



(51) International Patent Classification:

G01N 30/34 (2006.01) G01N 33/50 (2006.01)
G01N 30/96 (2006.01) C07K 1/18 (2006.01)

(21) International Application Number:

PCT/US2013/070415

(22) International Filing Date:

15 November 2013 (15.11.2013)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/727,051 15 November 2012 (15.11.2012) US
61/780,707 13 March 2013 (13.03.2013) US

(71) Applicant: GENENTECH, INC. [US/US]; 1 DNA Way,
South San Francisco, CA 94080-4990 (US).

(72) Inventors: WANG, Yajun; 1 DNA Way, South San Francisco, CA 94080-4990 (US). MORENO, George, T.; 1 DNA Way, South San Francisco, CA 94080-4990 (US). ZHANG, Boyan; 1 DNA Way, South San Francisco, CA 94080-4990 (US). ZHANG, Liangyi; 1 DNA Way, South San Francisco, CA 94080-4990 (US). FARNAN, Dell; 1 DNA Way, South San Francisco, CA 94080-4990 (US). PATAPOFF, Tom; 1 DNA Way, South San Francisco, CA 94080-4990 (US).

(74) Agents: DONAHUE, Brian, A. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

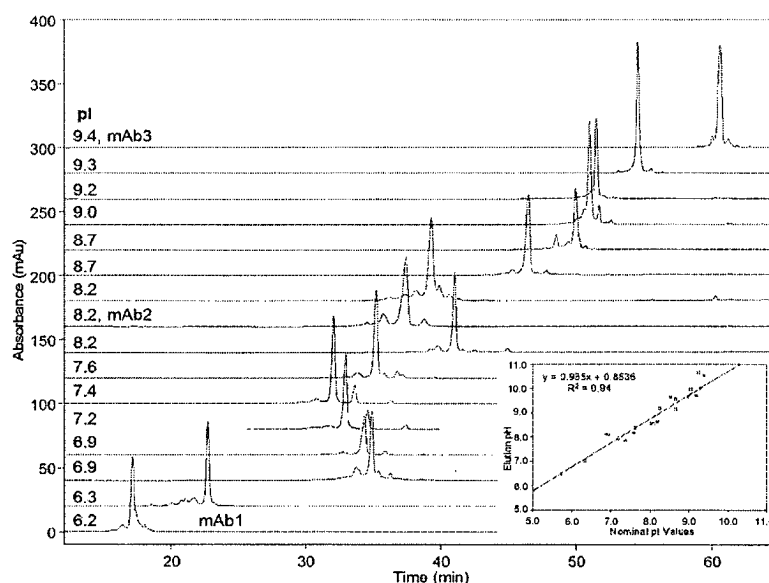
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: IONIC STRENGTH-MEDIATED pH GRADIENT ION EXCHANGE CHROMATOGRAPHY

Figure 4



(57) Abstract: The present invention provides methods for analyzing compositions of polypeptides such as antibodies by ionic strength-mediated pH gradient ion exchange chromatography. In some aspects, the methods use a combination of pH gradients and ionic strength gradients to separate the polypeptide from charge variants of the polypeptide. In some aspects, the methods use a stable ionic strength to optimize the pH gradient separation window to separate the polypeptide from charge variants. Such methods are useful for analyzing polypeptide, e.g. antibodies, with a pI greater than 9 or a pI less than 7. In some aspects, the invention provides a multiproduct method for the analysis of polypeptides of varying pI's.

IONIC STRENGTH-MEDIATED pH GRADIENT ION EXCHANGE CHROMATOGRAPHY

FIELD OF THE INVENTION

[0001] The present invention provides methods for analyzing preparations of polypeptides using ionic strength-mediated pH gradient ion exchange chromatography.

BACKGROUND OF THE INVENTION

[0002] Proteins like monoclonal antibodies (mAbs) have mostly charged and polar amino acids at the surface in an aqueous environment (Barlow, DJ and Thornton, JM (1986) *Biopolymers* 25:1717). Because of molecular interaction with the solution components, the surface residues can undergo multiple chemical and enzymatic modifications, leading to a heterogeneous mixture of protein variants with slight differences on their electrostatic surface (Dick, LW *et al.*, (2009) *J. Chromatogr. B* 877:3841; Liu, HW *et al.*, (2008) *Rapid Commun. Mass Spectrom.* 22:4081; Miller, AK, *et al.*, (2011) *J. Pharm. Sci.* 100:2543; Wang, WR *et al.*, (2011) *Mol. Immunol.* 48:860). Cation-exchange chromatography (CEC) is considered to be the gold standard to profile the charge heterogeneity of protein therapeutics according to a recent review by Vlasak, J and Ionescu, R (2008 *Curr. Pharm. Biotechnol.* 9:468). The charge sensitive separation method is typically required by the regulatory agencies to ensure the production consistency during manufacturing and to monitor the degradation level of protein therapeutics (Miller, AK, *et al.*, (2011) *J. Pharm. Sci.* 100:2543; He, XPZ (2009) *Electrophoresis* 30:714; Sasic, Z *et al.*, (2008) *Electrophoresis* 29:4368; Kim, J *et al.*, (2010) *J. Chromatogr. B* 878:1973; Teshima, G *et al.*, (2010) *J. Chromatogr. A* 1218:2091).

[0003] Analytical ion exchange chromatography (IEC) methods using a pH gradient have emerged as alternative techniques to conventional salt gradient IEC for profiling the charge heterogeneity of therapeutic proteins (Farnan, D and Moreno, GT (2009) *Anal. Chem.* 81:8846; Tsonev, LI and Hirsh, AG (2008) *J. Chromatogr. A* 1200:166; Nordborg, A *et al.*, (2009) *J. Sep. Sci.* 32:2668; Rozhkova, A (2009) *J. Chromatogr. A* 1216:5989; Rea, JC *et al.* (2010) *J. Pharm. Biomed. Anal.* 54:317). In this technique, proteins that are typically loaded on a cation-exchange stationary phase are eluted by increasing the pH of the mobile phase. It has recently been demonstrated that a pH gradient IEC (pH-IEC) method with a relatively broad pH window from 6.0 to 9.5 not only provided better resolution than traditional salt-gradient IEC, but also offered

multi-product capability through the analysis of 12 monoclonal antibodies (mAbs) with pI from 7.3 to 9.0 (Farnan, D and Moreno, GT (2009) *Anal. Chem.* 81:8846). That pH-IEC method is also highly tolerant to sample matrix with varied ionic strengths (0 to 250 mM NaCl) and pH values (5.0 to 8.5) (Farnan, D and Moreno, GT (2009) *Anal. Chem.* 81:8846). Furthermore, the reported pH-IEC method is not evidently impacted by the column length and chemistry and fast separation with a shorter column can be achieved to improve the throughput of protein variant analysis. According to a recent validation report (Rea, JC *et al.* (2010) *J. Pharm. Biomed. Anal.* 54:317), the developed pH-IEC method has shown excellent precision at different chromatography conditions and good linearity at different column loads. Thus the reported pH-IEC method is suitable for routine testing in the biotechnology industry.

[0004] Despite the many advantages, the reported pH-IEC method was intended primarily for the mAbs with pI values in the studied range of 7.3 to 9.0. The fact that the elution profile of a mAb can vary with different buffer compositions and concentration, and the pH values at which the mAbs elutes indicates that pH-gradient IEC involves a combined ionic-strength and pH-gradient elution mechanism (Farnan, D and Moreno, GT (2009) *Anal. Chem.* 81:8846). This is also consistent with Anderson and coworkers' report on pH-gradient anion-exchange chromatography (pH-AIEC) (Anderson, DJ and Shan, L (2001) *Clin. Chem.* 47:128; Shan, L and Anderson, DJ (2001) *J. Chromatogr. A* 909:191; Shan, L and Anderson, DJ (2002) *Anal. Chem.* 74:5641). With an increasing number of mAbs in the development phase in the biotechnology industry, especially more low-pI mAbs that show potentially longer half-life based on the animal studies (Igawa, T. (2010) *Protein Eng. Des. Sel.* 23:385) there is a need to expand the applicability of pH-IEC methods to a broader range of therapeutic mAbs.

[0005] All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

BRIEF SUMMARY

[0006] The invention provides a method for analyzing a composition comprising the polypeptide and one or more contaminants, the method comprising a) binding the polypeptide and one of more contaminants to an ion-exchange chromatography material using a loading buffer, wherein the loading buffer is at a first pH and comprises a first ionic strength; b) eluting the polypeptide and one or more contaminants from the ion-exchange chromatography material using an elution buffer wherein the pH of the elution buffer is altered in a pH gradient and the ionic strength of

the elution buffer is altered in an ionic strength gradient, wherein the polypeptide and the one or more contaminants are separated by the combination of pH gradient and ionic strength gradient; c) detecting the polypeptide and the one or more contaminants. In some embodiments of the invention, polypeptides with pI's ranging from 6.0 to 9.5 can be analyzed using the same methods.

[0007] In some embodiments, the polypeptide is an antibody or immunoadhesin or fragment thereof. In further embodiments, the polypeptide is a monoclonal antibody or fragment thereof. In yet further embodiments, the antibody is a human antibody. In other embodiments, the antibody is a humanized antibody. In other embodiments, the antibody is a chimeric antibody. In some embodiments, the antibody is an antibody fragment.

[0008] In some embodiments of the above embodiments, the contaminant is a variant of the polypeptide. In further embodiments, the contaminant is a degradation product of the polypeptide.

[0009] In some embodiments, the polypeptide has a pI greater than about 9.0. In further embodiments, the chromatography material is a cation exchange chromatography material. In yet further embodiments, the cation exchange chromatography material is a sulfonated chromatography material or a carboxylated chromatography material.

[0010] In further embodiments of any of the above embodiments, the pH gradient is a linear gradient. In other embodiments of any one of the above embodiments, the pH gradient is a step gradient. In further embodiments, the pH gradient comprises an increase from about pH 5 to about pH 11. In some embodiments of the above embodiments, the pH gradient is generated using one or more buffers. In further embodiments, the one or more buffers is piperazine, imidazole, tris, phosphate, or CAPS.

[0011] In some embodiments of any one of embodiments, the ionic strength gradient is a linear gradient. In other embodiments, the ionic strength gradient is a step gradient. In some embodiments, the ionic strength gradient comprises an increase in salt concentration from about 0 mM to about 200 mM. In further embodiments, the ionic strength gradient is an NaCl gradient, a KCl gradient, or an Na₂SO₄ gradient.

[0012] In some embodiments of the above embodiments, the polypeptide has a pI less than about 7.0. In further embodiments, the chromatography material is an anion exchange chromatography material. In yet further embodiments, the anion exchange chromatography material is a quarternary amine chromatography material or a tertiary amine chromatography

material. In some embodiments, the pH gradient is a linear gradient. In other embodiments, the pH gradient is a step gradient. In some embodiments, the pH gradient comprises a decrease from about pH 8 to about pH 5. In some embodiments, the pH gradient is generated using one or more buffers. In yet further embodiments, the one or more buffers is piperazine, imidazole or Tris. In some embodiments, the ionic strength gradient is a linear gradient. In other embodiments, the ionic strength gradient is a step gradient. In yet further embodiments, the ionic strength gradient comprises an increase in salt concentration from about 0 mM to about 200 mM. In some embodiments, the ionic strength gradient is a NaCl gradient, a KCl gradient, or a Na₂SO₄ gradient.

[0013] In some aspects, the invention provides a method for analyzing a composition comprising the polypeptide and one or more contaminants, the method comprising a) binding the polypeptide and one of more contaminants to an ion-exchange chromatography material using a loading buffer, wherein the loading buffer is at an initial pH and comprises an initial ionic strength; b) eluting the polypeptide and one or more contaminants from the ion-exchange chromatography material using an elution buffer wherein the pH of the elution buffer is altered in a pH gradient and wherein the ionic strength of the elution buffer is essentially the same as the initial ionic strength of the loading buffer, wherein the polypeptide and the one or more contaminants are separated by pH gradient at about the initial ionic strength; c) detecting the polypeptide and the one or more contaminants. In some embodiments of the invention, polypeptides with pI's ranging from 6.0 to 9.5 can be analyzed using essentially the same methods.

[0014] In some embodiments of the above aspect, the polypeptide is an antibody or immunoadhesin or fragment thereof. In further embodiments, the polypeptide is a monoclonal antibody or fragment thereof. In further embodiments of embodiments, the antibody is a human antibody. In other embodiments, the antibody is a humanized antibody. In yet other embodiments, the antibody is a chimeric antibody. In some embodiments, the antibody is an antibody fragment.

[0015] In some embodiments of the above aspect, the contaminant is a variant of the polypeptide. In some embodiments, the contaminant is a degradation product of the polypeptide.

[0016] In some embodiments of the above aspect, the polypeptide has a pI greater than about 9.0. In further embodiments, the chromatography material is a cation exchange chromatography

material. In yet further embodiments, the cation exchange chromatography material is a sulfonated chromatography material or a carboxylated chromatography material.

[0017] In some embodiments of the above aspect, the pH gradient is a linear gradient. In other embodiments, the pH gradient is a step gradient. In some embodiments, the pH gradient comprises an increase from about pH 5 to about pH 11. In some embodiments, the pH gradient is generated using one or more buffers. In further embodiments, the one or more buffers is piperazine, imidazole, tris, phosphate, or CAPS.

[0018] In some embodiments of the above aspect, the ionic strength of the elution buffer is from about 0 mM to about 100 mM. In further embodiments, the elution buffer comprises about 0 mM NaCl to about 100 mM NaCl, about 0 mM KCl to about 100 mM KCl, or about 0 mM Na₂SO₄ to about 100 mM Na₂SO₄.

[0019] In some embodiments of the above aspect, the polypeptide has a pI less than about 7.0. In further embodiments, the chromatography material is an anion exchange chromatography material. In yet further embodiments, the anion exchange chromatography material is a quaternary amine chromatography material or a tertiary amine chromatography material. In some embodiments of the above aspect, the pH gradient is a linear gradient. In other embodiments, the pH gradient is a step gradient. In some embodiments of the above aspect, the pH gradient comprises a decrease from about pH 8 to about pH 5. In some embodiments, the pH gradient is generated using one or more buffers. In further embodiments, the one or more buffers is piperazine, imidazole or Tris. In some embodiments, the ionic strength of the elution buffer is from about 0 mM to about 100 mM. In further embodiments, the elution buffer comprises about 10 mM NaCl to about 100 mM NaCl.

[0020] In some embodiments of any one of the above embodiments, the analysis is by high performance liquid chromatography.

[0021] In some aspects, the invention provides a method of determining the purity of a polypeptide in a composition comprising analyzing the composition according to any one of the methods of the above embodiments and determining the ratio of polypeptide to contaminants in the composition.

[0022] In some aspects, the invention provides a method of determining the stability of a polypeptide in a composition comprising the polypeptide, the method comprising, a) incubating the composition comprising the polypeptide at 0 °C to 40 °C for one to six weeks, b) analyzing the composition of step a) by any of the methods of embodiments 1 to 63, and c) determining the

ratio of variants to polypeptide in the composition, wherein an increase in the ratio of variants to polypeptide in the composition compared to a composition that was not incubated indicates the degradation of the polypeptide in the composition.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] Figure 1 shows the charge heterogeneity profiles on three monoclonal antibodies with different pIs (mAb1, mAb2 and mAb3) obtained with a pH-gradient ion exchange chromatography (IEC) method.

[0024] Figure 2 shows the ionic strength and pH profiles at column exit (panel A) and the column back pressure in the pH gradient (panel B) before optimization and the ionic strength and pH profiles at column exit (panel C) and the column back pressure in the pH gradient (panel D) after optimization. Modeled and experimental pH profiles and conductivity profiles are shown in panels A and C.

[0025] Figure 3 shows the ionic strength mediated pH IEC chromatograms of mAb1 (panel A) and mAb2 (panel B) obtained with four different buffer concentrations. The full-width at half-maxima (FWHM) of the main peak in the chromatograms are plotted against buffer concentration (panel C) and salt concentration (panel D).

[0026] Figure 4 shows the charge heterogeneity profiles obtained with ionic strength-mediated pH gradient IEC of sixteen monoclonal antibodies with pI's ranging from 6.2 to 9.4. Insert shows a graph of nominal pI values plotted against elution pH.

[0027] Figure 5 shows the chromatograms obtained with ionic strength-mediated pH gradient IEC of native mAb1 (0 wk) and thermally stressed mAb1 (3 wk and 6 wk at 40 °C).

[0028] Figure 6 shows chromatograms of mAb1 with 10, 20, 50, 100 and 200 µg of column load obtained with the ionic strength-mediated pH gradient IEC.

[0029] Figure 7 shows the buffering through amino functional groups of a piperazine, imidazole, Tris (PIT) buffer solution in a semi-linear pH gradient from pH 6 to pH 9.5.

[0030] Figure 8 shows chromatograms of mAbs with pI's ranging from pI 6.2 to pI 9.4 using ionic strength mediated IEC. The column was a Propac WCX-10, 4 × 250 mm.

[0031] Figure 9 shows that similar profiles are observed between original pH-IEC and ionic strength-mediated pH-IEC when using a 4 × 250 mm column.

[0032] Figure 10 shows a model linear pH gradient from pH 6 to pH 11 using phosphate to maintain conductivity as pH increases.

[0033] Figure 11 are graphs showing that observed pH (Panel A) and conductivity (Panel C) profiles are consistent with model predictions (Panels B and D).

[0034] Figure 12 shows that shorter runs are possible using TPP buffers. Top chromatogram shows results with a 4 × 50 mm Propac WCX-10HT column with a 16 minute gradient and bottom chromatogram shows results with a 4 × 250 mm column with a 58 minute gradient.

[0035] Figure 13 is a graph demonstrating the charge state of different molecules at different pH using a modeling tool that calculates the charge of monoclonal antibodies with different pI's. The charge on a monoclonal antibody is neutral when it crosses the X-axis. The suitable pH-IEC separation window corresponds to the pH range where the curve is relatively flat.

[0036] Figure 14 is a graph showing that at high pI, molecules have less time to resolve as pH increases.

[0037] Figure 15 shows the results of a study to determine the optimal salt concentration for the best charge shielding effect of an antibody with a pI > 9.0. Salt concentrations of 0 mM, 10 mM, 20 mM, 30 mM, 40 mM and 50 mM were tested.

[0038] Figure 16 shows the results of a study to determine the optimal salt concentrations for the best charge shielding effects for antibodies with pI's ranging from 8.9 to 9.1.

[0039] Figure 17 shows the results of a study to determine the optimal salt concentrations for the best charge shielding effects for antibodies with pI's ranging from 7.6 to 8.7.

[0040] Figure 18 is a graph showing that adding salt to shield extra charges moves the charge state into a suitable separation window.

[0041] Figure 19 is a graph showing that using a shallow pH gradient may improve peak resolution using a MAb with a pI of 7.6. Gradients tested were as follows: PIT (2.4 mM Tris, 1.5 mM imidazole, 11.6 mM piperazine, pH 6-11); salt mediated PIT (4 mM Tris, 4 mM imidazole, 4 mM piperazine, pH 6-11, 0-16 mM NaCl gradient); salt mediated TPP (5 mM Tris, 5 mM piperazine, 5 mM phosphate, pH 6-11, 0-30 mM NaCl gradient); hybrid pH-IEC, TPP (5 mM Tris, 5 mM piperazine, 5 mM phosphate, pH 6-9, 0 mM NaCl); TIC (5 mM Tris, 5 mM piperazine, 5 mM CAPS, pH 6-9, 10 mM NaCl). Runs were 20-22 minutes.

[0042] Figure 20 is a graph showing that using a shallow pH gradient may improve peak resolution using a MAb with a pI of 8.6-9.3. Gradients tested were as follows: PIT (2.4 mM Tris, 1.5 mM imidazole, 11.6 mM piperazine, pH 6-11); salt mediated PIT (4 mM Tris, 4 mM imidazole, 4 mM piperazine, pH 5-11, 16 mM NaCl); salt mediated TPP (5 mM Tris, 5 mM piperazine, 5 mM phosphate, pH 6-11, 20 mM NaCl gradient); TPP (5 mM Tris, 5 mM

piperazine, 5 mM phosphate, pH 6-9, 20 mM NaCl); TIC (5 mM Tris, 5 mM piperazine, 5 mM CAPS, pH 7-10, 30 mM NaCl). Runs were 20-22 minutes.

[0043] Figure 21 is a graph showing that using a shallow pH gradient may improve peak resolution using a MAb with a pI of 9.0. Gradients tested were as follows: PIT (2.4 mM Tris, 1.5 mM imidazole, 11.6 mM piperazine, pH 6-11); salt mediated PIT (4 mM Tris, 4 mM imidazole, 4 mM piperazine, pH 5-11, 16 mM NaCl); TPP (5 mM Tris, 5 mM piperazine, 5 mM phosphate, pH 6-11, 30 mM NaCl gradient); TPP (5 mM Tris, 5 mM piperazine, 5 mM phosphate, pH 7-10, 20 mM NaCl); TIC (5 mM Tris, 5 mM piperazine, 5 mM CAPS, pH 7-10, 30 mM NaCl). Runs were 20-22 minutes.

[0044] Figure 22 is a graph showing that run times of fifteen minutes are possible. The Mab has a pI of 8.8. Two chromatograms are shown, one with a pH gradient from 6 to 10 in 20 mM NaCl in 22 minutes, and one with a pH gradient from 7 to 10 in 20 mM NaCl in 15 minutes.

[0045] Figure 23 is a graph showing that run times of fifteen minutes are possible. The Mab has a pI of 9.0. Two chromatograms are shown, one with a pH gradient from 6 to 10 in 20 mM NaCl in 22 minutes, and one with a pH gradient from 7 to 10 in 20 mM NaCl in 15 minutes.

[0046] Figure 24 is a graph showing overlaid chromatograms of duplicate analysis of three monoclonal antibodies at the target condition.

[0047] Figure 25 is a graph showing overlaid chromatograms of MAb3 at different chromatography conditions. Main peaks are aligned.

[0048] Figure 26 is a distribution plot showing the effect of salt concentration on chromatography performance. Circles represent main peak percentage, diamonds represent acidic variants percentage, × represents basic variant percentage, triangles represent resolution 1, and * represents resolution 2.

[0049] Figure 27 shows distribution plots showing the effects of other parameters on chromatography performance. Top left shows the effect of buffer concentration (mM). Top right shows the effect of starting pH. Bottom left shows the effect of column temperature in °C. Bottom right shows the effect of flow rate (ml/min).

DETAILED DESCRIPTION OF THE INVENTION

[0050] The invention provides methods of analyzing a composition comprising a polypeptide and one or more contaminants, *e.g.* polypeptide variants, comprising binding the polypeptide and one or more contaminants to a ion exchange chromatography material using a loading buffer

with an initial pH and an initial ionic strength, eluting the polypeptide and one or more contaminants from the ion-exchange column using an elution buffer wherein the pH of the elution buffer is altered by a pH gradient and the ionic strength of the elution buffer is altered by an ionic strength gradient such that the polypeptides and the one or more contaminants elute from the chromatography material as distinct separate entities. The methods of the invention can be used to analyze polypeptides with isoelectric points that are not in the neutral pH range. In some embodiments, the methods may be used to effectively separate polypeptides with a pI greater than 9 from contaminants. In other embodiments, the methods may be used to effectively separation polypeptides with a pI less than 7 from contaminants. In some embodiments, the method effectively separates one or more contaminants from the polypeptide, wherein the polypeptide has a pI ranging from about 7.0 to about 12. In some embodiments, the method can be used to effectively separate one or more contaminants from the polypeptide, wherein the polypeptide has a pI ranging from about 7.0 to about 12. Examples of polypeptides include, but are not limited to, antibodies and antibody fragments. Examples of contaminants include, but are not limited to, antibody variants such as antibody charge variants.

