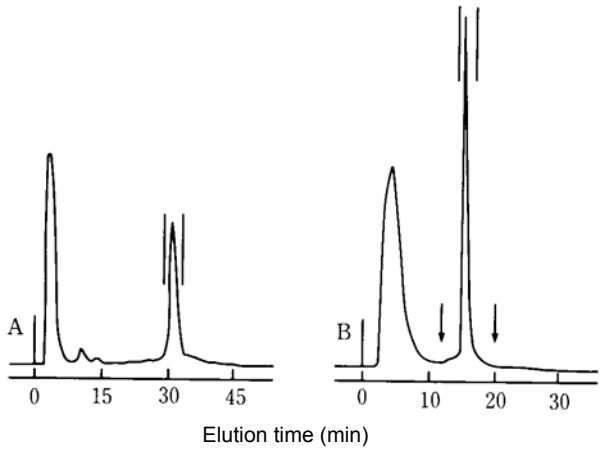

Figure 7 Separation of large amount of monoclonal antibody by HIC

Column: TSKgel Ether-5PW, 7.5mm ID × 7.5cm
Eluent: A: Same as Figure 6
B: a: 0.1mol/L sodium phosphate buffer (pH 7.0) + 1.0mol/L ammonium sulfate
b: 0.1mol/L sodium phosphate buffer (pH 7.0)
a → a/b (50/50) (12 min.) → b (20 min.) step-wise gradient
Flow rate: 1.0mL/min
Temperature: 25°C
Detection: UV@280nm
Sample: Anti-fowl 14K lectin (IgG_1),
Mouse ascites (90mg in 3.0mL)

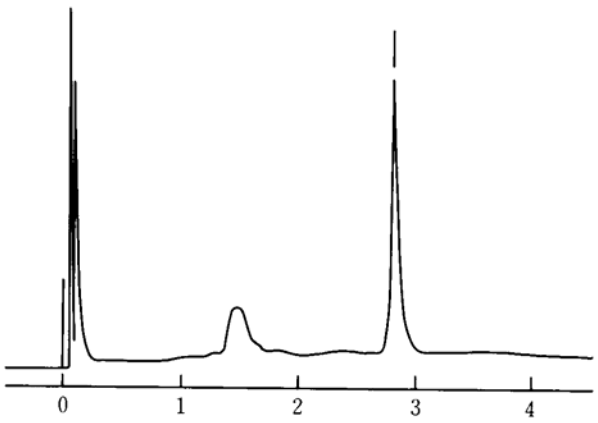

Figure 8 Separation of monoclonal antibody by HIC

Column: TSKgel Butyl-NPR, 4.6mm ID × 3.5cm
Eluent: A: 0.1mol/L sodium phosphate buffer (pH 7.0) + 1.5mol/L ammonium sulfate
B: 0.1mol/L sodium phosphate buffer (pH 7.0)
A → B linear gradient (10 min.)
Flow rate: 1.0mL/min
Temperature: 25°C
Detection: UV@280nm
Sample: Anti-fowl 14K lectin (IgG_1)
Mouse ascites (38μg in 2.5μl)

3.4 Affinity Chromatography (AFC)

AFC using Protein A is often employed for MAb separation, however, it is also possible to separate monoclonal antibodies using Immobilized Metal Ion Affinity Chromatography (IMAC). Figures 10 and 11 demonstrate MAb purification from two matrices, mouse ascites and culture supernatant, on a TSKgel Chelate-5PW column loaded with Zn^{2+} ion. Figure 11 shows that MAb (IgG_1) is eluted in about 32 minutes by pH gradient elution and is well separated from other impurities.

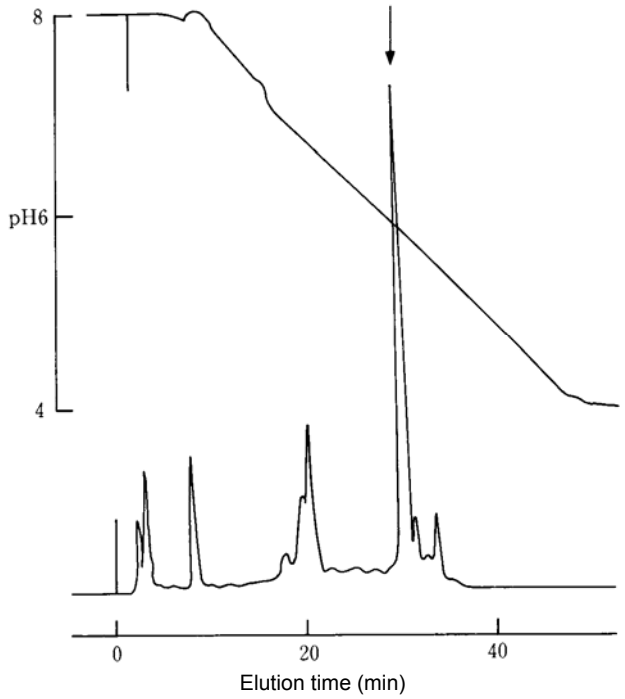

Figure 10 Separation of monoclonal antibody by AFC

Column:	TSKgel Chelate-5PW Glass (Zn^{2+}), 8mm ID × 7.5cm
Eluent:	A: 20mmol/L HEPES-MES-acetate buffer (pH 8.0) + 0.5mol/L NaCl B: 20mmol/L HEPES-MES-acetate buffer (pH 4.0) + 0.5mol/L NaCl A → B linear gradient (40 min.)
Flow rate:	1.0mL/min
Temperature:	25°C
Detection:	UV@280nm
Sample:	Anti-fowl 14K lectin (IgG_1), Mouse ascites (50µl)

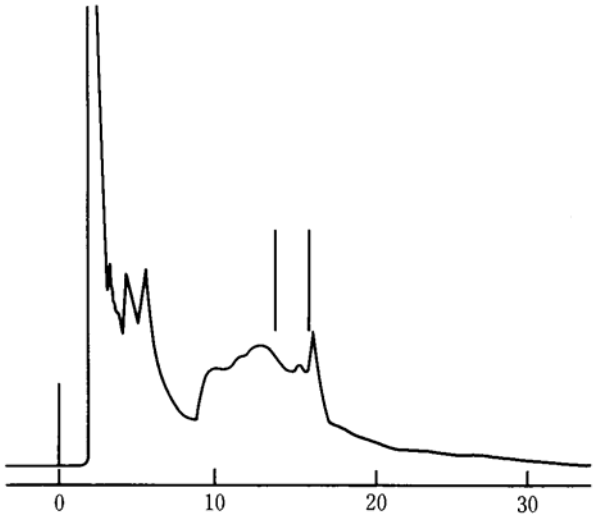

Figure 11 Separation of monoclonal antibody by AFC

Column:	TSKgel Chelate-5PW (Zn^{2+}), 7.5mm ID × 7.5cm
Eluent:	A: 20mmol/L Tris-HCl (pH 8.0) + 0.5mol/L NaCl B: A + 200mmol/L glycine A → B linear gradient (30 min.)
Flow rate:	1.0mL/min
Temperature:	25°C
Detection:	UV@280nm
Sample:	Anti-HLA-A, B, C (IgG_1), NS-1 cultured supernatant

4. Conclusio n

As discussed in this document, there are several different separation modes for the purification of monoclonal antibodies and each separation mode has its advantages. Therefore, it is important in the purification of monoclonal antibodies to select the most appropriate separation mode and column with consideration of various factors for purification, such as resolution, sample load, operability, cost, and purity.

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