[0051] In other aspects, the invention provides methods of analyzing a composition comprising a polypeptide and one or more contaminants, *e.g.* polypeptide variants, comprising binding the polypeptide and one or more contaminants to a ion exchange chromatography material using a loading buffer with an initial pH and an initial ionic strength, eluting the polypeptide and one or more contaminants from the ion-exchange column using an elution buffer wherein the pH of the elution buffer is altered by a pH gradient and the ionic strength essentially remains the same such that the polypeptides and the one or more contaminants elute from the chromatography material as distinct separate entities. The methods of the invention can be used to analyze polypeptides with isoelectric points that are not in the neutral pH range. In some embodiments, the methods may be used to effectively separate polypeptides with a pI greater than 9 from contaminants. In other embodiments, the methods may be used to effectively separate polypeptides with a pI less than 7 from contaminants. Examples of polypeptides include, but are not limited to, antibodies and antibody fragments. Examples of contaminants include, but are not limited to, antibody variants such as antibody charge variants.

[0052] In some aspects, the invention provides methods of analyzing compositions comprising a polypeptide and one or more contaminants, *e.g.* polypeptide variants, comprising binding the polypeptide and one or more contaminants to a ion exchange chromatography material using a

loading buffer with an initial pH and an initial ionic strength such that the charge state of the polypeptide in the solution falls within an optimal pH gradient ion exchange separation window. The polypeptide and the one or more contaminants are then eluted from the chromatography material as distinct separate entities.

[0053] In some embodiments of the invention, the contaminants are polypeptide charge variants including acidic variants, *i.e.* variants with a retention time less than that of the main peak in the cation exchange mode. Examples of acidic variants include but are not limited to polypeptides where one or more glutamine and/or asparagine residues have been deamidated. In some embodiments of the invention, the contaminants are polypeptide charge variants including basic variants, *i.e.* variants with a retention time greater than that of the main peak in the cation exchange mode. Examples include but are not limited to variants where an aspartic acid residue has undergone modification to a succinimide moiety.

[0054] In some embodiments, the invention provides methods of analyzing a composition comprising a polypeptide and one or more contaminants, wherein essentially the same methods can be used to analyze polypeptides with different pI. For example, the method may be used to analyze polypeptides with pI's ranging from 6.0 to 9.5. In some embodiments, the polypeptides are antibodies, or fragments thereof. In some embodiments, the contaminants are antibody variants or variants of antibody fragments. In some embodiments, the contaminants are antibody charge variants or charge variants of antibody fragments. In some embodiments, the invention provides a method to analyze compositions of antibodies or antibody fragments for the presence of charge variants (*e.g.* acidic variants and/or basic variants) wherein the method can be used to analyze different compositions comprising an antibody wherein the antibody has a pI ranging from 6.0 to 9.5.

I. Definitions

[0055] The term “polypeptide” or “protein” are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid

(including, for example, unnatural amino acids, *etc.*), as well as other modifications known in the art. The terms "polypeptide" and "protein" as used herein specifically encompass antibodies.

[0056] The term "polypeptide charge variant" as used herein refers to polypeptide that has been modified from its native state such that the charge of the polypeptide is altered. In some examples, charge variants are more acidic than the parent polypeptide; *i.e.* have a lower pI than the parent polypeptide. In other examples, charge variants are more basic than the parent polypeptide; *i.e.* have a higher pI than the parent polypeptide. Such modifications may be engineered or the result of natural processes such as oxidation, deamidation, C-terminal processing of lysine residues, N-terminal pyroglutamate formation, and glycation. In some examples, a polypeptide charge variant is a glycoprotein where the glycan attached to the protein is modified such that the charge of the glycoprotein is altered compared to parent glycoprotein, for example, by addition of sialic acid or its derivatives. An "antibody charge variant" as used herein is an antibody or fragment thereof wherein the antibody or fragment thereof has been modified from its native state such that the charge of the antibody or fragment thereof is altered.

[0057] "Purified" polypeptide (*e.g.*, antibody or immunoadhesin) means that the polypeptide has been increased in purity, such that it exists in a form that is more pure than it exists in its natural environment and/or when initially synthesized and/or amplified under laboratory conditions.

Purity is a relative term and does not necessarily mean absolute purity.

[0058] The term "antagonist" is used in the broadest sense, and includes any molecule that partially or fully blocks, inhibits, or neutralizes a biological activity of a native polypeptide. In a similar manner, the term "agonist" is used in the broadest sense and includes any molecule that mimics a biological activity of a native polypeptide. Suitable agonist or antagonist molecules specifically include agonist or antagonist antibodies or antibody fragments, fragments or amino acid sequence variants of native polypeptides, *etc.* Methods for identifying agonists or antagonists of a polypeptide may comprise contacting a polypeptide with a candidate agonist or antagonist molecule and measuring a detectable change in one or more biological activities normally associated with the polypeptide.

[0059] A polypeptide "which binds" an antigen of interest, *e.g.* a tumor-associated polypeptide antigen target, is one that binds the antigen with sufficient affinity such that the polypeptide is useful as a diagnostic and/or therapeutic agent in targeting a cell or tissue expressing the antigen, and does not significantly cross-react with other polypeptides. In such embodiments, the extent of binding of the polypeptide to a "non-target" polypeptide will be less than about 10% of the

binding of the polypeptide to its particular target polypeptide as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

[0060] With regard to the binding of a polypeptide to a target molecule, the term “specific binding” or “specifically binds to” or is “specific for” a particular polypeptide or an epitope on a particular polypeptide target means binding that is measurably different from a non-specific interaction. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding can be determined by competition with a control molecule that is similar to the target, for example, an excess of non-labeled target. In this case, specific binding is indicated if the binding of the labeled target to a probe is competitively inhibited by excess unlabeled target.

[0061] The term “antibody” herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.* bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity. The term “immunoglobulin” (Ig) is used interchangeable with antibody herein.

[0062] Antibodies are naturally occurring immunoglobulin molecules which have varying structures, all based upon the immunoglobulin fold. For example, IgG antibodies have two “heavy” chains and two “light” chains that are disulphide-bonded to form a functional antibody. Each heavy and light chain itself comprises a “constant” (C) and a “variable” (V) region. The V regions determine the antigen binding specificity of the antibody, whilst the C regions provide structural support and function in non-antigen-specific interactions with immune effectors. The antigen binding specificity of an antibody or antigen-binding fragment of an antibody is the ability of an antibody to specifically bind to a particular antigen.

[0063] The antigen binding specificity of an antibody is determined by the structural characteristics of the V region. The variability is not evenly distributed across the 110-amino acid span of the variable domains. Instead, the V regions consist of relatively invariant stretches called framework regions (FRs) of 15-30 amino acids separated by shorter regions of extreme variability called “hypervariable regions” that are each 9-12 amino acids long. The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β -sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β -sheet structure. The hypervariable regions in each chain are

held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (*see Kabat et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

[0064] Each V region typically comprises three complementarity determining regions (“CDRs”, each of which contains a “hypervariable loop”), and four framework regions. An antibody binding site, the minimal structural unit required to bind with substantial affinity to a particular desired antigen, will therefore typically include the three CDRs, and at least three, preferably four, framework regions interspersed there between to hold and present the CDRs in the appropriate conformation. Classical four chain antibodies have antigen binding sites which are defined by V_H and V_L domains in cooperation. Certain antibodies, such as camel and shark antibodies, lack light chains and rely on binding sites formed by heavy chains only. Single domain engineered immunoglobulins can be prepared in which the binding sites are formed by heavy chains or light chains alone, in absence of cooperation between V_H and V_L.

[0065] The term “variable” refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β -sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β -sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (*see Kabat et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

[0066] The term “hypervariable region” when used herein refers to the amino acid residues of an antibody that are responsible for antigen binding. The hypervariable region may comprise amino acid residues from a “complementarity determining region” or “CDR” (e.g., around about residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the V_L , and around about 31-35B (H1), 50-65 (H2) and 95-102 (H3) in the V_H (Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a “hypervariable loop” (e.g. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the V_L , and 26-32 (H1), 52A-55 (H2) and 96-101 (H3) in the V_H (Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)).

[0067] “Framework” or “FR” residues are those variable domain residues other than the hypervariable region residues as herein defined.

[0068] “Antibody fragments” comprise a portion of an intact antibody, preferably comprising the antigen binding region thereof. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; tandem diabodies (taDb), linear antibodies (e.g., U.S. Patent No. 5,641,870, Example 2; Zapata *et al.*, *Protein Eng.* 8(10):1057-1062 (1995)); one-armed antibodies, single variable domain antibodies, minibodies, single-chain antibody molecules; multispecific antibodies formed from antibody fragments (e.g., including but not limited to, Db-Fc, taDb-Fc, taDb-CH3, (scFV)₄-Fc, di-scFv, bi-scFv, or tandem (di,tri)-scFv); and Bi-specific T-cell engagers (BiTEs).

[0069] Papain digestion of antibodies produces two identical antigen-binding fragments, called “Fab” fragments, each with a single antigen-binding site, and a residual “Fc” fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')₂ fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

[0070] “Fv” is the minimum antibody fragment that contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H - V_L dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

[0071] The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group. F(ab')₂ antibody fragments originally were produced as pairs of Fab' fragments that have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

[0072] The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

[0073] Depending on the amino acid sequence of the constant domain of their heavy chains, antibodies can be assigned to different classes. There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy chain constant domains that correspond to the different classes of antibodies are called α , δ , ϵ , γ , and μ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

[0074] "Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. In some embodiments, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains that enables the scFv to form the desired structure for antigen binding. For a review of scFv *see* Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

[0075] The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

[0076] The term "multispecific antibody" is used in the broadest sense and specifically covers an antibody that has polyepitopic specificity. Such multispecific antibodies include, but are not

limited to, an antibody comprising a heavy chain variable domain (V_H) and a light chain variable domain (V_L), where the V_HV_L unit has polyepitopic specificity, antibodies having two or more V_L and V_H domains with each V_HV_L unit binding to a different epitope, antibodies having two or more single variable domains with each single variable domain binding to a different epitope, full length antibodies, antibody fragments such as Fab, Fv, dsFv, scFv, diabodies, bispecific diabodies, triabodies, tri-functional antibodies, antibody fragments that have been linked covalently or non-covalently. "Polyepitopic specificity" refers to the ability to specifically bind to two or more different epitopes on the same or different target(s). "Monospecific" refers to the ability to bind only one epitope. According to one embodiment the multispecific antibody is an IgG antibody that binds to each epitope with an affinity of 5 μ M to 0.001 pM, 3 μ M to 0.001 pM, 1 μ M to 0.001 pM, 0.5 μ M to 0.001 pM, or 0.1 μ M to 0.001 pM.

[0077] The expression "single domain antibodies" (sdAbs) or "single variable domain (SVD) antibodies" generally refers to antibodies in which a single variable domain (V_H or V_L) can confer antigen binding. In other words, the single variable domain does not need to interact with another variable domain in order to recognize the target antigen. Examples of single domain antibodies include those derived from camelids (lamas and camels) and cartilaginous fish (*e.g.*, nurse sharks) and those derived from recombinant methods from humans and mouse antibodies (Nature (1989) 341:544-546; Dev Comp Immunol (2006) 30:43-56; Trend Biochem Sci (2001) 26:230-235; Trends Biotechnol (2003):21:484-490; WO 2005/035572; WO 03/035694; Febs Lett (1994) 339:285-290; WO00/29004; WO 02/051870).

[0078] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variants that may arise during production of the monoclonal antibody, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the methods provided herein may be made by the

hybridoma method first described by Kohler *et al.*, *Nature* 256:495 (1975), or may be made by recombinant DNA methods (*see, e.g.*, U.S. Patent No. 4,816,567). The “monoclonal antibodies” may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, *Nature* 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991), for example.

[0079] The monoclonal antibodies herein specifically include “chimeric” antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)). Chimeric antibodies of interest herein include “primatized” antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (*e.g.* Old World Monkey, such as baboon, rhesus or cynomolgus monkey) and human constant region sequences (US Pat No. 5,693,780).

[0080] “Humanized” forms of non-human (*e.g.*, murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence, except for FR substitution(s) as noted above. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region, typically that of a human immunoglobulin. For further details, *see* Jones *et al.*, *Nature* 321:522-525 (1986); Riechmann *et al.*, *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

[0081] For the purposes herein, an “intact antibody” is one comprising heavy and light variable domains as well as an Fc region. The constant domains may be native sequence constant domains (*e.g.* human native sequence constant domains) or amino acid sequence variant thereof. Preferably, the intact antibody has one or more effector functions.

[0082] “Native antibodies” are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

[0083] A “naked antibody” is an antibody (as herein defined) that is not conjugated to a heterologous molecule, such as a cytotoxic moiety or radiolabel.

[0084] In some embodiments, antibody “effector functions” refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors.

[0085] “Antibody-dependent cell-mediated cytotoxicity” and “ADCC” refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (*e.g.* Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express $Fc\gamma RIII$ only, whereas monocytes express $Fc\gamma RI$, $Fc\gamma RII$ and $Fc\gamma RIII$. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may

be assessed *in vivo*, *e.g.*, in an animal model such as that disclosed in Clynes *et al.*, *Proc. Natl. Acad. Sci. (USA)* 95:652-656 (1998).

[0086] “Human effector cells” are leukocytes that express one or more FcRs and perform effector functions. In some embodiments, the cells express at least FcγRIII and carry out ADCC effector function. Examples of human leukocytes that mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred.

[0087] “Complement dependent cytotoxicity” or “CDC” refers to the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (*e.g.* polypeptide (*e.g.*, an antibody)) complexed with a cognate antigen. To assess complement activation, a CDC assay, *e.g.* as described in Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996), may be performed.

[0088] The terms “Fc receptor” or “FcR” are used to describe a receptor that binds to the Fc region of an antibody. In some embodiments, the FcR is a native sequence human FcR. Moreover, a preferred FcR is one that binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an “activating receptor”) and FcγRIIB (an “inhibiting receptor”), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (*see* Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991); Capel *et al.*, *Immunomethods* 4:25-34 (1994); and de Haas *et al.*, *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term “FcR” herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al.*, *J. Immunol.* 117:587 (1976) and Kim *et al.*, *J. Immunol.* 24:249 (1994)).

[0089] “Contaminants” refer to materials that are different from the desired polypeptide product. In some embodiments of the invention, contaminants include charge variants of the polypeptide. In some embodiments of the invention, contaminants include charge variants of an antibody or antibody fragment. In other embodiments of the invention, the contaminant includes, without

limitation: host cell materials, such as CHOP; leached Protein A; nucleic acid; a variant, fragment, aggregate or derivative of the desired polypeptide; another polypeptide; endotoxin; viral contaminant; cell culture media component, *etc.* In some examples, the contaminant may be a host cell protein (HCP) from, for example but not limited to, a bacterial cell such as an *E. coli* cell, an insect cell, a prokaryotic cell, a eukaryotic cell, a yeast cell, a mammalian cell, an avian cell, a fungal cell.

[0090] As used herein, the term “immunoadhesin” designates antibody-like molecules which combine the binding specificity of a heterologous polypeptide with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of an amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site of an antibody (*i.e.*, is “heterologous”), and an immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding site of a receptor or a ligand. The immunoglobulin constant domain sequence in the immunoadhesin may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA (including IgA-1 and IgA-2), IgE, IgD or IgM.

[0091] As used herein “essentially the same” indicates that a value or parameter has not been altered by a significant effect. For example, an ionic strength of a chromatography mobile phase at column exit is essentially the same as the initial ionic strength of the mobile phase if the ionic strength has not changed significantly. For example, an ionic strength at column exit that is within 10%, 5% or 1% of the initial ionic strength is essentially the same as the initial ionic strength.

[0092] Reference to “about” a value or parameter herein includes (and describes) variations that are directed to that value or parameter per se. For example, description referring to “about X” includes description of “X”.

[0093] As used herein and in the appended claims, the singular forms “a,” “or,” and “the” include plural referents unless the context clearly dictates otherwise. It is understood that aspects and variations of the invention described herein include “consisting” and/or “consisting essentially of” aspects and variations.

II. Methods of Chromatography

[0094] In some aspects, the invention provides methods of analyzing compositions comprising a polypeptide and one or more contaminants, *e.g.* polypeptide charge variants, comprising binding the polypeptide and one or more contaminants to a ion exchange chromatography material using a loading buffer with an initial pH and an initial ionic strength, eluting the polypeptide and one or more contaminants from the ion-exchange column using an elution buffer wherein the pH of the elution buffer is altered by a pH gradient and the ionic strength of the elution buffer is altered by an ionic strength gradient such that the polypeptides and the one or more contaminants elute from the chromatography material as distinct separate entities.

[0095] In other aspects, the invention provides methods of analyzing compositions comprising a polypeptide and one or more contaminants, *e.g.* polypeptide variants, comprising binding the polypeptide and one or more contaminants to a ion exchange chromatography material using a loading buffer with an initial pH and an initial ionic strength, eluting the polypeptide and one or more contaminants from the ion-exchange column using an elution buffer wherein the pH of the elution buffer is altered by a pH gradient and the ionic strength of the elution buffer is altered by an ionic strength gradient such that the polypeptides and the one or more contaminants elute from the chromatography material as distinct separate entities.

[0096] In some embodiments of any of the methods described herein, the chromatography material is a cation exchange material. In some embodiments, the cation exchange material is a solid phase that is negatively charged and has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. In some embodiments of any of the methods described herein, the cation exchange material may be a membrane, a monolith, or resin. In some embodiments, the cation exchange material may be a resin. The cation exchange material may comprise a carboxylic acid functional group or a sulfonic acid functional group such as, but not limited to, sulfonate, carboxylic, carboxymethyl sulfonic acid, sulfoisobutyl, sulfoethyl, carboxyl, sulphopropyl, sulphonyl, sulphoxyethyl, or orthophosphate. In some embodiments of the above, the cation exchange chromatography material is a cation exchange chromatography column. In some embodiments, a cation exchange chromatography material is used for a polypeptide, *e.g.* and antibody or fragment thereof, with a pI greater than about 9. For example, the antibody or fragment thereof may have a pI of 9-10, 9-11, 10-11, 9-12, 10-12, or 11-12.

[0097] In some embodiments of any of the methods described herein, the chromatography material is an anion exchange material. In some embodiments, the anion exchange chromatography material is a solid phase that is positively charged and has free anions for exchange with anions in an aqueous solution passed over or through the solid phase. In some embodiments of any of the methods described herein, the anion exchange material may be a membrane, a monolith, or resin. In an embodiment, the anion exchange material may be a resin. In some embodiments, the anion exchange material may comprise a primary amine, a secondary amine, a tertiary amine or a quaternary ammonium ion functional group, a polyamine functional group, or a diethylaminoethyl functional group. In some embodiments of the above, the anion exchange chromatography material is an anion exchange chromatography column. In some embodiments, an anion exchange chromatography material is used for a polypeptide, *e.g.* and antibody or fragment thereof, with a pI less than about 7. For example, the antibody or fragment thereof may have a pI of 6-7, 5-7, 5-6, 4-7, 4-6, or 4-5.

[0098] In some embodiments of any of the methods described herein, the ion exchange material may utilize a conventional chromatography material or a convective chromatography material. The conventional chromatography materials include, for example, perfusive materials (*e.g.*, poly(styrene-divinylbenzene) resin) and diffusive materials (*e.g.*, cross-linked agarose resin). In some embodiments, the poly(styrene-divinylbenzene) resin can be Poros resin. In some embodiments, the cross-linked agarose resin may be sulphopropyl-Sepharose Fast Flow (“SPSFF”) resin. The convective chromatography material may be a membrane (*e.g.*, polyethersulfone) or monolith material (*e.g.* cross-linked polymer). The polyethersulfone membrane may be Mustang. The cross-linked polymer monolith material may be cross-linked poly(glycidyl methacrylate-co-ethylene dimethacrylate).

[0099] In some embodiments of any of the methods of the invention, the chromatography material is in a chromatography column; for example a cation exchange chromatography column or an anion exchange chromatography column. In some embodiments, the chromatography column is used for liquid chromatography. In some embodiments, the chromatography column is used for high performance liquid chromatography (HPLC). In some embodiments, the chromatography column is an HPLC chromatography column; for example, a cation exchange HPLC column or an anion exchange HPLC column.

[0100] Examples of cation exchange materials are known in the art include, but are not limited to Mustang S, Sartobind S, SO3 Monolith, S Ceramic HyperD, Poros XS, Poros HS50, Poros

HS20, SPSFF, SP-Sepharose XL (SPXL), CM Sepharose Fast Flow, Capto S, Fractogel Se HiCap, Fractogel SO3, or Fractogel COO. In some embodiments of any of the methods described herein, the cation exchange material is Poros HS50. In some embodiments, the Poros HS resin may be Poros HS 50 μm or Poros HS 20 μm particles. Examples of cation exchange chromatography columns for use in the methods of the invention include, but are not limited to ProPac WCX-10 and ProPac WCX-10HT.

[0101] Examples of anion exchange materials are known in the art and include, but are not limited to Poros HQ 50, Poros PI 50, Poros D, Mustang Q, Q Sepharose FF, and DEAE Sepharose. Examples of anion exchange chromatography columns for use in the methods of the invention include, but are not limited to Dionex ProPac 10 SAX and Tosoh GSKgel Q STAT 7 μM WAX.

[0102] An exemplary HPLC procedure that may be used for the methods of the invention is as follows; however, the methods of the invention are not construed to be bound by these procedures. Samples are added to autosampler and are refrigerated (5 ± 3 °C). Columns are placed in the column compartment and a temperature control feature may be employed to keep the compartment temperature within a narrow range (± 1 °C) from the set point during analysis. Column effluent is monitored at 280 nm.

[0103] Samples are diluted with mobile phase to a target polypeptide concentration of approximately 1-2 mg/mL. In some embodiments, the polypeptide may be digested with carboxypeptidase B (CpB), added in a ratio of 1:100 (w/w) and incubated at 37 °C for 20 min. Samples may be stored at 5 °C until analysis.

[0104] The instrument may include a low-pressure quaternary gradient pump, a rapid separation auto-sampler with temperature control capability, a thermal-controlled column compartment and a diode array UV detector. At the outlet of the detector, a PCM-3000 pH and conductivity monitor may be connected to collect pH and conductivity data in real time. Instrument control, data acquisition, and data analysis can be performed; for example, by using Thermo Scientific Dionex Chromeleon software, version 6.8.

[0105] Non-limiting examples of mobile phase preparations are as follows. Individual stock buffer solutions of tris and imidazole are prepared at 1.0 M and a solution of CAPS is prepared at a concentration of 0.1 M, without adjusting the pH value and stored at room temperature. Individual components are diluted to a final concentration of 10 mM in approximately 90% of

the target volume using deionized water and allowed to mix. Once the solution is quenched to the final volume, the mixture is divided into two equal aliquots.

[0106] The pH values of the buffers are adjusted by hydrochloric acid to 6.0 (Buffer A) and sodium hydroxide to 11.0 (Buffer B). Sodium chloride is prepared as a 0.1 M solution with deionized water (Buffer C). MilliQ water (18 MOhms) is dispensed into a separate container (Buffer D). All mobile phases are then individually filtered through a 0.2 μm nylon filter prior to use.

[0107] A non-limiting example of cation-exchange chromatography is as follows. Monoclonal antibody samples are diluted to 1 mg/mL with a 1:1 mixture of buffers A and D and are kept at 5 ± 3 °C in the auto-sampler. A Propac WCX-10HT, 4 x 50 mm column is placed in the column compartment with the temperature setting at 40 ± 1 °C. For each chromatographic run, 20 μL of protein (20 μg) is injected. The mobile phase flow rate is 1.0 mL/min. Proteins are detected by ultraviolet (UV) absorbance at 280 nm.

[0108] In some examples, a hybrid pH gradient is established by using a quaternary gradient formed on the quaternary pump using buffers A, B, C and D. This arrangement offers the flexibility of adjusting 1) the starting and ending pH, using buffers A and B and 2) the amount of salt for each gradient, using buffers C and D. For example, a pH gradient from 6 to 10, with a constant salt concentration of 10 mM, is established by an increase of buffer B from 0 to 40%, while maintaining buffers C and D at 10% and 40%, respectively. Examples of hybrid gradients are provided in Table 2 in the examples below.

[0109] Various buffers which can be employed depending, for example, on the desired pH of the buffer, the desired conductivity of the buffer, the characteristics of the protein of interest, and the analytical method.

[0110] Elution, as used herein, is the removal of the product, *e.g.* polypeptide, and or contaminants from the chromatography material. Elution buffer is the buffer used to elute the polypeptide or other product of interest from a chromatography material. In some embodiments, the elution buffer is part to the mobile phase of the chromatography. In some embodiments, the composition comprising the polypeptide and the contaminants is applied to the chromatography material as part of the mobile phase. The mobile phase is then altered to allow for separation of the polypeptide from contaminants as the polypeptide and contaminants are eluted from the chromatography material. In many cases, an elution buffer has a different physical characteristic than the load buffer. For example, the elution buffer may have a different pH than load buffer

and/or a different ionic strength than the load buffer. In some embodiments, the polypeptides and contaminants are eluted from the chromatography material by altering the pH and the ionic strength of the elution buffer. In some embodiments the pH of the elution buffer and the ionic strength of the elution buffer are increased over the course of the elution compared to the load buffer. In some embodiments the pH of the elution buffer is increased over the course of the elution compared to the load buffer and the ionic strength of the elution buffer remains essentially the same.

[0111] In some embodiments of the invention, a polypeptide with a $pI > 9$ is applied to a cation exchange chromatography material and the polypeptide is eluted from the cation exchange chromatography material by increasing the pH and the ionic strength of the mobile phase of the chromatography. In some embodiments of the invention, a polypeptide with a $pI > 9$ is applied to a cation exchange chromatography material and the polypeptide is eluted from the cation exchange chromatography material by increasing the pH of the mobile phase of the chromatography while maintaining the ionic strength of the mobile phase.

[0112] In some embodiments of the invention, a polypeptide with a $pI < 7$ is applied to an anion exchange chromatography material and the polypeptide is eluted from the anion exchange chromatography material by decreasing the pH of the mobile phase of the chromatography and increasing the ionic strength of the mobile phase of the chromatography. In some embodiments of the invention, a polypeptide with a $pI < 7$ is applied to an anion exchange chromatography material and the polypeptide is eluted from the anion exchange chromatography material by decreasing the pH of the mobile phase of the chromatography while maintaining the ionic strength of the mobile phase.

[0113] In some aspects of any of the above embodiments, the pH of the elution buffer changed from the load buffer by linear gradient or by step gradient.

[0114] In some embodiments of the invention, the polypeptide is eluted from the chromatography material by increasing the pH of the elution buffer in the mobile phase. In some embodiments, the pH of the elution buffer increases from about pH 5 to about pH 11. In some embodiments, the pH of the elution buffer increases from about pH 6 to about pH 9. In some embodiments, the pH of the elution buffer increases from about pH 6 to about pH 10. In some embodiments, the pH of the elution buffer increases from about pH 6 to about pH 11. In some embodiments, the pH of the elution buffer increases from about pH 7 to about pH 9. In some embodiments, the pH of the elution buffer increases from about pH 7 to about pH 10. In

some embodiments, the pH of the elution buffer increases from about pH 7 to about pH 11. In some embodiments, the pH of the elution buffer increases from about pH 8 to about pH 9. In some embodiments, the pH of the elution buffer increases from about pH 8 to about pH 10. In some embodiments, the pH of the elution buffer increases from about pH 8 to about pH 11. In some embodiments, the pH of the elution buffer increases from about pH 9 to about pH 11. In any of the above embodiments, the increase in pH of the elution buffer is combined with an increase in the ionic strength of the elution buffer. In other embodiments of any of the above embodiments, the pH of the elution buffer is increased but the ionic strength of the elution buffer remains essentially the same. In any of the above embodiments, the pH gradient is established over more than any of about 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min, 45 min, 50 min, 55 min, or 60 min. In any of the above embodiments, the chromatography material is a cation exchange chromatography material. In any of the above embodiments, the polypeptide has a $pI > 9$. In any of the above embodiments, the polypeptide is an antibody or fragment thereof.

[0115] In some embodiments of the invention, the polypeptide is eluted from the chromatography material by decreasing the pH of the elution buffer in the mobile phase. In some embodiments, the pH of the elution buffer decreases from about pH 9 to about pH 5. In some embodiments, the pH of the elution buffer decreases from about pH 9 to about pH 6. In some embodiments, the pH of the elution buffer decreases from about pH 9 to about pH 7. In some embodiments, the pH of the elution buffer decreases from about pH 8 to about pH 5. In some embodiments, the pH of the elution buffer decreases from about pH 8 to about pH 6. In some embodiments, the pH of the elution buffer decreases from about pH 8 to about pH 7. In some embodiments, the pH of the elution buffer decreases from about pH 7 to about pH 5. In any of the above embodiments, the decrease in pH of the elution buffer is combined with an increase in the ionic strength of the elution buffer. In other embodiments of any of the above embodiments, the pH of the elution buffer is decreased but the ionic strength of the elution buffer remains essentially the same. In any of the above embodiments, the pH gradient is established over more than any of about 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min, 45 min, 50 min, 55 min, or 60 min. In any of the above embodiments, the chromatography material is an anion exchange chromatography material. In any of the above embodiments, the polypeptide has a $pI < 7$. In any of the above embodiments, the polypeptide is an antibody or fragment thereof.

[0116] An elution buffer can be prepared with a specific pH using buffers known in the art. Examples of buffers include but are not limited to piperazine, imidazole, Tris, phosphate, and *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS). In some embodiments, the pH of the buffer may be adjusted, for example, by adding HCl to make a buffer more acidic or adding NaOH to make a buffer more basic. In some embodiments, the elution buffer comprises a combination of buffers. In some embodiments, the elution buffer comprises a combination of piperazine, imidazole and Tris (*e.g.* a PIT buffer). In some embodiments, the elution buffer comprises 11.6 mM piperazine, 1.5 mM imidazole and 2.4 mM Tris. In some embodiments, the elution buffer comprises 4 mM piperazine, 4 mM imidazole and 4 mM Tris. In some embodiments of the invention, an elution buffer comprising a combination of piperazine, imidazole and Tris is used in the mobile phase of cation exchange chromatography of a polypeptide. In some embodiments of the invention, an elution buffer comprising a combination of piperazine, imidazole and Tris is used in the mobile phase of cation exchange chromatography of a polypeptide with a pI >9. In some embodiments of the invention, an elution buffer comprising a combination of piperazine, imidazole and Tris is used in the mobile phase of cation exchange chromatography of an antibody. In some embodiments of the invention, an elution buffer comprising a combination of piperazine, imidazole and Tris is used in the mobile phase of cation exchange chromatography of an antibody with a pI >9. In some embodiments of the invention, an elution buffer comprising a combination of piperazine, imidazole and Tris is used in the mobile phase of anion exchange chromatography of a polypeptide. In some embodiments of the invention, an elution buffer comprising a combination of piperazine, imidazole and Tris is used in the mobile phase of anion exchange chromatography of a polypeptide with a pI <7. In some embodiments of the invention, an elution buffer comprising a combination of piperazine, imidazole and Tris is used in the mobile phase of anion exchange chromatography of an antibody. In some embodiments of the invention, an elution buffer comprising a combination of piperazine, imidazole and Tris is used in the mobile phase of anion exchange chromatography of an antibody with a pI <7. In some embodiments, the elution buffer comprises a combination of Tris, piperazine and phosphate (*e.g.* a TPP buffer). In some embodiments, the elution buffer comprises 5 mM Tris, 5 mM piperazine and 5 mM phosphate. In some embodiments, the elution buffer comprises 10 mM Tris, 10 mM piperazine and 10 mM phosphate. In some embodiments of the invention, an elution buffer comprising a combination of Tris, piperazine and phosphate is used in the mobile phase of cation exchange chromatography of a polypeptide. In some embodiments of the

invention, an elution buffer comprising a combination of Tris, piperazine and phosphate is used in the mobile phase of cation exchange chromatography of a polypeptide with a pI >9. In some embodiments of the invention, an elution buffer comprising a combination of Tris, piperazine and phosphate is used in the mobile phase of cation exchange chromatography of an antibody. In some embodiments of the invention, an elution buffer comprising a combination of Tris, piperazine and phosphate is used in the mobile phase of cation exchange chromatography of an antibody with a pI >9. In some embodiments, the elution buffer comprises a combination of Tris, imidazole and CAPS (*e.g.* a TIC buffer). In some embodiments, the elution buffer comprises 5 mM Tris, 5 mM imidazole and 5 mM CAPS. In some embodiments of the invention, an elution buffer comprising a combination of Tris, imidazole and CAPS is used in the mobile phase of cation exchange chromatography of a polypeptide. In some embodiments of the invention, an elution buffer comprising a combination of Tris, imidazole and CAPS is used in the mobile phase of cation exchange chromatography of a polypeptide with a pI >9. In some embodiments of the invention, an elution buffer comprising a combination of Tris, imidazole and CAPS is used in the mobile phase of cation exchange chromatography of an antibody. In some embodiments of the invention, an elution buffer comprising a combination of Tris, imidazole and CAPS is used in the mobile phase of cation exchange chromatography of an antibody with a pI >9.

[0117] In some embodiments of the invention, the polypeptide is eluted from the chromatography material by increasing the pH of the elution buffer as described above, and by increasing the ionic strength of the elution buffer in the mobile phase thereby forming a pH gradient and an ionic strength gradient. In some embodiments, the ionic strength of the elution buffer is increased by increasing the salt concentration of the mobile phase of the chromatography. In other embodiments of the invention, the polypeptide is eluted from the chromatography material by increasing the pH of the elution buffer where the ionic strength of the elution buffer remains essentially the same over the course of the elution by maintaining the salt concentration over the course of the elution. In some embodiments, the salt concentration is chosen such that the charge state of the polypeptide provides an optimal pH gradient ion exchange chromatography separation window. The charge state of the polypeptide can be determined by modeling procedures known in the art. Examples of salts include but are not limited to NaCl, KCl and Na₂SO₄.

[0118] In some embodiments, the ionic strength gradient is a salt gradient. In some embodiments the salt gradient is a gradient from about 0 mM salt to about 200 mM salt. In some embodiments, the salt gradient is any of from about 0 mM to about 100 mM, 0 mM to about 60 mM, 0 mM to about 50 mM, 0 mM to about 40 mM, 0 mM to about 30 mM, 0 mM to about 20 mM, 0 mM to about 10 mM, 10 mM to about 200 mM, 10 mM to about 100 mM, 10 mM to about 50 mM, 10 mM to about 40 mM, 10 mM to about 30 mM, 10 mM to about 20 mM, 20 mM to about 200 mM, 20 mM to about 100 mM, 20 mM to about 50 mM, 20 mM to about 30 mM, 30 mM to about 200 mM, 30 mM to about 100 mM, and 30 mM to about 50 mM.

[0119] In some embodiments of the above embodiments, the polypeptide and/or the one or more contaminants are eluted from the chromatography material by a combination of pH gradient and ionic strength gradient. In some embodiments, the pH gradient is from pH 5 to pH 10.8 and the ionic strength gradient is from 0 mM salt to 16 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 11 and the ionic strength gradient is from 0 mM salt to 60 mM salt. In some embodiments, the pH gradient is from pH 5 to pH 9.5 and the ionic strength gradient is from 0 mM salt to 16 mM salt. In some embodiments, the pH gradient is from pH 5 to pH 9.5 and the ionic strength gradient is from 0 mM salt to 30 mM salt. In any of the above embodiments, the salt is NaCl, KCl or Na₂SO₄. In any of the above embodiments, the chromatography material is a cation exchange chromatography material. In any of the above embodiments, the polypeptide has a pI >9. In any of the above embodiments, the polypeptide is an antibody with a pI >9.

[0120] In some embodiments of the invention, a polypeptide and/or one or more contaminants are analyzed by ion exchange chromatography where the polypeptide and/or one or more contaminants is eluted from the chromatography material by a combination of pH gradient and ionic strength gradient. In some embodiments, the pH gradient is from pH 7 to pH 5 and the ionic strength gradient is from 0 mM salt to 25 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 5 and the ionic strength gradient is from 0 mM salt to 100 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 5 and the ionic strength gradient is from 0 mM salt to 200 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 5 and the ionic strength gradient is from 0 mM salt to 300 mM salt. In any of the above embodiments, the salt is NaCl, KCl or Na₂SO₄. In any of the above embodiments, the chromatography material is an anion exchange chromatography material. In any of the above embodiments, the polypeptide has a pI <7. In any of the above embodiments, the polypeptide is an antibody with a pI <7.

[0121] In some embodiments of the invention, a polypeptide and/or one or more contaminants are analyzed by ion exchange chromatography where the polypeptide and/or one or more contaminants is eluted from the chromatography material by pH gradient where the ionic strength of the mobile phase remains essentially the same over the elution. In some embodiments, the pH gradient is from pH 6 to pH 11 and the ionic strength of the mobile phase is about 10 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 11 and the ionic strength of the mobile phase is about 20 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 11 and the ionic strength of the mobile phase is about 30 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 11 and the ionic strength of the mobile phase is about 40 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 11 and the ionic strength of the mobile phase is about 50 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 10 and the ionic strength of the mobile phase is about 10 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 10 and the ionic strength of the mobile phase is about 20 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 10 and the ionic strength of the mobile phase is about 30 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 10 and the ionic strength of the mobile phase is about 40 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 10 and the ionic strength of the mobile phase is about 50 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 9 and the ionic strength of the mobile phase is about 10 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 9 and the ionic strength of the mobile phase is about 20 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 9 and the ionic strength of the mobile phase is about 30 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 9 and the ionic strength of the mobile phase is about 40 mM salt. In some embodiments, the pH gradient is from pH 6 to pH 9 and the ionic strength of the mobile phase is about 50 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 11 and the ionic strength of the mobile phase is about 10 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 11 and the ionic strength of the mobile phase is about 20 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 11 and the ionic strength of the mobile phase is about 30 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 11 and the ionic strength of the mobile phase is about 40 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 11 and the ionic strength of the mobile phase is about 50 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 10 and the ionic strength of the mobile phase is about 10 mM salt. In some

embodiments, the pH gradient is from pH 7 to pH 10 and the ionic strength of the mobile phase is about 20 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 10 and the ionic strength of the mobile phase is about 30 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 10 and the ionic strength of the mobile phase is about 40 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 10 and the ionic strength of the mobile phase is about 50 mM salt. In any of the above embodiments, the salt is NaCl, KCl or Na₂SO₄. In any of the above embodiments, the chromatography material is a cation exchange chromatography material. In any of the above embodiments, the polypeptide has a pI >9. In any of the above embodiments, the polypeptide is an antibody with a pI >9.

[0122] In some embodiments of the invention, a polypeptide and/or one or more contaminants are analyzed by ion exchange chromatography where the polypeptide and/or one or more contaminants is eluted from the chromatography material by a combination of pH gradient where the ionic strength of the mobile phase remains essentially the same over the elution. In some embodiments, the pH gradient is from pH 8 to pH 5 and the ionic strength of the mobile phase is about 10 mM salt. In some embodiments, the pH gradient is from pH 8 to pH 5 and the ionic strength of the mobile phase is about 20 mM salt. In some embodiments, the pH gradient is from pH 8 to pH 5 and the ionic strength of the mobile phase is about 30 mM salt. In some embodiments, the pH gradient is from pH 8 to pH 5 and the ionic strength of the mobile phase is about 40 mM salt. In some embodiments, the pH gradient is from pH 8 to pH 5 and the ionic strength of the mobile phase is about 50 mM salt. In some embodiments, the pH gradient is from pH 8 to pH 5 and the ionic strength of the mobile phase is about 100 mM salt. In some embodiments, the pH gradient is from pH 8 to pH 5 and the ionic strength of the mobile phase is about 200 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 5 and the ionic strength of the mobile phase is about 10 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 5 and the ionic strength of the mobile phase is about 20 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 5 and the ionic strength of the mobile phase is about 30 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 5 and the ionic strength of the mobile phase is about 40 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 5 and the ionic strength of the mobile phase is about 50 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 5 and the ionic strength of the mobile phase is about 100 mM salt. In some embodiments, the pH gradient is from pH 7 to pH 5 and the ionic strength of the mobile phase is about 200 mM salt. In any of the above embodiments, the salt is

NaCl, KCl or Na₂SO₄. In any of the above embodiments, the chromatography material is an anion exchange chromatography material. In any of the above embodiments, the polypeptide has a pI <7. In any of the above embodiments, the polypeptide is an antibody with a pI <7.

[0123] In some embodiments of the invention, ionic strength of the mobile phase, *e.g.* the elution buffer, is measured by conductivity of the mobile phase. Conductivity refers to the ability of an aqueous solution to conduct an electric current between two electrodes. In solution, the current flows by ion transport. Therefore, with an increasing amount of ions present in the aqueous solution, the solution will have a higher conductivity. The basic unit of measure for conductivity is the Siemen (or mho), mho (mS/cm), and can be measured using a conductivity meter, such as various models of Orion conductivity meters. Since electrolytic conductivity is the capacity of ions in a solution to carry electrical current, the conductivity of a solution may be altered by changing the concentration of ions therein. For example, the concentration of a buffering agent and/or the concentration of a salt (*e.g.* sodium chloride, sodium acetate, or potassium chloride) in the solution may be altered in order to achieve the desired conductivity. Preferably, the salt concentration of the various buffers is modified to achieve the desired conductivity.

[0124] In some embodiments, the mobile phase of the chromatography has an initial conductivity of more than about any of 0.0 mS/cm, 0.5 mS/cm, 1.0 mS/cm, 1.5 mS/cm, 2.0 mS/cm, 2.5 mS/cm, 3.0 mS/cm, 3.5 mS/cm, 4.0 mS/cm, 4.5 mS/cm, 5.0 mS/cm, 5.5 mS/cm, 6.0 mS/cm, 6.5 mS/cm, 7.0 mS/cm, 7.5 mS/cm, 8.0 mS/cm, 8.5 mS/cm, 9.0 mS/cm, 9.5 mS/cm, 10 mS/cm, 11 mS/cm, 12 mS/cm, 13 mS/cm, 14 mS/cm, 15 mS/cm, 16 mS/cm, 17.0 mS/cm, 18.0 mS/cm, 19.0 mS/cm, or 20.0 mS/cm. In some embodiments, the conductivity of the mobile phase is increased over the course of the chromatography, *e.g.* by an ionic strength gradient. In some embodiments, the conductivity of the mobile phase at the completion of elution is more than about any of 1.0 mS/cm, 1.5 mS/cm, 2.0 mS/cm, 2.5 mS/cm, 3.0 mS/cm, 3.5 mS/cm, 4.0 mS/cm, 4.5 mS/cm, 5.0 mS/cm, 5.5 mS/cm, 6.0 mS/cm, 6.5 mS/cm, 7.0 mS/cm, 7.5 mS/cm, 8.0 mS/cm, 8.5 mS/cm, 9.0 mS/cm, 9.5 mS/cm, 10 mS/cm, 11 mS/cm, 12 mS/cm, 13 mS/cm, 14 mS/cm, 15 mS/cm, 16 mS/cm, 17.0 mS/cm, 18.0 mS/cm, 19.0 mS/cm, or 20.0 mS/cm. In some embodiments, the conductivity of the mobile phase is increased by a linear gradient. In some embodiments, the conductivity of the mobile phase is increased by a step gradient comprising one or more steps.

[0125] In some embodiments, the initial conductivity of the mobile phase and the final conductivity of the mobile phase of the chromatography at elution of the polypeptide is essentially the same. In some embodiments, the conductivity of the mobile phase remains essentially at more than about any of 0.0 mS/cm, 0.5 mS/cm, 1.0 mS/cm, 1.5 mS/cm, 2.0 mS/cm, 2.5 mS/cm, 3.0 mS/cm, 3.5 mS/cm, 4.0 mS/cm, 4.5 mS/cm, 5.0 mS/cm, 5.5 mS/cm, 6.0 mS/cm, 6.5 mS/cm, 7.0 mS/cm, 7.5 mS/cm, 8.0 mS/cm, 8.5 mS/cm, 9.0 mS/cm, 9.5 mS/cm, 10 mS/cm, 11 mS/cm, 12 mS/cm, 13 mS/cm, 14 mS/cm, 15 mS/cm, 16 mS/cm, 17.0 mS/cm, 18.0 mS/cm, 19.0 mS/cm, or 20.0 mS/cm.

[0126] In some embodiments of any of the methods described herein, the composition comprising a polypeptide and one or more contaminants is loaded on the chromatography material at an amount of more than any one of about 1 μ g, 2 μ g, 3 μ g, 4 μ g, 5 μ g, 6 μ g, 7 μ g, 8 μ g, 9 μ g, 10 μ g, 15 μ g, 20 μ g, 25 μ g, or 50 μ g. In some embodiments, the composition is loaded onto the chromatography material at a concentration of more than any one of about 0.5 mg/mL, 1.0 mg/mL, 1.5 mg/mL, 2.0 mg/mL, 2.5 mg/mL, and 5.0 mg/mL. In some embodiments, the composition is diluted prior to loading onto the chromatography material; for example, diluted 1:1, 1:2, 1:5, 1:10 or greater than 1:10. In some embodiments, the composition is diluted into the mobile phase of the chromatography. In some embodiments, the composition is diluted into a loading buffer.

[0127] In some embodiments of any of the methods described herein, the flow rate is more than about any of 0.5 mL/min, 0.6 mL/min, 0.7 mL/min, 0.8 mL/min, 0.9 mL/min, 1.0 mL/min, 1.1 mL/min, 1.2 mL/min, 1.3 mL/min, 1.4 mL/min, 1.5 mL/min, 1.75 mL/min and 2.0 mL/min.

[0128] In some embodiments of the methods described herein, the chromatography material is in a column. In some embodiments the column is an HPLC column. In some embodiments the column has any one of the following dimensions: 4 $\times$ 50 mm, 4 $\times$ 100 mm, 4 $\times$ 150 mm, 4 $\times$ 200 mm, 4 $\times$ 250 mm, or 2 $\times$ 250 mm.

[0129] In some embodiments of the invention, the methods are robust; *i.e.* one or more of the running parameters can be perturbed without affecting the analytical results (*e.g.* relative percentages of the main peak and the contaminant peaks). In some embodiments, the concentration of the buffer in the loading buffer and/or the elution buffer varies from any one of about 10 mM to 50 mM, 10 mM to 40 mM, 10 mM to 30 mM, 10 mM to 20 mM, 20 mM to 50 mM, 20 mM to 40 mM, 20 mM to 30 mM, 30 mM to 50 mM, 30 mM to 40 mM, or 40 mM to 50 mM. In some embodiments, the concentration of the buffer in the loading buffer and/or the

elution buffer varies from about 10 mM to about 20 mM. In some embodiments, the first pH varies from any one of about pH 5.0 to pH 7.0, pH 5.0 to pH 6.5, pH 5.0 to pH 6.0, pH 5.0 to pH 5.5, pH 5.5 to pH 7.0, pH 5.5 to pH 6.5, pH 5.5 to pH 6.0, pH 6.0 to pH 7.0, pH 6.0 to pH 6.5 or pH 6.5 to pH 7.0. In some embodiments, the first pH varies from about pH 5.7 to about pH 6.3. In some embodiments, the temperature of the chromatography material varies from any one of about 20 °C to 50 °C, 25 °C to 50 °C, 30 °C to 50 °C, 35 °C to 50 °C, 40 °C to 50 °C, 45 °C to 50 °C, 20 °C to 45 °C, 25 °C to 45 °C, 30 °C to 45 °C, 35 °C to 45 °C, 40 °C to 45 °C, 20 °C to 40 °C, 25 °C to 40 °C, 30 °C to 40 °C, 35 °C to 40 °C, 20 °C to 35 °C, 25 °C to 35 °C, 30 °C to 35 °C, 20 °C to 30 °C, 25 °C to 30 °C, or 20 °C to 25 °C. In some embodiments, the temperature of the chromatography material varies from about 36 °C to about 44 °C. In some embodiments, the loading and elution are conducted at a flow rate varying from any one of about 0.5 ml/min to 2.0 ml/min, 0.8 ml/min to 2.0 ml/min, 1.0 ml/min to 2.0 ml/min, 1.2 ml/min to 2.0 ml/min, 1.5 ml/min to 2.0 ml/min, 1.8 ml/min to 2.0 ml/min, 0.5 ml/min to 1.8 ml/min, 0.8 ml/min to 1.8 ml/min, 1.0 ml/min to 1.8 ml/min, 1.2 ml/min to 1.8 ml/min, 1.5 ml/min to 1.8 ml/min, 0.5 ml/min to 1.5 ml/min, 0.8 ml/min to 1.5 ml/min, 1.0 ml/min to 1.5 ml/min, 1.2 ml/min to 1.5 ml/min, 0.5 ml/min to 1.2 ml/min, 0.8 ml/min to 1.2 ml/min, 1.0 ml/min to 1.2 ml/min, 0.5 ml/min to 1.0 ml/min, 0.8 ml/min to 1.0 ml/min, or 0.5 ml/min to 0.8 ml/min. In some embodiments, the loading and elution are conducted at a flow rate varying from about 0.8 ml/min to about 1.2 ml/min. In some embodiments, the loading and elution are conducted at a flow rate varying from about 1.5 ml/min to about 2.0 ml/min. In further embodiments, any combination of buffer concentration, starting pH, temperature of chromatography material and/or flow rate can vary according to the above embodiments.

Detection of charge variants

[0130] In some aspects, the invention provides methods of detecting variants of a polypeptide in a composition comprising the polypeptide and one or more variants in the composition of the polypeptide. The method comprising binding the polypeptide and one or more variants to a ion exchange chromatography material using a loading buffer with an initial pH and an initial ionic strength, eluting the polypeptide and one or more contaminants from the ion-exchange column using an elution buffer wherein the pH of the elution buffer is altered by a pH gradient and the ionic strength of the elution buffer is altered by an ionic strength gradient such that the polypeptides and the one or more contaminants elute from the chromatography material as

distinct separate entities. The eluents of the chromatography are then analyzed for the parent polypeptide and the presence of variants. Variants of the polypeptide may include acidic variants of the polypeptide and basic variants of the parent polypeptide. Examples of acidic variants, *i.e.* variants with a pI less than the pI of the parent polypeptide, include but are not limited to polypeptides where one or more glutamine and/or asparagine residues have been deamidated. Examples of basic polypeptide variants, *i.e.* variants with a pI greater than the pI of the parent polypeptide, include but are not limited to variants where an aspartic acid residue has undergone modification to a succinimide moiety. In some embodiments, the methods of the invention are used to detect variants of a polypeptide in a composition comprising a polypeptide with an isoelectric point that is not in the neutral pH range. In some embodiments, the methods may be used to effectively detect charge variants in a composition comprising a polypeptide with a pI greater than 9 from contaminants. In some embodiments, a cation exchange chromatography material is used to effectively detect charge variants in a composition comprising a polypeptide with a pI greater than 9. In other embodiments, the methods may be used to effectively detect charge variants in a composition comprising a polypeptide with a pI less than 7 from contaminants. In some embodiments, an anion exchange chromatography material is used to effectively detect charge variants in a composition comprising a polypeptide with a pI less than 7. Examples of polypeptides include, but are not limited to, antibodies and antibody fragments.

[0131] In some aspects, the invention provides methods of detecting variants of a polypeptide in a composition comprising the polypeptide and one or more variants of the polypeptide. The method comprising binding the polypeptide and one or more variants to a ion exchange chromatography material using a loading buffer with an initial pH and an initial ionic strength, eluting the polypeptide and one or more contaminants from the ion-exchange column using an elution buffer wherein the pH of the elution buffer is altered by a pH gradient and the ionic strength of the elution buffer remains essentially the same as the initial ionic strength such that the polypeptides and the one or more contaminants elute from the chromatography material as distinct separate entities. The eluents of the chromatography are then analyzed for the presence of the parent polypeptide and the presence of the variants. Variants of the polypeptide may include acidic variants of the polypeptide and basic variants of the parent polypeptide. Examples of acidic variants, *i.e.* variants with a pI less than the pI of the parent polypeptide, include but are not limited to polypeptides where one or more glutamine and/or asparagine

residues have been deamidated. Examples of basic polypeptide variants, *i.e.* variants with a pI greater than the pI of the parent polypeptide, include but are not limited to variants where an aspartic acid residue has undergone modification to a succinimide moiety. In some embodiments, the methods of the invention are used to detect variants of a polypeptide in a composition comprising a polypeptide with an isoelectric point that is not in the neutral pH range. In some embodiments, the methods may be used to effectively detect charge variants in a composition comprising a polypeptide with a pI greater than 9. In some embodiments, a cation exchange chromatography material is used to effectively detect charge variants in a composition comprising a polypeptide with a pI greater than 9. In other embodiments, the methods may be used to effectively detect charge variants in a composition comprising a polypeptide with a pI less than 7. In some embodiments, an anion exchange chromatography material is used to effectively detect charge variants in a composition comprising a polypeptide with a pI less than 7. Examples of polypeptides include, but are not limited to, antibodies and antibody fragments. Determining the purity of a polypeptide in a composition

[0132] In some aspects, the invention provides methods of determining the purity of a polypeptide in a composition comprising the polypeptide. The method comprising binding the polypeptide and one or more contaminants to a ion exchange chromatography material using a loading buffer with an initial pH and an initial ionic strength, eluting the polypeptide and one or more contaminants from the ion-exchange column using an elution buffer wherein the pH of the elution buffer is altered by a pH gradient and the ionic strength of the elution buffer is altered by an ionic strength gradient such that the polypeptides and the one or more contaminants elute from the chromatography material as distinct separate entities. The purity of the polypeptide can be assessed by determining the ratio of the amount of polypeptide eluting from the chromatography material to the total amount of contaminants, *e.g.* charge variants, eluting from the chromatography material. In some embodiments, the methods of the invention are used to determine the purity of a polypeptide in a composition comprising a polypeptide with an isoelectric point that is not in the neutral pH range. In some embodiments, the methods may be used to effectively determine the purity of a polypeptide in a composition comprising a polypeptide with a pI greater than 9 from contaminants. In some embodiments, a cation exchange chromatography material is used to effectively determine the purity of a polypeptide in a composition comprising a polypeptide with a pI greater than 9. In other embodiments, the methods may be used to effectively determine the purity of a polypeptide in a composition

comprising a polypeptide with a pI less than 7 from contaminants. In some embodiments, an anion exchange chromatography material is used to effectively determine the purity of a polypeptide in a composition comprising a polypeptide with a pI less than 7. Examples of polypeptides include, but are not limited to, antibodies and antibody fragments.

[0133] In some aspects, the invention provides methods of determining the purity of a polypeptide in a composition comprising the polypeptide. The method comprising binding the polypeptide and one or more contaminants to a ion exchange chromatography material using a loading buffer with an initial pH and an initial ionic strength, eluting the polypeptide and one or more contaminants from the ion-exchange column using an elution buffer wherein the pH of the elution buffer is altered by a pH gradient and the ionic strength of the elution buffer remains essentially the same as the initial ionic strength such that the polypeptides and the one or more contaminants elute from the chromatography material as distinct separate entities. The purity of the polypeptide can be assessed by determining the ratio of the amount of polypeptide eluting from the chromatography material to the total amount of contaminants, *e.g.* charge variants, eluting from the chromatography material. In some embodiments, the methods of the invention are used to determine the purity of a polypeptide in a composition comprising a polypeptide with an isoelectric point that is not in the neutral pH range. In some embodiments, the methods may be used to effectively determine the purity of a polypeptide in a composition comprising a polypeptide with a pI greater than 9 from contaminants. In some embodiments, a cation exchange chromatography material is used to effectively determine the purity of a polypeptide in a composition comprising a polypeptide with a pI greater than 9. In other embodiments, the methods may be used to effectively determine the purity of a polypeptide in a composition comprising a polypeptide with a pI less than 7 from contaminants. In some embodiments, an anion exchange chromatography material is used to effectively determine the purity of a polypeptide in a composition comprising a polypeptide with a pI less than 7. Examples of polypeptides include, but are not limited to, antibodies and antibody fragments.

Determining the stability of a polypeptide in a composition

[0134] In some aspects, the invention provides methods for determining the stability of a polypeptide in a composition comprising the polypeptide. In some embodiments, samples of the composition comprising the polypeptide are analyzed over time. In some embodiments, the composition is incubated at various times before analysis. In some embodiments, the composition is incubated at more than any one of about 0°C, 20°C, 37°C or 40°C prior to

analysis. In some embodiments, the composition is incubated for one or more of 1 day, 2 days, 3 days, 5 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 2 months, 3 months, 6 months, 1 year prior to analysis. The composition is then analyzed by binding the polypeptide and one or more contaminants in the composition to a ion exchange chromatography material using a loading buffer with an initial pH and an initial ionic strength, eluting the polypeptide and one or more contaminants from the ion-exchange column using an elution buffer wherein the pH of the elution buffer is altered by a pH gradient and the ionic strength of the elution buffer is altered by an ionic strength gradient such that the polypeptides and the one or more contaminants elute from the chromatography material as distinct separate entities. In other embodiments, the composition is analyzed by binding the polypeptide and one or more contaminants in the composition to a ion exchange chromatography material using a loading buffer with an initial pH and an initial ionic strength, eluting the polypeptide and one or more contaminants from the ion-exchange column using an elution buffer wherein the pH of the elution buffer is altered by a pH gradient and the ionic strength of the elution buffer remains essentially the same as the initial ionic strength such that the polypeptides and the one or more contaminants elute from the chromatography material as distinct separate entities. For either of the above embodiments, the change in the ratio of polypeptide to contaminants indicates the stability of the polypeptide in the composition. For example, if the ratio of polypeptide to contaminants does not change over time, the polypeptide may be considered stable whereas the rapid accumulation of contaminants with a concomitant decrease in the amount of protein in the composition indicates the polypeptide in the composition is less stable. In some embodiments, the methods of the invention are used to determine the stability of a polypeptide in a composition comprising a polypeptide with an isoelectric point that is not in the neutral pH range. In some embodiments, the methods may be used to effectively determine the stability of a polypeptide in a composition comprising a polypeptide with a pI greater than 9 from contaminants. In some embodiments, a cation exchange chromatography material is used to determine the stability of a polypeptide in a composition comprising a polypeptide with a pI greater than 9. In other embodiments, the methods may be used to determine the stability of a polypeptide in a composition comprising a polypeptide with a pI less than 7 from contaminants. In some embodiments, an anion exchange chromatography material is used to determine the stability of a polypeptide in a composition comprising a polypeptide with a pI less than 7. Examples of polypeptides include, but are not limited to, antibodies and antibody fragments.

Purification of polypeptides

[0135] In some aspects, the invention provides methods of purifying a polypeptide from a composition comprising the polypeptide and one or more variants of the polypeptide. The method comprising binding the polypeptide and one or more variants to a ion exchange chromatography material using a loading buffer with an initial pH and an initial ionic strength, eluting the polypeptide and one or more contaminants from the ion-exchange column using an elution buffer wherein the pH of the elution buffer is altered by a pH gradient and the ionic strength of the elution buffer is altered by an ionic strength gradient such that the polypeptides and the one or more contaminants elute from the chromatography material as distinct separate entities. Fractions are collected during the elution phase of the chromatography and fractions that contain polypeptide with no or minimal contaminants are pooled for further processing or for pharmaceutical formulation. In some embodiments, the methods of the invention are used to purify a polypeptide in a composition comprising a polypeptide with an isoelectric point that is not in the neutral pH range. In some embodiments, the methods may be used to purify a polypeptide with a pI greater than 9. In some embodiments, a cation exchange chromatography material is used to purify a polypeptide with a pI greater than 9. In other embodiments, the methods may be used to purify a polypeptide with a pI less than 7 from contaminants. In some embodiments, an anion exchange chromatography material is used to purify a polypeptide with a pI less than 7. Examples of polypeptides include, but are not limited to, antibodies and antibody fragments.

[0136] In some aspects, the invention provides methods of purifying a polypeptide from a composition comprising the polypeptide and one or more variants of the polypeptide. The method comprising binding the polypeptide and one or more variants to a ion exchange chromatography material using a loading buffer with an initial pH and an initial ionic strength, eluting the polypeptide and one or more contaminants from the ion-exchange column using an elution buffer wherein the pH of the elution buffer is altered by a pH gradient and the ionic strength of the elution buffer is essentially the same as the initial ionic strength such that the polypeptides and the one or more contaminants elute from the chromatography material as distinct separate entities. Fractions are collected during the elution phase of the chromatography and fractions that contain polypeptide but not the contaminants are pooled for further processing or for pharmaceutical formulation. In some embodiments, the methods of the invention are used

purify a polypeptide in a composition comprising a polypeptide with an isoelectric point that is not in the neutral pH range. In some embodiments, the methods may be used to purify a polypeptide with a pI greater than 9. In some embodiments, a cation exchange chromatography material is used to purify a polypeptide with a pI greater than 9. In other embodiments, the methods may be used to purify a polypeptide with a pI less than 7 from contaminants. In some embodiments, an anion exchange chromatography material is used to purify a polypeptide with a pI less than 7. Examples of polypeptides include, but are not limited to, antibodies and antibody fragments.

III. Polypeptides

[0137] Polypeptides are provided for use in any of the methods of ionic-strength-mediated pH gradient ion exchange chromatography described herein. In some embodiments of the invention, compositions of a polypeptide are analyzed by ionic strength mediated pH gradient ion exchange chromatography. Such methods are useful in identifying charge variants of the polypeptide within the composition. In some embodiments, the polypeptide is an antibody or fragment thereof.

[0138] In some embodiments, the polypeptide is a therapeutic polypeptide. The therapeutic polypeptide may inhibit the growth of tumor cells, induce apoptosis, and/or induce cell death. In some embodiments, the polypeptide is an antagonist. In some embodiments, the polypeptide is an agonist. In some embodiments, the polypeptide is an antibody. In some embodiments, the polypeptide is an immunoadhesin.

[0139] In some embodiments, the polypeptide has a molecular weight of greater than about any of 5,000 Daltons, 10,000 Daltons, 15,000 Daltons, 25,000 Daltons, 50,000 Daltons, 75,000 Daltons, 100,000 Dalton, 125,000 Daltons, or 150,000 Daltons. The polypeptide may have a molecular weight between about any of 50,000 Daltons to 200,000 Daltons or 100,000 Daltons to 200,000 Daltons. Alternatively, the polypeptide for use herein may have a molecular weight of about 120,000 Daltons or about 25,000 Daltons.

[0140] pI is the isoelectric point and is the pH at which a particular molecule or surface carries no net electrical charge. In some embodiments of any of the methods described herein, the pI of the polypeptide, *e.g.* an antibody, may be greater than about 9. In some embodiments, the polypeptide has a pI of about any of 9, 9.5, 10, 10.5, 11, 11.5, or 12. In some embodiments, the polypeptide has a pI between about 9 and 12. In some embodiments, the polypeptide has a pI

between about 9 and 11. In some embodiments, the polypeptide has a pI between about 9 and 10. In some embodiments, the polypeptide has a pI between about 10 and 12. In some embodiments, the polypeptide has a pI between about 10 and 11. In some embodiments, the polypeptide has a pI between about 11 and 12.

[0141] In some embodiments of any of the methods described herein, the pI of the polypeptide, *e.g.* an antibody, may be less than about 7. In some embodiments, the polypeptide has a pI of about any of 7, 6.5, 6, 5.5, 5, 4.5, or 4. In some embodiments, the polypeptide has a pI between about 4 and 7. In some embodiments, the polypeptide has a pI between about 4 and 6. In some embodiments, the polypeptide has a pI between about 4 and 5. In some embodiments, the polypeptide has a pI between about 5 and 7. In some embodiments, the polypeptide has a pI between about 5 and 6.

[0142] In embodiments of any of the methods described herein, the one or more contaminants in a composition comprising a polypeptide and one or more contaminants are polypeptide charge variants. In some embodiments, the polypeptide charge variant is a polypeptide that has been modified from its native state such that the charge of the polypeptide is altered. In some embodiments, the charge variants are more acidic than the parent polypeptide; *i.e.* have a lower pI than the parent polypeptide. In other embodiments, the charge variants are more basic than the parent polypeptide; *i.e.* have a higher pI than the parent polypeptide. In some embodiments, the polypeptide charge variants are engineered. In some embodiments, the polypeptide charge variant is the result of natural processes; for example, oxidation, deamidation, C-terminal processing of lysine residues, N-terminal pyroglutamate formation, and glycation. In some embodiments, the polypeptide charge variant is a glycoprotein where the glycan attached to the protein is modified such that the charge of the glycoprotein is altered compared to parent glycoprotein; for example, by addition of sialic acid or its derivatives. In some embodiments, the polypeptide charge variant is an antibody charge variant.

[0143] The polypeptides to be analyzed using the methods described herein is generally produced using recombinant techniques. Methods for producing recombinant proteins are described, *e.g.*, in U.S. Pat Nos. 5,534,615 and 4,816,567, specifically incorporated herein by reference. In some embodiments, the protein of interest is produced in a CHO cell (*see, e.g.* WO 94/11026). When using recombinant techniques, the polypeptides can be produced intracellularly, in the periplasmic space, or directly secreted into the medium.

[0144] The polypeptides may be recovered from culture medium or from host cell lysates. Cells employed in expression of the polypeptides can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents. If the polypeptide is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Carter *et al.*, *Bio/Technology* 10: 163-167 (1992) describe a procedure for isolating polypeptides which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the polypeptide is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available polypeptide concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[0145] In some embodiments, the polypeptide in the composition comprising the polypeptide and one or more contaminants has been purified or partially purified prior to analysis by the methods of the invention. For example, the polypeptide of the methods is in an eluent from an affinity chromatography, a cation exchange chromatography, an anion exchange chromatography, a mixed mode chromatography and a hydrophobic interaction chromatography. In some embodiments, the polypeptide is in an eluent from a Protein A chromatography.

[0146] Examples of polypeptides that may be analyzed by the methods of the invention include but are not limited to immunoglobulins, immunoadhesins, antibodies, enzymes, hormones, fusion proteins, Fc-containing proteins, immunoconjugates, cytokines and interleukins.

[0147] In some embodiments, the polypeptide is in a pharmaceutical composition. In some embodiments the polypeptide is an antibody, or antigen binding fragment thereof, in a pharmaceutical composition. In some embodiments, the pharmaceutical composition comprises the polypeptide and a pharmaceutically acceptable carrier including, but not limited to, a buffer, excipient, stabilizer, or preservative. In some embodiments, the pharmaceutical composition comprises an antibody, or antigen binding fragment thereof, and a pharmaceutically acceptable carrier including, but not limited to, a buffer, excipient, stabilizer, or preservative.

(A) Antibodies

[0148] In some embodiments of any of the methods described herein, the polypeptide for use in any of the methods of analyzing polypeptides and formulations comprising the polypeptides by the methods described herein is an antibody.

[0149] Molecular targets for antibodies include CD proteins and their ligands, such as, but not limited to: (i) CD3, CD4, CD8, CD19, CD11a, CD20, CD22, CD34, CD40, CD79 α (CD79a), and CD79 β (CD79b); (ii) members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; (iii) cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and α v/ β 3 integrin, including either alpha or beta subunits thereof (e.g., anti-CD11a, anti-CD18 or anti-CD11b antibodies); (iv) growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; *mpl* receptor; CTLA-4; protein C, BR3, c-met, tissue factor, β 7 etc; and (v) cell surface and transmembrane tumor-associated antigens (TAA), such as those described in U.S. Patent No. 7,521,541.

[0150] Other exemplary antibodies include those selected from, and without limitation, anti-estrogen receptor antibody, anti-progesterone receptor antibody, anti-p53 antibody, anti-HER-2/neu antibody, anti-EGFR antibody, anti-cathepsin D antibody, anti-Bcl-2 antibody, anti-E-cadherin antibody, anti-CA125 antibody, anti-CA15-3 antibody, anti-CA19-9 antibody, anti-c-erbB-2 antibody, anti-P-glycoprotein antibody, anti-CEA antibody, anti-retinoblastoma protein antibody, anti-ras oncoprotein antibody, anti-Lewis X antibody, anti-Ki-67 antibody, anti-PCNA antibody, anti-CD3 antibody, anti-CD4 antibody, anti-CD5 antibody, anti-CD7 antibody, anti-CD8 antibody, anti-CD9/p24 antibody, anti-CD10 antibody, anti-CD11a antibody, anti-CD11c antibody, anti-CD13 antibody, anti-CD14 antibody, anti-CD15 antibody, anti-CD19 antibody, anti-CD20 antibody, anti-CD22 antibody, anti-CD23 antibody, anti-CD30 antibody, anti-CD31 antibody, anti-CD33 antibody, anti-CD34 antibody, anti-CD35 antibody, anti-CD38 antibody, anti-CD41 antibody, anti-LCA/CD45 antibody, anti-CD45RO antibody, anti-CD45RA antibody, anti-CD39 antibody, anti-CD100 antibody, anti-CD95/Fas antibody, anti-CD99 antibody, anti-CD106 antibody, anti-ubiquitin antibody, anti-CD71 antibody, anti-c-myc antibody, anti-cytokeratins antibody, anti-vimentins antibody, anti-HPV proteins antibody, anti-kappa light chains antibody, anti-lambda light chains antibody, anti-melanosomes antibody, anti-prostate specific antigen antibody, anti-S-100 antibody, anti-tau antigen antibody, anti-fibrin antibody, anti-keratins antibody and anti-Tn-antigen antibody.

(i) *Monoclonal antibodies*

[0151] In some embodiments, the antibodies are monoclonal antibodies. Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical and/or bind the same epitope except for possible variants that arise during production of the monoclonal antibody, such variants generally being present in minor amounts. Thus, the modifier “monoclonal” indicates the character of the antibody as not being a mixture of discrete or polyclonal antibodies.

[0152] For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, *Nature* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

[0153] In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as herein described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the polypeptide used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

[0154] The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

[0155] In some embodiments, the myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, in some embodiments, the myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.* 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications* pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

[0156] Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. In some embodiments, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

[0157] The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.* 107:220 (1980).

[0158] After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice* pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

[0159] The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, polypeptide A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[0160] DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). In some embodiments, the hybridoma cells serve as a source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin polypeptide, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al.*, *Curr. Opinion in Immunol.* 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-188 (1992).

[0161] In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, *Nature* 348:552-554 (1990). Clackson *et al.*, *Nature* 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range)

human antibodies by chain shuffling (Marks *et al.*, *Bio/Technology* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, *Nuc. Acids. Res.* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

[0162] The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison *et al.*, *Proc. Natl Acad. Sci. USA* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

[0163] Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

[0164] In some embodiments of any of the methods described herein, the antibody is IgA, IgD, IgE, IgG, or IgM. In some embodiments, the antibody is an IgG monoclonal antibody.

(ii) Humanized antibodies

[0165] In some embodiments, the antibody is a humanized antibody. Methods for humanizing non-human antibodies have been described in the art. In some embodiments, a humanized antibody has one or more amino acid residues introduced into it from a source that is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, *Nature* 321:522-525 (1986); Riechmann *et al.*, *Nature* 332:323-327 (1988); Verhoeven *et al.*, *Science* 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[0166] The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence that is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims *et al.*, *J. Immunol.* 151:2296 (1993); Chothia *et al.*, *J. Mol. Biol.* 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chain variable regions. The same framework may be used for several different humanized antibodies (Carter *et al.*, *Proc. Natl. Acad. Sci. USA* 89:4285 (1992); Presta *et al.*, *J. Immunol.* 151:2623 (1993)).

[0167] It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, in some embodiments of the methods, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available that illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

(iii) *Human antibodies*

[0168] In some embodiments, the antibody is a human antibody. As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy chain joining

region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. *See, e.g.,* Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA* 90:2551 (1993); Jakobovits *et al.*, *Nature* 362:255-258 (1993); Bruggermann *et al.*, *Year in Immuno.* 7:33 (1993); and US Patent Nos. 5,591,669; 5,589,369; and 5,545,807.

[0169] Alternatively, phage display technology (McCafferty *et al.*, *Nature* 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat polypeptide gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats; for their review *see, e.g.,* Johnson, Kevin S. and Chiswell, David J., *Current Opinion in Structural Biology* 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson *et al.*, *Nature* 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991), or Griffith *et al.*, *EMBO J.* 12:725-734 (1993). *See also*, US Patent Nos. 5,565,332 and 5,573,905.

[0170] Human antibodies may also be generated by *in vitro* activated B cells (*see* US Patents 5,567,610 and 5,229,275).

(iv) *Antibody fragments*

[0171] In some embodiments, the antibody is an antibody fragment. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (*see, e.g.,* Morimoto *et al.*, *Journal of*

Biochemical and Biophysical Methods 24:107-117 (1992) and Brennan *et al.*, *Science* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')₂ fragments (Carter *et al.*, *Bio/Technology* 10:163-167 (1992)). According to another approach, F(ab')₂ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; US Patent No. 5,571,894; and US Patent No. 5,587,458. The antibody fragment may also be a "linear antibody," *e.g.*, as described in US Patent 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

[0172] In some embodiments, fragments of the antibodies described herein are provided. In some embodiments, the antibody fragment is an antigen binding fragment. In some embodiments, the antigen binding fragment is selected from the group consisting of a Fab fragment, a Fab' fragment, a F(ab')₂ fragment, a scFv, a Fv, and a diabody.

(v) *Bispecific antibodies*

[0173] In some embodiments, the antibody is a bispecific antibody. Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes. Alternatively, a bispecific antibody binding arm may be combined with an arm that binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.* CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the cell. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* F(ab')₂ bispecific antibodies).

[0174] Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al.*, *Nature* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the

product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker *et al.*, *EMBO J.*, 10:3655-3659 (1991).

[0175] According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. In some embodiments, the fusion is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. In some embodiments, the first heavy chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

[0176] In some embodiments of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies *see*, for example, Suresh *et al.*, *Methods in Enzymology* 121:210 (1986).

[0177] According to another approach described in US Patent No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers that are recovered from recombinant cell culture. In some embodiments, the interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.*

alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

[0178] Bispecific antibodies include cross-linked or “heteroconjugate” antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

[0179] Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al.*, *Science* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')₂ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

[0180] Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al.*, *J. Immunol.* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The “diabody” technology described by Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) by a linker that is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making

bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, *J. Immunol.* 152:5368 (1994).

[0181] Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al.*, *J. Immunol.* 147: 60 (1991).

(v) *Multivalent Antibodies*

[0182] In some embodiments, the antibodies are multivalent antibodies. A multivalent antibody may be internalized (and/or catabolized) faster than a bivalent antibody by a cell expressing an antigen to which the antibodies bind. The antibodies provided herein can be multivalent antibodies (which are other than of the IgM class) with three or more antigen binding sites (*e.g.*, tetravalent antibodies), which can be readily produced by recombinant expression of nucleic acid encoding the polypeptide chains of the antibody. The multivalent antibody can comprise a dimerization domain and three or more antigen binding sites. The preferred dimerization domain comprises (or consists of) an Fc region or a hinge region. In this scenario, the antibody will comprise an Fc region and three or more antigen binding sites amino-terminal to the Fc region. The preferred multivalent antibody herein comprises (or consists of) three to about eight, but preferably four, antigen binding sites. The multivalent antibody comprises at least one polypeptide chain (and preferably two polypeptide chains), wherein the polypeptide chain(s) comprise two or more variable domains. For instance, the polypeptide chain(s) may comprise VD1-(X1)_n-VD2-(X2)_n-Fc, wherein VD1 is a first variable domain, VD2 is a second variable domain, Fc is one polypeptide chain of an Fc region, X1 and X2 represent an amino acid or polypeptide, and n is 0 or 1. For instance, the polypeptide chain(s) may comprise: VH-CH1-flexible linker-VH-CH1-Fc region chain; or VH-CH1-VH-CH1-Fc region chain. The multivalent antibody herein preferably further comprises at least two (and preferably four) light chain variable domain polypeptides. The multivalent antibody herein may, for instance, comprise from about two to about eight light chain variable domain polypeptides. The light chain variable domain polypeptides contemplated here comprise a light chain variable domain and, optionally, further comprise a CL domain.

[0183] In some embodiments, the antibody is a multispecific antibody. Example of multispecific antibodies include, but are not limited to, an antibody comprising a heavy chain variable domain (V_H) and a light chain variable domain (V_L), where the V_HV_L unit has polyepitopic specificity, antibodies having two or more V_L and V_H domains with each V_HV_L unit binding to a different epitope, antibodies having two or more single variable domains with each

single variable domain binding to a different epitope, full length antibodies, antibody fragments such as Fab, Fv, dsFv, scFv, diabodies, bispecific diabodies, triabodies, tri-functional antibodies, antibody fragments that have been linked covalently or non-covalently. In some embodiment that antibody has polyepitopic specificity; for example, the ability to specifically bind to two or more different epitopes on the same or different target(s). In some embodiments, the antibodies are monospecific; for example, an antibody that binds only one epitope. According to one embodiment the multispecific antibody is an IgG antibody that binds to each epitope with an affinity of 5 μ M to 0.001 pM, 3 μ M to 0.001 pM, 1 μ M to 0.001 pM, 0.5 μ M to 0.001 pM, or 0.1 μ M to 0.001 pM.

(vi) *Other Antibody Modifications*

[0184] It may be desirable to modify the antibody provided herein with respect to effector function, *e.g.*, so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). *See Caron et al., J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. J., *Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al.*, *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement mediated lysis and ADCC capabilities. *See Stevenson et al., Anti-Cancer Drug Design* 3:219-230 (1989).

[0185] For increasing serum half the serum half life of the antibody, amino acid alterations can be made in the antibody as described in US 2006/0067930, which is hereby incorporated by reference in its entirety.

(B) *Polypeptide Variants and Modifications*

[0186] Amino acid sequence modification(s) of the polypeptides, including antibodies, described herein may be used in the methods of purifying polypeptides (*e.g.*, antibodies) described herein.

(i) *Variant Polypeptides*

[0187] “Polypeptide variant” means a polypeptide, preferably an active polypeptide, as defined herein having at least about 80% amino acid sequence identity with a full-length native sequence of the polypeptide, a polypeptide sequence lacking the signal peptide, an extracellular domain of a polypeptide, with or without the signal peptide. Such polypeptide variants include, for instance, polypeptides wherein one or more amino acid residues are added, or deleted, at the N or C-terminus of the full-length native amino acid sequence. Ordinarily, a TAT polypeptide variant will have at least about 80% amino acid sequence identity, alternatively at least about any of 85%, 90%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity, to a full-length native sequence polypeptide sequence, a polypeptide sequence lacking the signal peptide, an extracellular domain of a polypeptide, with or without the signal peptide. Optionally, variant polypeptides will have no more than one conservative amino acid substitution as compared to the native polypeptide sequence, alternatively no more than about any of 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative amino acid substitution as compared to the native polypeptide sequence.

[0188] The variant polypeptide may be truncated at the N-terminus or C-terminus, or may lack internal residues, for example, when compared with a full length native polypeptide. Certain variant polypeptides may lack amino acid residues that are not essential for a desired biological activity. These variant polypeptides with truncations, deletions, and insertions may be prepared by any of a number of conventional techniques. Desired variant polypeptides may be chemically synthesized. Another suitable technique involves isolating and amplifying a nucleic acid fragment encoding a desired variant polypeptide, by polymerase chain reaction (PCR). Oligonucleotides that define the desired termini of the nucleic acid fragment are employed at the 5' and 3' primers in the PCR. Preferably, variant polypeptides share at least one biological and/or immunological activity with the native polypeptide disclosed herein.

[0189] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme or a polypeptide which increases the serum half-life of the antibody.

[0190] For example, it may be desirable to improve the binding affinity and/or other biological properties of the polypeptide. Amino acid sequence variants of the polypeptide are prepared by

introducing appropriate nucleotide changes into the antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the polypeptide. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the polypeptide (*e.g.*, antibody), such as changing the number or position of glycosylation sites.

[0191] Guidance in determining which amino acid residue may be inserted, substituted or deleted without adversely affecting the desired activity may be found by comparing the sequence of the polypeptide with that of homologous known polypeptide molecules and minimizing the number of amino acid sequence changes made in regions of high homology.

[0192] A useful method for identification of certain residues or regions of the polypeptide (*e.g.*, antibody) that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells, *Science* 244:1081-1085 (1989). Here, a residue or group of target residues are identified (*e.g.*, charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably Alanine or Polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed antibody variants are screened for the desired activity.

[0193] Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in the Table 1 below under the heading of "preferred substitutions." If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in the Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 1.

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

[0194] Substantial modifications in the biological properties of the polypeptide are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, *Biochemistry* second ed., pp. 73-75, Worth Publishers, New York (1975)):

(1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)

(2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)

(3) acidic: Asp (D), Glu (E)

(4) basic: Lys (K), Arg (R), His(H)

[0195] Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

[0196] Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

[0197] Any cysteine residue not involved in maintaining the proper conformation of the antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the polypeptide to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

[0198] A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.*, a humanized antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (*e.g.*, 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.*, binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and target. Such contact residues and neighboring residues

are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

[0199] Another type of amino acid variant of the polypeptide alters the original glycosylation pattern of the antibody. The polypeptide may comprise non-amino acid moieties. For example, the polypeptide may be glycosylated. Such glycosylation may occur naturally during expression of the polypeptide in the host cell or host organism, or may be a deliberate modification arising from human intervention. By altering is meant deleting one or more carbohydrate moieties found in the polypeptide, and/or adding one or more glycosylation sites that are not present in the polypeptide.

[0200] Glycosylation of polypeptide is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

[0201] Addition of glycosylation sites to the polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

[0202] Removal of carbohydrate moieties present on the polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases.

[0203] Other modifications include deamidation of glutamyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino

groups of lysine, arginine, and histidine side chains, acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

(ii) Chimeric Polypeptides

[0204] The polypeptide described herein may be modified in a way to form chimeric molecules comprising the polypeptide fused to another, heterologous polypeptide or amino acid sequence. In some embodiments, a chimeric molecule comprises a fusion of the polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl-terminus of the polypeptide. The presence of such epitope-tagged forms of the polypeptide can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

[0205] In an alternative embodiment, the chimeric molecule may comprise a fusion of the polypeptide with an immunoglobulin or a particular region of an immunoglobulin. A bivalent form of the chimeric molecule is referred to as an “immunoadhesin.”

[0206] As used herein, the term “immunoadhesin” designates antibody-like molecules which combine the binding specificity of a heterologous polypeptide with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of an amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site of an antibody (*i.e.*, is “heterologous”), and an immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding site of a receptor or a ligand. The immunoglobulin constant domain sequence in the immunoadhesin may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA (including IgA-1 and IgA-2), IgE, IgD or IgM.

[0207] The Ig fusions preferably include the substitution of a soluble (transmembrane domain deleted or inactivated) form of a polypeptide in place of at least one variable region within an Ig molecule. In a particularly preferred embodiment, the immunoglobulin fusion includes the hinge, CH₂ and CH₃, or the hinge, CH₁, CH₂ and CH₃ regions of an IgG1 molecule.

(iii) Polypeptide Conjugates

[0208] The polypeptide for use in polypeptide formulations may be conjugated to a cytotoxic agent such as a chemotherapeutic agent, a growth inhibitory agent, a toxin (*e.g.*, an

enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (*i.e.*, a radioconjugate).

[0209] Chemotherapeutic agents useful in the generation of such conjugates can be used. In addition, enzymatically active toxins and fragments thereof that can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated polypeptides. Examples include ^{212}Bi , ^{131}I , ^{131}In , ^{90}Y , and ^{186}Re . Conjugates of the polypeptide and cytotoxic agent are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis(p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.*, *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the polypeptide.

[0210] Conjugates of a polypeptide and one or more small molecule toxins, such as a calicheamicin, maytansinoids, a trichothene, and CC1065, and the derivatives of these toxins that have toxin activity, are also contemplated herein.

[0211] Maytansinoids are mitototic inhibitors which act by inhibiting tubulin polymerization. Maytansine was first isolated from the east African shrub *Maytenus serrata*. Subsequently, it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters. Synthetic maytansinol and derivatives and analogues thereof are also contemplated. There are many linking groups known in the art for making polypeptide-maytansinoid conjugates, including, for example, those disclosed in U.S. Pat. No. 5,208,020. The linking groups include disulfide groups, thioether groups, acid labile groups, photolabile

groups, peptidase labile groups, or esterase labile groups, as disclosed in the above-identified patents, disulfide and thioether groups being preferred.

[0212] The linker may be attached to the maytansinoid molecule at various positions, depending on the type of the link. For example, an ester linkage may be formed by reaction with a hydroxyl group using conventional coupling techniques. The reaction may occur at the C-3 position having a hydroxyl group, the C-14 position modified with hydroxymethyl, the C-15 position modified with a hydroxyl group, and the C-20 position having a hydroxyl group. In a preferred embodiment, the linkage is formed at the C-3 position of maytansinol or a maytansinol analogue.

[0213] Another conjugate of interest comprises a polypeptide conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. For the preparation of conjugates of the calicheamicin family, *see, e.g.*, U.S. Pat. No. 5,712,374. Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I , α_2^I , α_3^I , N-acetyl- γ_1^I , PSAG and θ_1^I . Another anti-tumor drug that the antibody can be conjugated is QFA which is an antifolate. Both calicheamicin and QFA have intracellular sites of action and do not readily cross the plasma membrane. Therefore, cellular uptake of these agents through polypeptide (*e.g.*, antibody) mediated internalization greatly enhances their cytotoxic effects.

[0214] Other antitumor agents that can be conjugated to the polypeptides described herein include BCNU, streptozocin, vincristine and 5-fluorouracil, the family of agents known collectively LL-E33288 complex, as well as esperamicins.

[0215] In some embodiments, the polypeptide may be a conjugate between a polypeptide and a compound with nucleolytic activity (*e.g.*, a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

[0216] In yet another embodiment, the polypeptide (*e.g.*, antibody) may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pre-targeting wherein the polypeptide receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.*, avidin) which is conjugated to a cytotoxic agent (*e.g.*, a radionucleotide).

[0217] In some embodiments, the polypeptide may be conjugated to a prodrug-activating enzyme which converts a prodrug (*e.g.*, a peptidyl chemotherapeutic agent) to an active anti-

cancer drug. The enzyme component of the immunoconjugate includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

[0218] Enzymes that are useful include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β -lactamase useful for converting drugs derivatized with β -lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as “abzymes”, can be used to convert the prodrugs into free active drugs.

(iv) Other

[0219] Another type of covalent modification of the polypeptide comprises linking the polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol. The polypeptide also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's *Pharmaceutical Sciences*, 18th edition, Gennaro, A.R., Ed., (1990).

IV. Obtaining Polypeptides for Use in the Formulations and Methods

[0220] The polypeptides used in the methods of analysis described herein may be obtained using methods well-known in the art, including the recombination methods. The following sections provide guidance regarding these methods.

(A) Polynucleotides

[0221] "Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA.

[0222] Polynucleotides encoding polypeptides may be obtained from any source including, but not limited to, a cDNA library prepared from tissue believed to possess the polypeptide mRNA and to express it at a detectable level. Accordingly, polynucleotides encoding polypeptide can be conveniently obtained from a cDNA library prepared from human tissue. The polypeptide-encoding gene may also be obtained from a genomic library or by known synthetic procedures (*e.g.*, automated nucleic acid synthesis).

[0223] For example, the polynucleotide may encode an entire immunoglobulin molecule chain, such as a light chain or a heavy chain. A complete heavy chain includes not only a heavy chain variable region (V_H) but also a heavy chain constant region (C_H), which typically will comprise three constant domains: C_{H1} , C_{H2} and C_{H3} ; and a "hinge" region. In some situations, the presence of a constant region is desirable.

[0224] Other polypeptides which may be encoded by the polynucleotide include antigen-binding antibody fragments such as single domain antibodies ("dAbs"), Fv, scFv, Fab' and $F(ab')_2$ and "minibodies." Minibodies are (typically) bivalent antibody fragments from which the C_{H1} and C_K or C_L domain has been excised. As minibodies are smaller than conventional antibodies they should achieve better tissue penetration in clinical/diagnostic use, but being bivalent they should retain higher binding affinity than monovalent antibody fragments, such as dAbs. Accordingly, unless the context dictates otherwise, the term "antibody" as used herein encompasses not only whole antibody molecules but also antigen-binding antibody fragments of the type discussed above. Preferably each framework region present in the encoded polypeptide will comprise at least one amino acid substitution relative to the corresponding human acceptor framework. Thus, for example, the framework regions may comprise, in total, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or fifteen amino acid substitutions relative to the acceptor framework regions.

VI. Exemplary embodiments

[0225] 1. In one embodiment, the invention provides a method for analyzing a composition comprising a polypeptide and one or more contaminants, the method comprising a) binding the polypeptide and one of more contaminants to an ion-exchange chromatography material using a loading buffer, wherein the loading buffer is at a first pH and comprises a first ionic strength; b)

eluting the polypeptide and one or more contaminants from the ion-exchange chromatography material using an elution buffer wherein the pH of the elution buffer is altered in a pH gradient and the ionic strength of the elution buffer is altered in an ionic strength gradient, wherein the polypeptide and the one or more contaminants are separated by the combination of pH gradient and ionic strength gradient; c) detecting the polypeptide and the one or more contaminants. In some aspects of this embodiment, the invention provides methods for analyzing polypeptides in compositions comprising a polypeptide and one or more contaminants, wherein the method separates one or more contaminants from the polypeptide, the method comprising a) binding the polypeptide and one of more contaminants to an ion-exchange chromatography material using a loading buffer, wherein the loading buffer is at a first pH and comprises a first ionic strength; b) eluting the polypeptide and one or more contaminants from the ion-exchange chromatography material using an elution buffer wherein the pH of the elution buffer is altered in a pH gradient and the ionic strength of the elution buffer is altered in an ionic strength gradient, wherein the polypeptide and the one or more contaminants are separated by the combination of pH gradient and ionic strength gradient; c) detecting the polypeptide and the one or more contaminants, wherein the method is used to analyze polypeptides having a pI ranging from about 7.0 to about 9.5.

[0226] 2. In a further embodiment of embodiment 1, the polypeptide is an antibody or immunoadhesin or fragment thereof.

[0227] 3. In a further embodiment of embodiments 1 or 2, the polypeptide is a monoclonal antibody or fragment thereof.

[0228] 4. In a further embodiment of embodiments 2 or 3, the antibody is a human antibody.

[0229] 5. In a further embodiment of embodiments 2 or 3, the antibody is a humanized antibody.

[0230] 6. In a further embodiment of embodiments 2 or 3, the antibody is a chimeric antibody.

[0231] 7. In a further embodiment of any one of embodiments 2-6 the antibody is an antibody fragment.

[0232] 8. In a further embodiment of any one of embodiments 1-7 the contaminant is a variant of the polypeptide.

[0233] 9. In a further embodiment of any one of embodiments 1-7, the contaminant is a degradation product of the polypeptide. For example, a charge variant.

- [0234] 10. In a further embodiment of any one of embodiments 1-9 the polypeptide has a pI greater than about 9.0.
- [0235] 11. In a further embodiment of any one of embodiments 1-10, the chromatography material is a cation exchange chromatography material.
- [0236] 12. In a further embodiment of embodiment 11, the cation exchange chromatography material is a sulfonated chromatography material or a carboxylated chromatography material.
- [0237] 13. In a further embodiment of any one of embodiments 1-12, the pH gradient is a linear gradient.
- [0238] 14. In a further embodiment of any one of embodiments 1-12, the pH gradient is a step gradient.
- [0239] 15. In a further embodiment of embodiments 13 or 14, the pH gradient comprises an increase from about pH 5 to about pH 11.
- [0240] 16. In a further embodiment of any one of embodiments 1-15, the pH gradient is generated using one or more buffers.
- [0241] 17. In a further embodiment of embodiment 15, the one or more buffers selected from piperazine, imidazole, tris, phosphate, or CAPS.
- [0242] 18. In a further embodiment of any one of embodiments 1-17, the ionic strength gradient is a linear gradient.
- [0243] 19. In a further embodiment of any one of embodiments 1-17, the ionic strength gradient is a step gradient.
- [0244] 20. In a further embodiment of embodiments 18 or 19, the ionic strength gradient comprises an increase in salt concentration from about 0 mM to about 200 mM.
- [0245] 21. In a further embodiment of any one of embodiments 18-20, the ionic strength gradient is an NaCl gradient, a KCl gradient, or an Na₂SO₄ gradient.
- [0246] 22. In a further embodiment of any one of embodiments 1-9, the polypeptide has a pI less than about 7.0.
- [0247] 23. In a further embodiment of embodiment 22, the chromatography material is an anion exchange chromatography material.
- [0248] 24. In a further embodiment of embodiment 23, the anion exchange chromatography material is a quarternary amine chromatography material or a tertiary amine chromatography material.

[0249] 25. In a further embodiment of any one of embodiments 22-24, the pH gradient is a linear gradient.

[0250] 26. In a further embodiment of any one of embodiments 22-24, the pH gradient is a step gradient.

[0251] 27. In a further embodiment of embodiments 25 or 26, the pH gradient comprises a decrease from about pH 8 to about pH 5.

[0252] 28. In a further embodiment of any one of embodiments 22-27, the pH gradient is generated using one or more buffers.

[0253] 29. In a further embodiment of embodiment 28, the one or more buffers selected from piperazine, imidazole or Tris.

[0254] 30. In a further embodiment of any one of embodiments 22-29, the ionic strength gradient is a linear gradient.

[0255] 31. In a further embodiment of any one of embodiments 22-29, the ionic strength gradient is a step gradient.

[0256] 32. In a further embodiment of embodiment 30 or 31, the ionic strength gradient comprises an increase in salt concentration from about 0 mM to about 200 mM.

[0257] 33. In a further embodiment of any one of embodiments 30-32, the ionic strength gradient is a NaCl gradient, a KCl gradient, or an Na₂SO₄ gradient.

[0258] 34. In one embodiment, the invention provides a method for analyzing a composition comprising the polypeptide and one or more contaminants, the method comprising a) binding the polypeptide and one or more contaminants to an ion-exchange chromatography material using a loading buffer, wherein the loading buffer is at an initial pH and comprises an initial ionic strength; b) eluting the polypeptide and one or more contaminants from the ion-exchange chromatography material using an elution buffer wherein the pH of the elution buffer is altered in a pH gradient and wherein the ionic strength of the elution buffer is essentially the same as the initial ionic strength of the loading buffer, wherein the polypeptide and the one or more contaminants are separated by pH gradient at about the initial ionic strength; c) detecting the polypeptide and the one or more contaminants. In some aspects of this embodiment, the invention provides method for analyzing polypeptides in compositions comprising a polypeptide and one or more contaminants, the method comprising a) binding the polypeptide and one or more contaminants to an ion-exchange chromatography material using a loading buffer, wherein the loading buffer is at an initial pH and comprises an initial ionic strength; b) eluting the

polypeptide and one or more contaminants from the ion-exchange chromatography material using an elution buffer wherein the pH of the elution buffer is altered in a pH gradient and wherein the ionic strength of the elution buffer is essentially the same as the initial ionic strength of the loading buffer, wherein the polypeptide and the one or more contaminants are separated by pH gradient at about the initial ionic strength; c) detecting the polypeptide and the one or more contaminants, wherein the method is used to analyze polypeptides having a pI ranging from about 7.0 to about 9.5.

[0259] 35. In a further embodiment of embodiment 34, the polypeptide is an antibody or immunoadhesin or fragment thereof.

[0260] 36. In a further embodiment of embodiments 34 or 35, the polypeptide is a monoclonal antibody or fragment thereof.

[0261] 37. In a further embodiment of embodiments 35 or 36, the antibody is a human antibody.

[0262] 38. In a further embodiment of embodiment 35 or 36, the antibody is a humanized antibody.

[0263] 39. In a further embodiment of embodiments 35 or 36, the antibody is a chimeric antibody.

[0264] 40. In a further embodiment of any one of embodiments 35-39, the antibody is an antibody fragment.

[0265] 41. In a further embodiment of any one of embodiments 34-40, the contaminant is a variant of the polypeptide.

[0266] 42. In a further embodiment of any one of embodiments 34-40, the contaminant is a degradation product of the polypeptide.

[0267] 43. In a further embodiment of any one of embodiments 34-42, the polypeptide has a pI greater than about 9.0.

[0268] 44. In a further embodiment of any one of embodiments 34-43, the chromatography material is a cation exchange chromatography material.

[0269] 45. In a further embodiment of embodiment 44, the cation exchange chromatography material is a sulfonated chromatography material or a carboxylated chromatography material.

[0270] 46. In a further embodiment of any one of embodiments 34-45, the pH gradient is a linear gradient.

[0271] 47. In a further embodiment of any one of embodiments 34-45, the pH gradient is a step gradient.

[0272] 48. In a further embodiment of embodiment 46 or 47, the pH gradient comprises an increase from about pH 5 to about pH 11.

[0273] 49. In a further embodiment of any one of embodiments 34-48, the pH gradient is generated using one or more buffers.

[0274] 50. In a further embodiment of embodiment 49, the one or more buffers selected from piperazine, imidazole, tris, phosphate, or CAPS.

[0275] 51. In a further embodiment of any one of embodiments 34-50, the ionic strength of the elution buffer is from about 0 mM to about 100 mM.

[0276] 52. In a further embodiment of embodiment 51, the elution buffer comprise about 0 mM NaCl to about 100 mM NaCl, about 0 mM KCl to about 100 mM KCl, or about 0 mM Na₂SO₄ to about 100 mM Na₂SO₄.

[0277] 53. In a further embodiment of any one of embodiments 34-42, the polypeptide has a pI less than about 7.0.

[0278] 54. In a further embodiment of embodiment 53, the chromatography material is an anion exchange chromatography material.

[0279] 55. In a further embodiment of embodiment 54, the anion exchange chromatography material is a quarternary amine chromatography material or a tertiary amine chromatography material.

[0280] 56. In a further embodiment of any one of embodiments 53-55, the pH gradient is a linear gradient.

[0281] 57. In a further embodiment of any one of embodiments 53-55, the pH gradient is a step gradient.

[0282] 58. In a further embodiment of embodiments 56 or 57, the pH gradient comprises a decrease from about pH 8 to about pH 5.

[0283] 59. In a further embodiment of any one of embodiments 53-58, the pH gradient is generated using one or more buffers.

[0284] 60. In a further embodiment of embodiment 59, the one or more buffers selected from piperazine, imidazole or Tris.

[0285] 61. In a further embodiment of any one of embodiments 53-60, the ionic strength of the elution buffer is from about 0 mM to about 100 mM.

- [0286] 62. In a further embodiment of embodiment 61, the elution buffer comprise about 10 mM NaCl to about 100 mM NaCl.
- [0287] 63. In a further embodiment of any one of embodiments 1-62, the analysis is by high performance liquid chromatography.
- [0288] 64. In a further embodiment of any one of embodiments 1-63, the concentration of the buffer in the loading buffer and/or the elution buffer varies from 10 mM to about 50 mM.
- [0289] 65. In a further embodiment of any one of embodiments 1-64, the first pH varies from about pH 5.0 to about pH 7.0.
- [0290] 66. In a further embodiment of any one of embodiments 1-65, the temperature of the chromatography material varies from about 20 °C to about 50 °C.
- [0291] 67. In a further embodiment of any one of embodiments 1-66, the loading and elution are conducted at a flow rate varying from about 0.5 ml/min to about 2.0 ml/min.
- [0292] 68. In another embodiment, the invention provides a method of determining the purity of a polypeptide in a composition comprising analyzing the composition according to any one of the methods of embodiments 1 to 67 and determining the ratio of polypeptide to contaminants in the composition.
- [0293] 69. In another embodiment, the invention provides a method of determining the stability of a polypeptide in a composition comprising the polypeptide, the method comprising, a) incubating the composition comprising the polypeptide at 0 °C to 40 °C for one to six weeks, b) analyzing the composition of step a) by any of the methods of embodiments 1 to 67, and c) determining the ratio of variants to polypeptide in the composition, wherein an increase in the ratio of variants to polypeptide in the composition compared to a composition that was not incubated indicates the degradation of the polypeptide in the composition.
- [0294] All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.
- [0295] Further details of the invention are illustrated by the following non-limiting Examples. The disclosures of all references in the specification are expressly incorporated herein by reference.

EXAMPLES

[0296] The examples below are intended to be purely exemplary of the invention and should therefore not be considered to limit the invention in any way. The following examples and detailed description are offered by way of illustration and not by way of limitation.

Materials and Methods for Examples

[0297] The following materials and methods were used for the examples unless otherwise noted.

Materials

[0298] All mAbs were manufactured in-house at Genentech (South San Francisco, CA) using stable Chinese Hamster Ovary (CHO) cell lines. The pI values for the mAbs used were determined experimentally using an icIEF protocol from the instrument manufacturer (Wu, J and Huang, T (2006) *Electrophoresis* 27:3584) employing seven pI markers. Thermal stressed samples were obtained by incubating mAbs at 40°C for 3 and 6 weeks, respectively. The stressed mAbs were stored at -80 °C before chromatographic analysis.

[0299] Propac WCX-10 columns were purchased from Dionex. Imidazole was obtained from EMD Biosciences or from Fluka. Piperazine (anhydrous) was acquired from Tokyo Chemical Industry Co. LTD. Trisma (Tris) were obtained from Mallinckrodt Baker Inc. or Sigma (St. Louis, MO)., Trizma base and CAPS was obtained from Sigma. Sodium chloride, sodium hydroxide (10 N) and hydrochloric acid (12 N) were obtained from Mallinckrodt Baker Inc.. Phosphoric acid (85%) was obtained from EMD Millipore.

HPLC Set up

[0300] Cation-exchange chromatography experiments were primarily performed on a Waters 2796 BioAlliance liquid chromatography instrument or an UltiMate 3000 Quaternary Rapid Separation LC (Thermo Scientific Dionex). The instrument included a low-pressure quaternary gradient pump, an auto-sampler with temperature control capability, a thermal column compartment for precise temperature control, and a dual-wavelength diode array UV detector. At the outlet of the column, an in-line pH sensor (Model S450CD from Sensorex) and a conductivity sensor (Model 529 from Amber Scince, Eugene, OR) were connected in tandem. The pH sensor was controlled by a model Seven Multi pH meter from Mettler Toledo; the conductivity sensor was controlled by a model 1056 digital conductivity meter from Amber

Science. The pH and conductivity readings from the two meters were collected into Chromeleon through a Dionex UCI 50 analog/digital convertor. Instrument control, data acquisition, and data analysis were performed with Dionex Chromeleon software, version 6.8.

Mobile Phase Preparation

[0301] Individual stock buffer solutions of tris and imidazole were prepared at 1.0 M and a solution of CAPS was prepared at a concentration of 0.1 M, without adjusting the pH value and stored at room temperature. A stock buffer solution containing 40 mM of piperazine, 40 mM imidazole, and 40 mM Tris (all free bases) was first prepared without adjusting the pH value and stored at room temperature. Prior to chromatographic experiments, a series of the mobile phase buffers containing equimolar concentration of piperazine, imidazole and Tris at 1, 2, 4 or 8 mM, were each made by diluting the buffer stock solution with deionized water. The pH values of the buffers were then adjusted using hydrochloric acid to 5.0 (Buffer A) and 10.8 (Buffer B), respectively. Sodium chloride solution of 0.5 M was prepared with deionized water (Salt Solution). The mobile phases were then individually filtered through a 0.2 μ M nylon filter prior to use.

[0302] The mobile phase buffers with 11.6 mM piperazine, 1.5 mM imidazole and 2.4 mM Tris were prepared as reported in literature (Farnan, D and Moreno, GT (2009) *Anal. Chem.* 81:8846; Rea, JC *et al.* (2010) *J. Pharm. Biomed. Anal.* 54:317). A ten-fold concentrated stock solution containing 116 mM piperazine, 15 mM imidazole and 24 mM Tris was first prepared and stored at room temperature. Before each experiment, two aliquots of the stock solution were diluted 10-fold with deionized water and their pH values were subsequently adjusted using hydrochloric acid to 5.0 (Buffer C) and 9.5 (Buffer D). The mobile phases were then individually filtered through a 0.2 μ M nylon filter prior to use.

Cation-Exchange Chromatography

[0303] Unless stated otherwise, the chromatographic conditions were as follows. mAb samples (control and stressed) were diluted to 2 mg/mL with deionized water and were kept at 5 ± 3 °C in the auto-sampler. Alternatively, mAb samples were diluted to 1 mg/mL with a 1:1 mixture of buffers A and D and were kept at 5 ± 3 °C in the auto-sampler. The Propac WCX-10HT, 4 x 50 mm column was placed in the column compartment with the temperature setting at 40 ± 1 °C. A 4x250 mm Dionex Propac WCX column was used for chromatographic separation and placed in the column compartment with the temperature setting at 40 ± 1 °C. For each chromatographic run, 10 μ L of protein (20 μ g) was injected.

[0304] The salt-mediated pH gradient was established by using a ternary gradient formed on the quaternary pump using buffer A, B and the Salt Solution (0.5 M NaCl). A linear gradient from 100% A to 96.8% B and 3.2% salt solution in 58 minutes was delivered to establish a pH gradient from 5.0 to 10.8 (0.1 pH unit/min) and a mediating salt gradient from 0 to 16 mM NaCl (0.28 mM/min). The final gradient (min, %B and C) was as follows: 0, 100% A; 2, 100% A; 60, 96.8% B and 3.2% C; 64, 96.8% B and 3.2% C; 65, 100% A; 75, 100% A. The mobile phase flow rate was 1.0 mL/min.

[0305] The reported pH gradient from 5.0 to 9.5 (Farnan, D and Moreno, GT (2009) *Anal. Chem.* 81:8846) was established by buffer C and D. A linear increase of buffer D from 0 to 100% in 45 minutes was delivered to establish a pH gradient from 5.0 to 9.5 (0.1 pH unit/min). The mobile phase flow rate was 1.0 mL/min. Proteins were detected by ultraviolet (UV) absorbance at 280 nm.

[0306] A hybrid pH gradient was established by using a quaternary gradient formed on the quaternary pump using buffers A, B, C and D. This arrangement offered the flexibility of adjusting 1) the starting and ending pH, using buffers A and B and 2) the amount of salt for each gradient, using buffers C and D. An example of the methods used in these experiments, a pH gradient from 6 to 10, with a constant salt concentration of 10 mM, was established by an increase of buffer B from 0 to 40%, while maintaining buffers C and D at 10% and 40%, respectively. The gradients used are listed in the Table 2.

Modeling of the pH-IEC

[0307] The pH of the linearly mixing gradient of two pH buffers was estimated using the Henderson–Hasselbalch (H-H) equation for each of the components based on ideal solution model. The number of available/dissociable protons was first determined for each starting buffer and subsequently for each pH value between the two buffers at a step of 0.1 pH unit. Based on the required number of protons, the molar ratio of the two buffers was derived. The percent of each buffer to attain a pH point in the gradient was obtained. At each pH point, ionic strength was calculated using the estimated ionic components.

Example 1. Assessment of a pH-IEC Method

[0308] The performance of a pH-IEC method was assessed. Although the reported pH-IEC method shows the capability of profiling the charge heterogeneity of multiple mAbs, it is intended primarily for mAbs with pI values from about 7 to 9. For mAbs beyond this range

($pI < 7$ or $pI > 9$, also referred to as extreme pI values), the pH-IEC method often yields unacceptable charge heterogeneity profiles. To assess the method, a pH gradient from 5.0 to 9.5 was produced following the procedure that was previously reported (Farnan, D and Moreno, GT (2009) *Anal. Chem.* 81:8846; Rea, JC *et al.* (2010) *J. Pharm. Biomed. Anal.* 54:317). The buffers were composed of 11.4 mM piperazine, 1.5 mM imidazole and 2.4 mM Tris and pH adjusted to 5.0 and 9.5, respectively. Three mAbs spanning a wide range of pI (6.2, 8.2 and 9.4) were analyzed and the resulting chromatograms are shown in Figure 1. Of these mAbs, only mAb2 (pI 8.2) showed an acceptable charge heterogeneity profile characterized by a good separation of charge variants. The charge variants of the low pI mAb1 ($pI=6.2$) were not well separated; the high pI mAb3 (pI 9.4) did not elute during the pH gradient. Even though mAb3 was eluted when the pH gradient was extended to pH 10.8, the column back pressure was close to the upper pressure limit of the column and the chromatography profiles were inconsistent between different runs. This experiment clearly demonstrated that although the reported pH-IEC method worked well for mAbs with pI values between 7 and 9, it was not able to profile the charge heterogeneity of mAbs with the extreme pI values.

Table 2. Sample Gradients

For samples with pI values between 7.2-8.3:

pH 6-9, 10 mM NaCl; 22 min run				
Time, min	%B	%C	%D	curve
0.0	0.0	10	40	5
0.2	0.0	10	40	5
16.0	30.0	10	40	5
18.0	30.0	10	40	5
18.1	0.0	10	40	5
22.0	0.0	10	40	5

pH 6-9, 10 mM NaCl; 15 min run				
Time, min	%B	%C	%D	curve
0.0	0.0	10	40	5
0.2	0.0	10	40	5
10.0	30.0	10	40	5
12.0	30.0	10	40	5
12.1	0.0	10	40	5
15.0	0.0	10	40	5

For samples with pI values between 8.3-9.0:

pH 6-10, 20 mM NaCl; 22 min run				
Time, min	%B	%C	%D	curve
0.0	0.0	20	30	5
0.2	0.0	20	30	5
16.0	40.0	20	30	5
18.0	40.0	20	30	5
18.1	0.0	20	30	5
22.0	0.0	20	30	5

pH 6-10, 20 mM NaCl; 15 min run				
Time, min	%B	%C	%D	curve
0.0	0.0	20	30	5
0.2	0.0	20	30	5
10.0	40.0	20	30	5
12.0	40.0	20	30	5
12.1	0.0	20	30	5
15.0	0.0	20	30	5

For samples with pI values between 9.0-9.2:

pH 7-10, 50 mM NaCl; 22 min run				
Time, min	%B	%C	%D	curve
0.0	10.0	50	0	5
0.2	10.0	50	0	5
16.0	40.0	50	0	5
18.0	40.0	50	0	5
18.1	10.0	50	0	5
22.0	10.0	50	0	5

pH 7-10, 50 mM NaCl; 15 min run				
Time, min	%B	%C	%D	curve
0.0	10.0	50	0	5
0.2	10.0	50	0	5
10.0	40.0	50	0	5
12.0	40.0	50	0	5
12.1	10.0	50	0	5
15.0	10.0	50	0	5

For samples with pI values between 9.3-9.4:

pH 9-11, 10 mM NaCl; 22 min run				
Time, min	%B	%C	%D	curve
0.0	30.0	10	40	5
0.2	30.0	10	40	5
16.0	50.0	10	40	5
18.0	50.0	10	40	5
18.1	30.0	10	40	5
22.0	30.0	10	40	5

pH 9-11, 10 mM NaCl; 15 min run				
Time, min	%B	%C	%D	curve
0.0	30.0	10	40	5
0.2	30.0	10	40	5
10.0	50.0	10	40	5
12.0	50.0	10	40	5
12.1	30.0	10	40	5
15.0	30.0	10	40	5

[0309] pH at the column exit increased from 5.0 to 9.5, the conductivity of the solvent decreased in a near-linear fashion from 2700 to 800 $\mu\text{S/m}$ (note that the conductivity of 5 mM KCl is 720 $\mu\text{S/m}$ while the conductivity of deionized water is 5.5 $\mu\text{S/m}$). The three pH buffer components are all amines with pK_a over a broad range: piperazine with $\text{pK}_{a1} = 5.68$ and $\text{pK}_{a2} = 9.82$, imidazole with $\text{pK}_a = 6.95$ and Tris with $\text{pK}_a = 8.10$ (at room temperature). These compounds are protonated (or positively charged) when the solution pH is lower than its pK_a , but become neutral when the pH is above its pK_a . When the solvent pH increases, the buffer components gradually become neutral from protonated and thus the conductivity of the buffer

decreases. It is noteworthy that the pH profile was concave at pH around 6 because the piperazine was the most abundant component in the buffer so that the pH curve was relatively flat around its two pK_a of 5.68 and 9.82.

[0310] The pH and ionic strength profiles of the pH gradient were also calculated based on an ideal solution model shown as dashed lines in Figure 2A. The modeled pH curve is very similar to the experimental pH profile except for that the experimental profile was delayed by 5 minutes because of the system dwell volume and column volume. Likewise, the modeled ionic strength curve showed similar shape as the conductivity profile observed experimentally. The agreement between the modeling and experimental data suggests that the mixing of amine-based buffer components followed the ideal solution model. The established model can thus be used to estimate experimental pH and ionic strength profiles at other chromatography conditions.

[0311] Furthermore, the column back pressure during pH-IEC significantly increased with the pH of mobile phase (Figure 2B). This is attributed to the decrease of ionic strength, considering that the composition of mobile phase was constant during the pH gradient. When the ionic strength of mobile phase is low, the electrostatic potential on the stationary phase surface becomes high, according to the double layer model (Staahlberg, J (1994) *Anal. Chem.* 66:440; Stahlberg, J (1999) *J. Chromatogr. A* 855:3). The high electrostatic potential may change the conformation of the resin (*e.g.* swelling the resin to reduce the surface charge density), which likely increases the column back pressure (Product Manual for Propac WCX-10 and Propac SCX-10, 7th ed., Dionex Incorporation, Sunnyvale, CA, 2007).

[0312] The experimental conductivity and the modeled ionic strength profiles can be used to explain the poor charge heterogeneity profiles for mAbs with extreme pI values. Low-pI mAbs elute in the low pH region where the buffer components are protonated and the mobile phase has a relatively high ionic strength. Since the pH gradient IEC separation appears to involve a combination of ionic strength-based and pH-based elution mechanisms (Anderson, DJ and Shan, L (2001) *Clin. Chem.* 47:128; Shan, L and Anderson, DJ (2001) *J. Chromatogr. A* 909:191; Shan, L and Anderson, DJ (2002) *Anal. Chem.* 74:5641), the high ionic strength-based elution may convolute with the pH-based elution, leading to a poor resolution of these low-pI mAb charge variants. On the other hand, high-pI mAbs typically elute in the high pH region where the buffer components become neutral. Because of the low ionic strength in mobile phase, these high-pI mAb are difficult to elute from cation exchange columns. In order to confirm that the ionic strength significantly affects the pH-IEC separation and improve the pH-IEC method for

mAbs with extreme pI values, the ionic strength of the pH buffer in the pH-gradient IEC method was modulated as described below.

Example 3. Improving the pH-Gradient IEC Method by Controlling Ionic Strength

[0313] The ionic strength during the course of a pH gradient was modulated in two ways.

First, the ionic strength at the low pH region was controlled by using different concentrations of buffers. A series of buffer concentrations were tested to assess their impact on pH gradient IEC as discussed below. Second, the ionic strength at high pH region was modulated by adding a salt gradient to the pH gradient. The impact of the salt concentration was also investigated. The new method is thus referred to as an “ionic strength-mediated pH-IEC” method.

[0314] Buffer Concentration: In this method, an equimolar pH-gradient is used rather than a mixed ratio pH gradient used in the reported method (Farnan, D and Moreno, GT (2009) *Anal. Chem.* 81:8846) based on two considerations: First, a near-linear pH gradient can be obtained by using equimolar concentrations of piperazine, imidazole and Tris (Figure 2C). The established linear gradient over a broad range of pH would not sacrifice the separation for a given pH region Tsonev, LI and Hirsh, AG (2008) *J. Chromatogr. A* 1200:166). Second, it provides for optimization of the gradient slope.

[0315] Four buffers consisting of equimolar concentrations of piperazine, imidazole and tris at 1, 2, 4 and 8 mM were investigated. These buffers were referred to as 1, 2, 4 and 8 mM buffers and each was mediated with a pH gradient from pH 5.0 to 10.8 and a linear salt gradient from 0 to 16 mM NaCl. The chromatograms of mAb1 (pI=6.2) with the four buffers are displayed in Figure 3A. The resolution between the charge variants evidently depended on the buffer concentration. With the 1 mM buffer, the charge variants were poorly separated. The resolution improved with the 2 mM buffer and peaked with the 4 mM buffer. However, the resolution significantly decreased with the 8 mM buffer. On the contrary, the resolutions for mAb2 (pI=8.2) were less sensitive to the buffer concentration than mAb1 (Figure 3B). Good resolution for mAb2 (pI = 8.2) was achieved with all four buffers even though the 4 mM buffer offered slightly better resolution than the other three buffers. Based on above visual inspection, the 4 mM buffer appeared to provide the best resolution for mAb1 and mAb2.

[0316] To better visualize the effect of buffer concentration on pH-IEC, the full width at half maximum (FWHM) of the main peak of mAbs plotted as a function of buffer concentration is shown in Figure 3C. The FWHM of the main peak generally correlates with the resolution of

pH-IEC in that the lower the FWHM represents the higher resolution. For both mAb1 and mAb2, the FWHM of the main peak with the 4 mM buffer was lowest among the four buffers, suggesting that the 4 mM buffer provided the narrowest peak width, thus good resolution. On the contrary for mAb3 (pI=9.4), the FWHM of the main peak slightly decreased when the buffer concentration increased from 1 to 8 mM. Thus the 8 mM buffer likely provided the best resolution for mAb3.

[0317] The effect of buffer concentration on pH-IEC of mAbs depended on the pI value of a mAb. The mAbs with low (6.2) and mid pI (8.2) values showed optimal separation with the 4 mM buffer, while the mAbs with high pI value (9.4) appeared to prefer higher concentration buffers. This is reasonable since the high pI mAbs strongly bind to column and thus may require more ionic strength-based elution than low and mid- pI mAbs to achieve optimal resolution. Since the buffer concentration and conductivity evidently impact the resolution of mAbs in pH-gradient IEC, these should be optimized for each individual mAb whenever high resolution is desired. An IEC method that can resolve acidic and basic variants from the main peak for mAbs over a wide pI range was developed. The 4 mM buffer appeared to meet this requirement and thus was chosen for the multi-product salt-mediated pH-IEC method.

[0318] Salt Concentration: To investigate how the ionic strength affects the pH-IEC separation, five different levels (0, 8, 16, 32 and 64 mM) of sodium chloride were added to the pH gradient (established by the 4 mM buffer) through a linear gradient from pH 5.0 to 10.8. mAb1, mAb2 and mAb3 were analyzed in parallel. The FWHM of the main peak of mAbs were plotted as a function of salt concentration as shown in Figure 3D. For mAb1, the FWHM of the main peak was highly sensitive to the salt concentration and it reached to the minimum with 8 mM NaCl. This suggests that 8 mM NaCl provided the best resolution for mAb1. For mAb2, the FWHM of the main peak was essentially flat across the entire range of salt concentrations, suggesting that the salt concentration did not evidently impact the resolution of mAb2. On the contrary for mAb3, the FWHM of the main peak decreased with the salt concentration increasing from 8 to 32 mM and remained unchanged between 32 mM and 64 mM of salt. This suggests that mAb3 required 32 mM of salt to achieve optimal resolution.

[0319] The effect of the ionic strength on pH-gradient IEC of mAbs also correlated with the mAb's pI. The low pI mAbs showed separation with 8 mM of salt; the high pI mAbs showed separation higher concentration of salt, while the resolution of mid-pI mAbs was independent of salt concentration. Because of the evident impact on resolution, the salt concentration may be

optimized for each individual mAb whenever high resolution is desired. Among the five salt concentrations, the pH-IEC method with a salt gradient of 16 mM NaCl provided acceptable resolution for mAbs with pI values over a broad range from 6.2 to 9.2 and thus it was chosen as the multi-product salt-mediated pH-IEC method in this work.

[0320] The Optimized ionic strength-mediated pH-IEC Method: The optimized salt-mediated pH-IEC method employed 4 mM piperazine, 4 mM imidazole, and 4 mM Tris to establish the pH gradient and was mediated with a linear salt gradient from 0 to 16 mM of NaCl. The pH and conductivity profiles of the method are shown as solid lines in Figure 2C. For comparison, the modeled pH and ionic strength are shown as dashed lines. The experimental pH at the column exit increased with the retention time in a roughly linear fashion, except for a small concave at pH from 8.5 to 9.0 and it is consistent with the modeled pH except for a delay time of 5 minutes due to the system void volume. With the salt mediation, the experimental conductivity of the mobile phase showed a slight increase during the pH gradient (from 1570 to 1800 μ S).

Likewise, the modeled ionic strength was essentially consistent during the pH gradient. The ionic strength of the amine-based pH gradient was successfully controlled by reducing the buffer concentration and adding a linear salt gradient. With the controlled ionic strength, the column back pressure was maintained below 95 bar at the high pH region (Figure 2D). The resulting chromatograms of mAb1-3 (not shown) were reproducible, indicating that the cation-exchange column was stable in this pressure range.

Example 4. Profiling the Charge Heterogeneity of 16 mAbs

[0321] To further demonstrate the multi-product capability of the new salt-mediated pH-IEC method, 16 mAbs with pI values from 6.2 to 9.4 were analyzed and their chromatograms are shown in Figure 4. Antibodies were eluted from the column with a pH gradient from pH 5 to pH 10.8 and a salt gradient from 0 mM NaCl to 16 mM NaCl. For both low pI mAb1 (6.2) and high pI mAb3 (9.4), the charge variants were well separated to yield acceptable charge heterogeneity profiles. This is a substantial improvement compared to the reported pH-IEC method (Figure 1). The charge variants of all 16 mAbs were well separated, indicating that the developed salt-mediated pH-IEC method was capable of profiling the charge heterogeneity of multiple mAb products without any additional method development effort.

[0322] In addition to broader applicability, the salt-mediated pH gradient offered better resolution than the reported pH-IEC method. For mAb2 (pI=8.2), the salt-mediated pH-IEC

method provided a baseline resolution between the charge variants (Figure 4). However, the resolution by previous pH-IEC method was much lower (Figure 1). Although the salt-mediated pH-gradient was longer (58 minutes) than the previous pH-gradient (45 minutes), the gradient slopes in the two methods were identical (0.1 pH unit/min). The improved resolution by the salt-mediated pH-IEC method was thus not a result of a change in gradient length, but rather from the effect of controlling the ionic strength.

Example 5. Monitoring the thermal stability of mAbs

[0323] Cation-exchange chromatography is commonly used to assess the degradation and lot-to-lot variation of biopharmaceutical proteins during manufacturing (Vlasak, J and Ionescu, R(2008) *Curr. Pharm. Biotechnol.* 9:468). To demonstrate the ability to monitor protein degradation, the developed ionic strength-mediated pH-IEC method was used to profile the charge heterogeneity of mAb1 after thermal stresses. mAb1 was chosen in this study because it has the lowest retention among the mAbs and its pH-IEC profile was most susceptible to changes in chromatography parameters. Antibody was eluted from the column with a pH gradient from pH 5 to pH 10.8 and a salt gradient from 0 mM NaCl to 16 mM NaCl.

[0324] The chromatograms of control and stressed materials of mAb1 are normalized with the main peak (Figure 5). After the thermal stresses, both acidic and basic variants increased. A shoulder also appeared to the right of the main peak for the stressed samples. These profile changes evidently indicate that mAb1 degraded after incubation at 40°C for 3 and 6 weeks. Likewise, the degradation of mAb2 and mAb3 under thermal stresses was also detected by the salt-mediated pH-IEC method (data not shown).

Example 6. Robustness testing of the ionic strength-mediated pH-IEC

[0325] Because of the complex elution process of the salt-mediated pH-IEC method, it is necessary to ensure its robustness for routine sample testing. As discussed above, we know that the pH buffer composition and the salt concentration affected the retention and resolution of mAbs. The purpose of further studies here was to investigate the variability originated from column, buffer lot, and instrument when the optimized pH buffer composition and salt concentration were used. Three columns, three buffer lots, two instruments were tested in four different days. The experimental design is shown Table 3. mAb1 was again chosen in the

studies because its pH-IEC profile was most susceptible to changes in chromatography parameters.

[0326] The chromatograms of mAb1 obtained with different columns and buffer lots were comparable, but those obtained with different instruments showed slightly different retention time. The difference in the delay volumes expected between instruments accounted for the variation in the retention time, but it doesn't impact the performance of the method. The quantitation of the charge variants of mAb1 is summarized in Table 4. For the 16 chromatograms obtained with two different instruments, three columns and three buffers preparations, the quantitation of the charge variants was consistent, indicating that the salt-mediated pH-IEC was robust at these chromatography conditions.

Table 3. The experimental design for the robustness test of the salt-mediated pH-IEC using mAb1

	Day1	Day2	Day3	Day4
Waters 2796	X	X	X	
Dionex U3000				X
Column 1	X			
Column 2		X	X	
Column 3				X
Buffer 1	X	X		
Buffer 2			X	
Buffer 3				X

Table 4. Summary of the robustness data (n=16) of the salt-mediated pH gradient obtained for mAb1

	Acidic Variants	Main Peak	Basic Variants
Average	12.32	78.99	8.69
Highest	13.20	79.92	9.54
Lowest	10.54	77.91	8.14
STD deviation	0.78	0.56	0.55
% RSD	6.3	0.7	6.4

[0327] The salt-mediated pH gradient IEC was also robust across a wide range of sample mass loadings on column. As shown in Figure 6, consistent elution profiles were observed when 5-

200 µg of mAb1 was loaded on the column. Although the main peak slightly broadened when the column load was over 50 µg, the quantitation of the charge variants was consistent among all the tested column loads (Table 5).

[0328] During the course of the robustness studies, sufficient data was obtained to cover most variables that are experienced in a typical HPLC experiment. The salt-mediated pH-IEC method provides comparable chromatograms and consistent quantitation results of charge variants for a typical mAb, demonstrating that the method is robust at all chromatography conditions studied here.

Table 5. The robustness of the charge heterogeneity of mAb1 at different column loads

Column Load (µg)	Acidic Variants	Main Peak	Basic Variants
5	12.66	79.04	8.30
10	13.04	78.84	8.12
50	13.27	78.14	8.59
100	13.58	77.80	8.62
200	13.55	77.86	8.59
Average	13.22	78.34	8.44
STD deviation	0.38	0.57	0.22
% RSD	2.9	0.7	2.6

Example 7. Reducing run time for ionic strength-mediated pH-IEC

[0329] Previously, pH-mediated IEC methods used piperazine, imidazole, tris (PIT) as buffering reagents (Farnan, D and Moreno), GT 2009 *Anal. Chem.* 81:884-8857). To generate a pH gradient, a semi linear pH curve was generated from pH 6 to 9.5. The amino functional group of each buffering agent maintains a positive charge when the mobile phase pH is less than the reagent's pKa and neutral when the mobile phase pH is greater, as shown in in Figure 7. The pH gradient was generated using Buffer A (pH 5.0) and Buffer B (pH 9.5) in a gradient from pH 5 to pH 9.5 in 35 minutes. The column was a Propac WCX-10, 4 × 250 mM. This buffer reagent system pH gradient works well for large range of molecules with pI values between 7 and 8.5. However, the charge variants for molecules outside this working range are not resolved as well. Preliminary work revealed that the concentration and ionic strength of the buffering reagents influenced the linearity of the pH curve and the retention times of sample. As shown in Example 2, ionic strength dramatically decreases as pH increased over time. Adjustments to buffer concentrations and the introduction of a simultaneous salt gradient provided a rather stable ionic

strength throughout the pH gradient. With a simultaneous salt gradient, pH-gradient separation can now resolve molecular variants with pI values from 6.2-9.4. As shown in Figure 8 using a long gradient of 58 min, where the pH increased from pH 5 to pH 10.8 at a slope of 0.1 pH unit/min and the salt concentration increased from 0 mM NaCl to 16 mM NaCl in a PIT buffer (4 mM piperazine, 4 mM imidazole, and 4 mM Tris) monoclonal antibodies with pI values of 6.2 (Mab1), 8.2 (Mab 2) and 9.4 (Mab3) are resolved. The improved method is referred to as an “ionic strength-mediated pH gradient” or a “salt-mediated pH gradient”.

[0330] To reduce run time and increase throughput, the salt-mediated pH gradient on a shorter column (4 × 50 mm) was evaluated. Farnan, D and Moreno, GT (2009) *Anal. Chem.* 81:8846-8857 indicated that similar elution profiles were obtained using pH-IEC regardless column length, provided it was the same pH-IEC separation mode (WCX or SCX). To determine if shorter columns could reduce elution times, the separation of three Mabs with different pI values using both the original pH-IEC and salt-mediated pH-IEC mobile phases using a WCX-10, 4 × 50 mm column were compared. The mAbs were Mab 1, pI 7.6; Mab 2, pI 8.6-9.3; and Mab 3, pI 9.1. The pH gradient was pH 6-11 in 0-16 min, held at pH 11 for 2 min, and followed by a 4 min re-equilibration at pH 6 for a total run time of 22 minutes. The buffer for the original pH-IEC was 2.4 mM Tris, 1.5 mM imidazole, and 11.6 mM piperazine. The buffer for the ionic strength-mediated pH-IEC was 4 mM Tris, 4 mM imidazole, and 4 mM piperazine with 16 mM NaCl. As seen in Figure 9, although similar peak profiles were obtained for these three Mabs, the resolution of charge variants decreased as molecule's pI increased. In order to achieve adequate separation for molecules with a wide pI range in less run time, an investigation into variant separation mechanism and different buffer system was initiated.

[0331] To assist in buffer screening, a pH and conductivity prediction tool may be used. The pH and conductivity curves of commonly used buffers and NaCl salt at different combinations, pH range and gradient times can be calculated and plotted based on buffer's pKa. The visualization prediction tool allows quick optimization of the buffer system which has desired properties, such as a linear pH curve, stable or increasing ionic strength over time, and lower buffer toxicity. Using this model, various buffer combinations were screened. The combination of Tris, piperazine and phosphate (TPP) buffers was found to form a linear pH gradient over 16 minute gradient (Figure 10). Buffers included piperazine pK1 with a pKa of 5.33 (range 5.0-6.0), phosphate pK2 with a pKa of 7.20 (range 5.8-8.0), Tris base with a pKa of 8.06 (range 7.5-9.0), piperazine pK2 with a pKa of 9.73 (range 9.5 to 9.8) and phosphate pK3 with a pKa of

12.33. The plot in Figure 10 elucidates that the phosphate molecule remains charged throughout the pH range, and compensates, to a certain extent, the ionic strength loss due to the deprotonation of amino functional groups in Tris and piperazine as the pH increases. The chromatography conditions were as follows:

Column: Propac WCX-10HT, 4 x 50 mm

pH gradient: 5.0 mM Tris, piperazine, phosphate (TPP)

Buffer A = pH 6.0

Buffer B = pH 11.0 Gradient: 6-11 in 16 min

Buffer C = 60 mM NaCl

Buffer D = MilliQ water

[0332] The predicted pH and conductivity curves (Figure 11 right panel) were consistent with those obtained experimentally (left panel). The buffer system contained 5 mM of Tris, piperazine, and phosphate, and a constant NaCl concentration at either 10 mM, 20 mM or 30 mM (used in model). The experimental flow rate was 1 mL/min or 2 mL/min.

Example 8. Determining optimal ionic strength ranges

[0333] Various samples were analyzed using the 16 minute gradient salt-mediated TPP method. An example of consistent profiles between 22 and 58 minute methods from 4 x 50 and 4 x 250 Propac WCX-10 columns, respectively is shown in Figure 12. The elution with the 4 x 50 column was with salt-mediated Tris/Piperazine/Phosphate (TPP), pH 6-11 and 0-30 mM NaCl over 16 min. The elution with the 4 x 250 column was with salt-mediated Piperazine/Imidazole/Tris (PIT) pH 5-10.8 and 0-16 mM NaCl over 58 min. It was determined that the salt-mediated TPP method was sufficient for molecules mainly in the pI range of 7-8.5, which was the similar range obtained with the original PIT method. It was concluded that the buffer reagents and ionic strength plays a part in separation but that other factors may be involved.

[0334] The net charge state of MAbs over a pH range was modeled. Figure 13 shows an overlay of net charges over pH for mAbs with different pI's. In the cation exchange mode, a mAb carries a positive charge until the pH decreases to reach its pI (x-intersect), and then is eluted from the column. As the pH increases, the mAb becomes negatively charged. The charge variant profile for a given protein molecule consists of a main peak, an acidic, and a basic region (as shown on the bottom panel). Usually, a charge variant is one or few charges apart from the

main peak, with the entire charge envelop from the most acidic to the most basic variant spanning across 5-7 charges. The optimal pH-gradient separation window may lie across curve's flat region between pH 6.5 to pH 8.5 (Figure 13); *e.g.* for molecules with pI values less than about 8.5, the net charge immediately below its theoretical pI changes very little and the curve is relatively flat between pH 6.5 and 8.5. In a pH gradient run, there is sufficient time for charge variants to be eluted off the column separately at different retention times as the pH increases over time.

[0335] Conversely, for molecules with pI values greater than 8.5 and less than 6.5, the net charge changes dramatically at its theoretical pI's (Figure 14). During a platform linear pH-gradient run, the charge per min slope is too steep at these pHs to allow adequate separation and one peak is observed (inset).

[0336] Since better peak separation and robustness was achieved in the relatively flat portion of the charge vs. pH curve, the question is how to elute protein charge variants in this region regardless their pI. This requires a pre-modification of charge state for higher pI molecules using factor other than pH. This factor is buffer ionic strength. With higher ionic strength in the mobile phase, more charges on the molecule surface are shielded from the stationary phase. Therefore, the apparent net charge decreases to allow the variants being eluted at pH below its pI.

[0337] In the work presented in Examples 1-6, charge variants of molecules with pI values greater than 9 (cation exchange mode) and less than 7 (anion exchange mode) were separated well by correcting the ionic strength deficiencies in the PIT mobile phases combined with a salt gradient (salt mediated pH-gradient). The combined driving force of pH and salt gradient allows for molecule's apparent charge state decreases faster over time as compares to the pH-gradient alone. This can be illustrated as the apparent charge vs. pH curve get stretched vertically, with a narrower flat region between pH 7-8, and a shallower slope at pH greater than 9. Therefore, for molecules with higher pIs, the separation took place at pH slightly below their pIs. Using a longer column (250 mm) and long run time (60 min), there was sufficient time for charge variants to elute. However, the salt mediated pH-gradient method failed to resolve charge variants for high pI molecules on a shorter column (50 mm) in a short run time (20 min).

[0338] Since short run time is critical, instead of gradually decreasing the apparent charge with a salt gradient over 60 min, the desired charge state can be reached at the beginning of the run by introducing salt early and kept constant throughout.

[0339] Figure 15 is an overlay of pH-gradient separations of a high pI mAb (pI 9.2) in the presence of NaCl salt at different concentrations. Using a quaternary system, a pH gradient from pH 6-11 was created using the A and B lines and NaCl salt concentrations of 0, 10, 20, 30, 40 or 50 mM was maintained using the C and D lines. Gradient conditions and run times were as follows: Instrument: U3000 2DLC; Mobile phases: 10 mM Tris, piperazine, phosphate, A) pH 6.0, B) pH 11.0; C) 100 mM NaCl, D) milliQ water; Column: Propac WCX-10, 4 × 50 mm, 10 µm; Column temp: 40°C; Flow rate: 1 mL/min; pH Gradient: 10-50% B; salt constant: 0 mM=0% C, 50% D; 10 mM=10% C, 40% D; 20 mM=20% C, 30% D, 30 mM=30% C, 20% D, 40 mM=40% C, 10% D, 50 mM=50% C, 0% D; Sample conc.: 1 µg/µL; Loaded volume: 20 µL.

[0340] At lower salt concentration, very little separation was observed. At subsequently higher salt concentrations, the separations improved while the elution time decreased. The decreasing elution time with increasing salt concentration supports that extra charges on the molecule surface are shielded from the stationary phase so that the apparent charge decreases to allow for variant separation at pH below its pI. In this case, an optimal separation was achieved at 40-50 mM NaCl, at which the apparent charge of this mAb presumably resided in the relatively flat portion of the charge vs. pH curve.

[0341] Analysis of various mAbs with different pIs were performed at different constant salt concentrations while applying the same pH gradient. Generally, the optimal separation was achieved at a constant salt concentration at such that the apparent charge state falls in the flat portion of the charge vs. pH curve. For example, mAbs in the pI range of 7-8.3 needed no salt, those in the pI range of 8.3-8.8 needed 20 mM salt and those in the pI range of 8.8-9.0 needed 50 mM salt (Figures 16 and 17). On the charge vs. pH curve, the addition of salt essentially moves the x-axis up to the desired charge state of the molecule to allow for optimal separation in the flat region of the curve. This type of separation is referred to as a “hybrid” pH-gradient (Figure 18).

[0342] Improved separations were achieved by altering gradient conditions. A shallow hybrid pH-IEC gradient (using TPP mobile phases) resulted in improved peak separation compared to either the original and salt mediated pH-IEC methods (Figures 19 and 20). Similar separation was achieved by using TIC buffer (Tris, Imidazole, CAPS) with 10 mM NaCl to replace piperazine, a hazardous disposal concern, and phosphate, a versatile biological buffer that may adversely induce assay artifact due to buffer-catalyzed post translational modification (Figure 21).

[0343] The short hybrid pH-IEC method was further challenged to determine if faster analysis times would be possible. The pH gradient was set from pH 7-10 in 10 minutes, with a 2 minute hold at pH 10 and a 3 min equilibration time for a total run time of 15 minutes.

[0344] Chromatography conditions were as follows: Instrument: U3000 2DLC; Mobile phases: 10 mM Tris, piperazine, phosphate, A) pH 6.0, B) pH 11.0; C) 100 mM NaCl, D) milliQ water; Column: Propac WCX-10HT, 4 × 50 mm, 10 µm; Column temp: 40°C; Flow rate: 1 mL/min; pH Gradient: 0-30% B; salt constant: 0 mM=0% C, 50% D; 10 mM=10% C, 40% D; 20 mM=20% C, 30% D, 30 mM=30% C, 20% D, 40 mM=40% C, 10% D, 50 mM=50% C, 0% D; Sample conc.: 1 µg/µL; Loaded volume: 20 µL.

[0345] As shown in Figures 22 and 23, profiles were similar between 15 and 22 min runs for two different MAbs with pI values of 8.8 and 9.0, respectively. However, as the run times became shorter, more product specific starting/ending pH values and salt concentrations were necessary.

[0346] When developing product specific method, optimal resolution can be achieved by carefully selecting the appropriate pH-gradient slope and salt concentration based on molecule's pI. Product specific methods would be beneficial if only one product is exclusively analyzed in a sequence. For laboratories that analyze a variety of mAbs, each with a different pI, using a quaternary system that can deliver a pH-gradient while maintaining a certain salt concentration is desirable. Otherwise, a longer run time of 22 min may be required as a platform method using a binary system that delivers the pH-gradient at a fixed salt concentration.

Example 9. Robustness of ionic strength-mediated pH gradient ion exchange chromatography

[0347] To demonstrate the robustness window and the target running conditions of an ionic strength-mediated pH gradient ion exchange chromatography suitable for analysis of multiple monoclonal antibody products with pI's across a wide range using a design of experiment (DOE) approach. The method is suitably robust if there is no significant change for the reportable values including the relative peak areas of the main (Main%), acidic variant (AV%) and basic variant (BV%) peaks. In addition, the general peak profile as measured by peak resolutions (Rs1 and Rs2). To test robustness, running parameters such as salt concentration, buffer concentrations, pH, column temperature, and flow rate are intentionally perturbed.

Materials and Methods

[0348] The following three antibodies were tested:

mAb1, pI = 8.2

mAb2, pI = 8.5

mAb3, pI = 9.0

[0349] A Waters 2796 Bioseparations Module equipped with an 8-port, 3-way switch valve to the column, 6-port solvent selector valves for lines C and D and Waters 2487 Dual λ UV detector was used for the chromatography.

[0350] The chromatography column was a Dionex ProPac WCX-10 HT, 4 × 50 mm column.

[0351] The buffer system was as follows: equal molar of imidazole, Tris, and CAPS for buffers A & B. Buffer C was 100 mM NaCl and buffer D was water.

[0352] For Buffer A the pH was as specified in the experiment design table (Table 6), the pH for buffer B was 10.0. The Total buffer strength and salt concentration that were used are shown in the experiment design table.

[0353] The gradient was as follows: 0-2 min, at starting pH; 2-16 min from starting pH to pH 10; 16-18 min, at pH 10; 18-22 min, at starting pH. Selected salt concentrations remained constant through gradient.

[0354] Column Temperature and flow rate were used as indicated in Table 6.

[0355] 3 mAbs:

mAb1, pI = 8.2

mAb2, pI = 8.5

mAb3, pI = 9.0

[0356] Waters 2796 Bioseparations Module equipped with an 8-port, 3-way switch valve to the column, 6-port solvent selector valves for lines C and D and Waters 2487 Dual λ UV detector.

[0357] Dionex ProPac WCX-10 HT, 4x50mm column

[0358] Buffer system: equal molar of imidazole, Tris, and CAPS for buffers A & B. Buffer C is 100 mM NaCl and buffer D is water.

[0359] Buffer A pH is specified in the experiment design table, pH for buffer B is 10.0. Total buffer strength and salt concentration are shown in the experiment design table.

[0360] Gradient: 0-2min, at starting pH; 2-16min from starting pH to 10; 16-18 min, at pH10, 18-22min, at starting pH. Selected salt conc. remains constant through gradient.

[0361] Column Temperature and flow rate: see experiment design table

Table 6. Experimental Design (Running Parameters)

Pattern	Salt (mM)	Buffer (mM)	Start pH	Column Temp. (°C)	Flow (ml/min)
+++--	25	20	6.3	36	0.8
--+--	15	10	6.3	36	0.8
-+---	15	20	5.7	36	0.8
+--++	25	10	6.3	36	1.2
-++-+	15	20	6.3	36	1.2
+---++	25	10	5.7	44	1.2
----+	15	10	5.7	36	1.2
0	20	15	6.0	40	1
+++++	25	20	6.3	44	1.2
++-+-	25	20	5.7	44	0.8
--+++	15	10	6.3	44	1.2
---+-	15	10	5.7	44	0.8
-+-++	15	20	5.7	44	1.2
+--+-	25	10	6.3	44	0.8
-+++-	15	20	6.3	44	0.8
++--+	25	20	5.7	36	1.2
+-----	25	10	5.7	36	0.8
0	20	15	6.0	40	1

[0362] The robustness of the chromatography procedure for the analysis of multiple monoclonal antibody products was tested by systematically perturbing the parameters from the target running conditions of 20 mM salt, 15 mM buffer, starting pH 6.0, column temperature 40 °C, and a flow rate of 1.0 ml/min (designated as 0 in Table 6). Three different MABs, with pI's of 8.2, 8.5 and 9.0 were tested. The resulting chromatograms of duplicate chromatographies at the target running condition are presented in Figure 24. An example of a test for the robustness of the target running condition for MAb3 by perturbing the running conditions according to Table 6 is presented in Figure 25. Although there are some minor differences in resolution, the general peak profiles, especially for the three regions (acidic, main, and basic) are maintained and allow quantitation.

[0363] The effects of running parameters on method performance can be visualized using a distribution plot (Figure 26). In this plot, the reportable values (Main%, AV% and BV%) for analyses at different conditions were distributed closely around those at the target condition (at the center of each panel, *i.e.* 20 mM salt). The results show that there was no significant effect on the reportable values due to salt concentration. However, there was a trend in resolutions with the increase of salt concentration: a slight downward trend for MAb1 (pI=8.2) and MAb2 (pI=8.5), and an upward trend for MAb3 (pI=9). It suggested that with the increase in molecules' pI, the resolution can be further improved by increasing salt concentration, for a product specific method. As a multi-product method, the salt concentration was optimized for mAbs with pI across a wide range. The results of these studies demonstrated that 20 mM is the useful salt concentration for analysis of antibodies with a pI ranging from 7.0 to 9.5.

[0364] The effects of the other running parameters were also plotted and are presented in Figure 27. In these plots, the reportable values (Main%, AV% and BV%) for analyses at different condition were also centered around those at the target conditions. The results demonstrated that there was no significant effect on the reportable values due to the intentional perturbation of running conditions. Thus, the multi-product method is suitably robust at the target running condition.

CLAIMS

WHAT IS CLAIMED IS:

1. A method for analyzing a composition comprising a polypeptide and one or more contaminants, the method comprising
 - a) binding the polypeptide and one of more contaminants to an ion-exchange chromatography material using a loading buffer, wherein the loading buffer is at a first pH and comprises a first ionic strength;
 - b) eluting the polypeptide and one or more contaminants from the ion-exchange chromatography material using an elution buffer wherein the pH of the elution buffer is altered in a pH gradient and the ionic strength of the elution buffer is altered in an ionic strength gradient, wherein the polypeptide and the one or more contaminants are separated by the combination of pH gradient and ionic strength gradient; and
 - c) detecting the polypeptide and the one or more contaminants.
2. The method of claim 1, wherein the polypeptide is an antibody or immunoadhesin or fragment thereof.
3. The method of claim 1 or 2, wherein the polypeptide is a monoclonal antibody or fragment thereof.
4. The method of claim 2 or 3, wherein the antibody is a human antibody.
5. The method of claim 2 or 3, wherein the antibody is a humanized antibody.
6. The method of claim 2 or 3, wherein the antibody is a chimeric antibody.
7. The method of any one of claims 2-6, wherein the antibody is an antibody fragment.
8. The method of any one of claims 1-7, wherein the contaminant is a variant of the polypeptide.

9. The method of any one of claims 1-7, wherein the contaminant is a degradation product of the polypeptide.
10. The method of any one of claims 1-9, wherein the polypeptide has a pI greater than about 9.0.
11. The method of any one of claims 1-10, wherein the chromatography material is a cation exchange chromatography material.
12. The method of claim 11, wherein the cation exchange chromatography material is a sulfonated chromatography material or a carboxylated chromatography material.
13. The method of any one of claims 1-12, wherein the pH gradient is a linear gradient.
14. The method of any one of claims 1-12, wherein the pH gradient is a step gradient.
15. The method of claim 13 or 14, wherein the pH gradient comprises an increase from about pH 5 to about pH 11.
16. The method of any one of claims 1-15, wherein the pH gradient is generated using one or more buffers.
17. The method of claim 16, wherein the one or more buffers selected from piperazine, imidazole, tris, phosphate, or CAPS.
18. The method of any one of claims 1-17, wherein the ionic strength gradient is a linear gradient.
19. The method of any one of claims 1-17, wherein the ionic strength gradient is a step gradient.

20. The method of claim 18 or 19, wherein the ionic strength gradient comprises an increase in salt concentration from about 0 mM to about 200 mM.
21. The method of any one of claims 18-20, wherein the ionic strength gradient is a NaCl gradient, a KCl gradient, or an Na₂SO₄ gradient.
22. The method of any one of claims 1-9, wherein the polypeptide has a pI less than about 7.0.
23. The method of claim 22, wherein the chromatography material is an anion exchange chromatography material.
24. The method of claim 23, wherein the anion exchange chromatography material is a quarternary amine chromatography material or a tertiary amine chromatography material.
25. The method of any one of claims 22-24, wherein the pH gradient is a linear gradient.
26. The method of any one of claims 22-24, wherein the pH gradient is a step gradient.
27. The method of claim 25 or 26, wherein the pH gradient comprises a decrease from about pH 8 to about pH 5.
28. The method of any one of claims 22-27 wherein the pH gradient is generated using one or more buffers.
29. The method of claim 28, wherein the one or more buffers selected from piperazine, imidazole or Tris.
30. The method of any one of claims 22-29, wherein the ionic strength gradient is a linear gradient.

31. The method of any one of claims 22-29, wherein the ionic strength gradient is a step gradient.
32. The method of claim 30 or 31, wherein the ionic strength gradient comprises an increase in salt concentration from about 0 mM to about 200 mM.
33. The method any one of claims 30-32, wherein the ionic strength gradient is a NaCl gradient, a KCl gradient, or an Na₂SO₄ gradient.
34. A method for analyzing a composition comprising the polypeptide and one or more contaminants, the method comprising
- a) binding the polypeptide and one of more contaminants to an ion-exchange chromatography material using a loading buffer, wherein the loading buffer is at an initial pH and comprises an initial ionic strength;
 - b) eluting the polypeptide and one or more contaminants from the ion-exchange chromatography material using an elution buffer wherein the pH of the elution buffer is altered in a pH gradient and wherein the ionic strength of the elution buffer is essentially the same as the initial ionic strength of the loading buffer, wherein the polypeptide and the one or more contaminants are separated by pH gradient at about the initial ionic strength; and
 - c) detecting the polypeptide and the one or more contaminants.
35. The method of claim 34, wherein the polypeptide is an antibody or immunoadhesin or fragment thereof.
36. The method of claim 34 or 35, wherein the polypeptide is a monoclonal antibody or fragment thereof.
37. The method of claim 35 or 36, wherein the antibody is a human antibody.
38. The method of claim 35 or 36, wherein the antibody is a humanized antibody.
39. The method of claim 35 or 36, wherein the antibody is a chimeric antibody.

40. The method of any one of claims 35-39, wherein the antibody is an antibody fragment.
41. The method of any one of claims 34-40, wherein the contaminant is a variant of the polypeptide.
42. The method of any one of claims 34-40, wherein the contaminant is a degradation product of the polypeptide.
43. The method of any one of claims 34-42, wherein the polypeptide has a pI greater than about 9.0.
44. The method of any one of claims 34-43, wherein the chromatography material is a cation exchange chromatography material.
45. The method of claim 44, wherein the cation exchange chromatography material is a sulfonated chromatography material or a carboxylated chromatography material.
46. The method of any one of claims 34-45, wherein the pH gradient is a linear gradient.
47. The method of any one of claims 34-45, wherein the pH gradient is a step gradient.
48. The method of claim 46 or 47, wherein the pH gradient comprises an increase from about pH 5 to about pH 11.
49. The method of any one of claims 34-48, wherein the pH gradient is generated using one or more buffers.
50. The method of claim 49, wherein the one or more buffers selected from piperazine, imidazole, tris, phosphate, or CAPS.

51. The method of any one of claims 34-50, wherein the ionic strength of the elution buffer is from about 0 mM to about 100 mM.
52. The method of claim 51, wherein the elution buffer comprises about 0 mM NaCl to about 100 mM NaCl, about 0 mM KCl to about 100 mM KCl, or about 0 mM Na₂SO₄ to about 100 mM Na₂SO₄.
53. The method of any one of claims 34-42, wherein the polypeptide has a pI less than about 7.0.
54. The method of claim 53, wherein the chromatography material is an anion exchange chromatography material.
55. The method of claim 54, wherein the anion exchange chromatography material is a quarternary amine chromatography material or a tertiary amine chromatography material.
56. The method of any one of claims 53-55, wherein the pH gradient is a linear gradient.
57. The method of any one of claims 53-55, wherein the pH gradient is a step gradient.
58. The method of claim 56 or 57, wherein the pH gradient comprises a decrease from about pH 8 to about pH 5.
59. The method of any one of claims 53-58, wherein the pH gradient is generated using one or more buffers.
60. The method of claim 59, wherein the one or more buffers selected from piperazine, imidazole or Tris.
61. The method of any one of claims 53-60, wherein the ionic strength of the elution buffer is from about 0 mM to about 100 mM.

62. The method of claim 61, wherein the elution buffer comprises about 0 mM NaCl to about 100 mM NaCl.

63. The method of any one of claims 1-62, wherein the analysis is by high performance liquid chromatography.

64. A method for analyzing a polypeptide in a composition comprising the polypeptide and one or more contaminants, wherein the method separates one or more contaminants from the polypeptide, the method comprising

a) binding the polypeptide and one of more contaminants to an ion-exchange chromatography material using a loading buffer, wherein the loading buffer is at a first pH and comprises a first ionic strength;

b) eluting the polypeptide and one or more contaminants from the ion-exchange chromatography material using an elution buffer wherein the pH of the elution buffer is altered in a pH gradient and the ionic strength of the elution buffer is altered in a ionic strength gradient, wherein the polypeptide and the one or more contaminants are separated by the combination of pH gradient and ionic strength gradient; and

c) detecting the polypeptide and the one or more contaminants, wherein the method is used to analyze polypeptides having a pI ranging from about 7.0 to about 9.5.

65. A method for analyzing a polypeptide in a composition comprising a polypeptide and one or more contaminants, the method comprising

a) binding the polypeptide and one of more contaminants to an ion-exchange chromatography material using a loading buffer, wherein the loading buffer is at an initial pH and comprises an initial ionic strength;

b) eluting the polypeptide and one or more contaminants from the ion-exchange chromatography material using an elution buffer wherein the pH of the elution buffer is altered in a pH gradient and wherein the ionic strength of the elution buffer is essentially the same as the initial ionic strength of the loading buffer, wherein the polypeptide and the one or more contaminants are separated by pH gradient at about the initial ionic strength; and

c) detecting the polypeptide and the one or more contaminants,

wherein the method is used to analyze polypeptides having a pI ranging from about 7.0 to about 9.5.

66. The method of claim 64 or 65, wherein the polypeptide is an antibody or immunoadhesin or fragment thereof.

67. The method of any one of claims 64-66, wherein the polypeptide is a monoclonal antibody or fragment thereof.

68. The method of claim 66 or 67, wherein the antibody is a human antibody.

69. The method of claim 66 or 67, wherein the antibody is a humanized antibody.

70. The method of claim 66 or 67, wherein the antibody is a chimeric antibody.

71. The method of any one of claims 65-70, wherein the antibody is an antibody fragment.

72. The method of any one of claims 64-71, wherein the contaminant is a variant of the polypeptide.

73. The method of any one of claims 64-72, wherein the contaminant is a degradation product of the polypeptide.

74. The method of any one of claims 64-73, wherein the contaminant is a charge variant of the polypeptide.

75. The method of any one of claims 1-74, wherein the concentration of the buffer in the loading buffer and/or the elution buffer varies from about 10 mM to about 50 mM.

76. The method of any one of claims 1-75, wherein the first pH varies from about pH 5.0 to about pH 7.0.

77. The method of any one of claims 1-76, wherein the temperature of the chromatography material varies from about 20 °C to about 50 °C.
78. The method of any one of claims 1-77, wherein the loading and elution are conducted at a flow rate varying from about 0.5 ml/min to about 2.0 ml/min.
79. A method of determining the purity of a polypeptide in a composition comprising analyzing the composition according to any one of the methods of claims 1 to 78 and determining the ratio of polypeptide to contaminants in the composition.
80. A method of determining the stability of a polypeptide in a composition comprising the polypeptide, the method comprising,
- a) incubating the composition comprising the polypeptide at 40 °C for three or six weeks,
 - b) analyzing the composition of step a) by any of the methods of claims 1 to 79, and
 - c) determining the ratio of variants to polypeptide in the composition, wherein an increase in the ratio of variants to polypeptide in the composition compared to a composition that was not incubated indicates the degradation of the polypeptide in the composition.

Figure 1

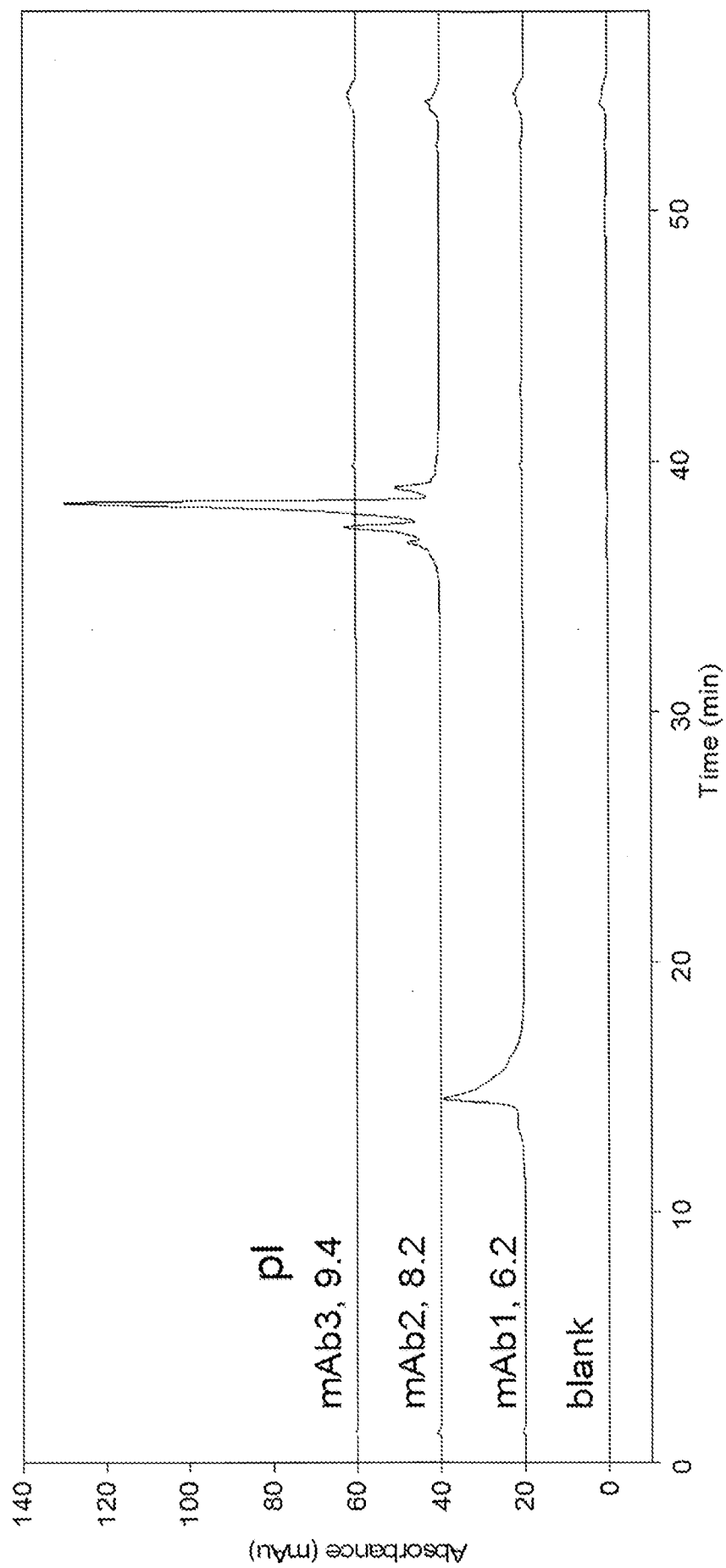


Figure 2A

A. before optimization

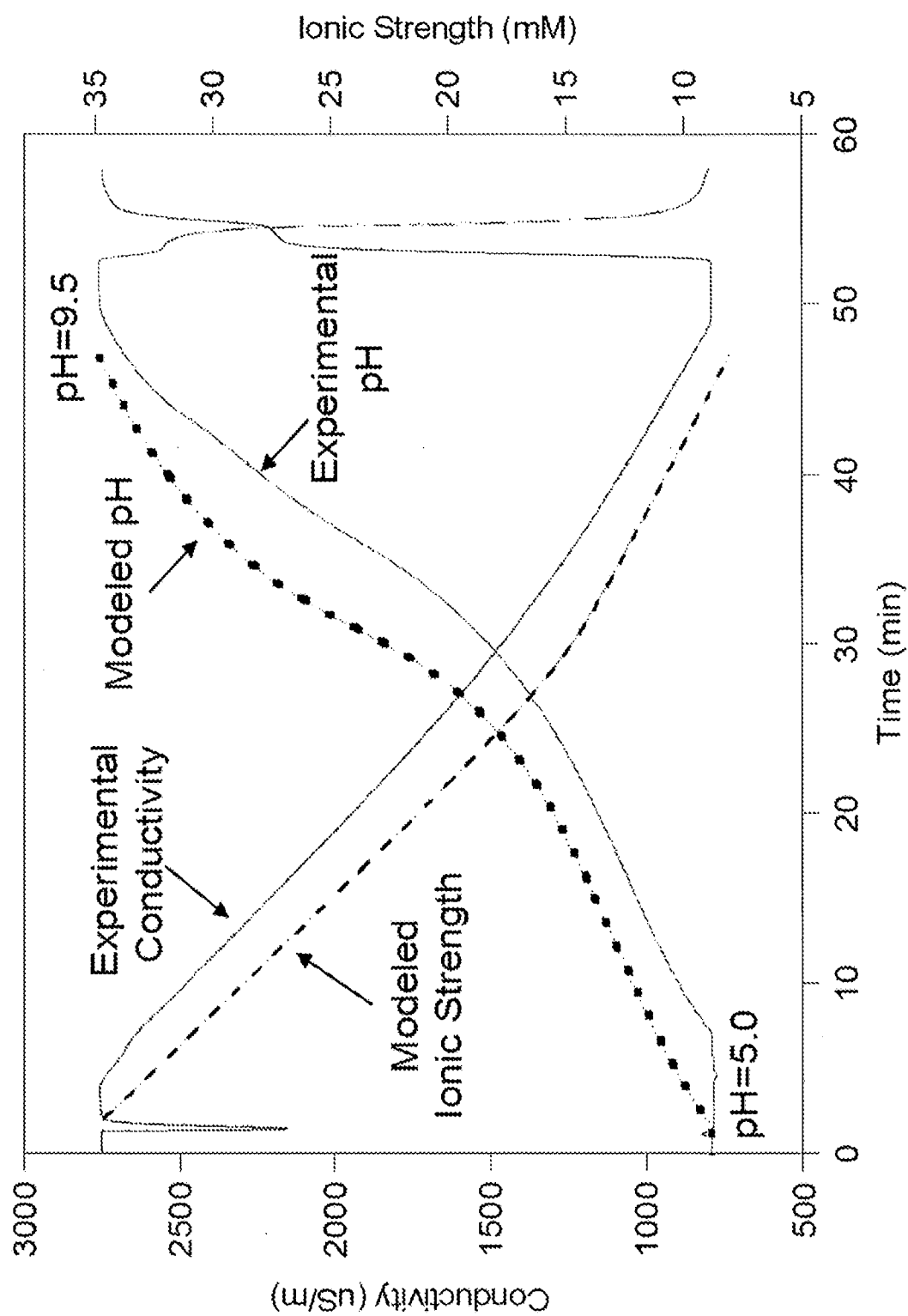


Figure 2B

B. before optimization

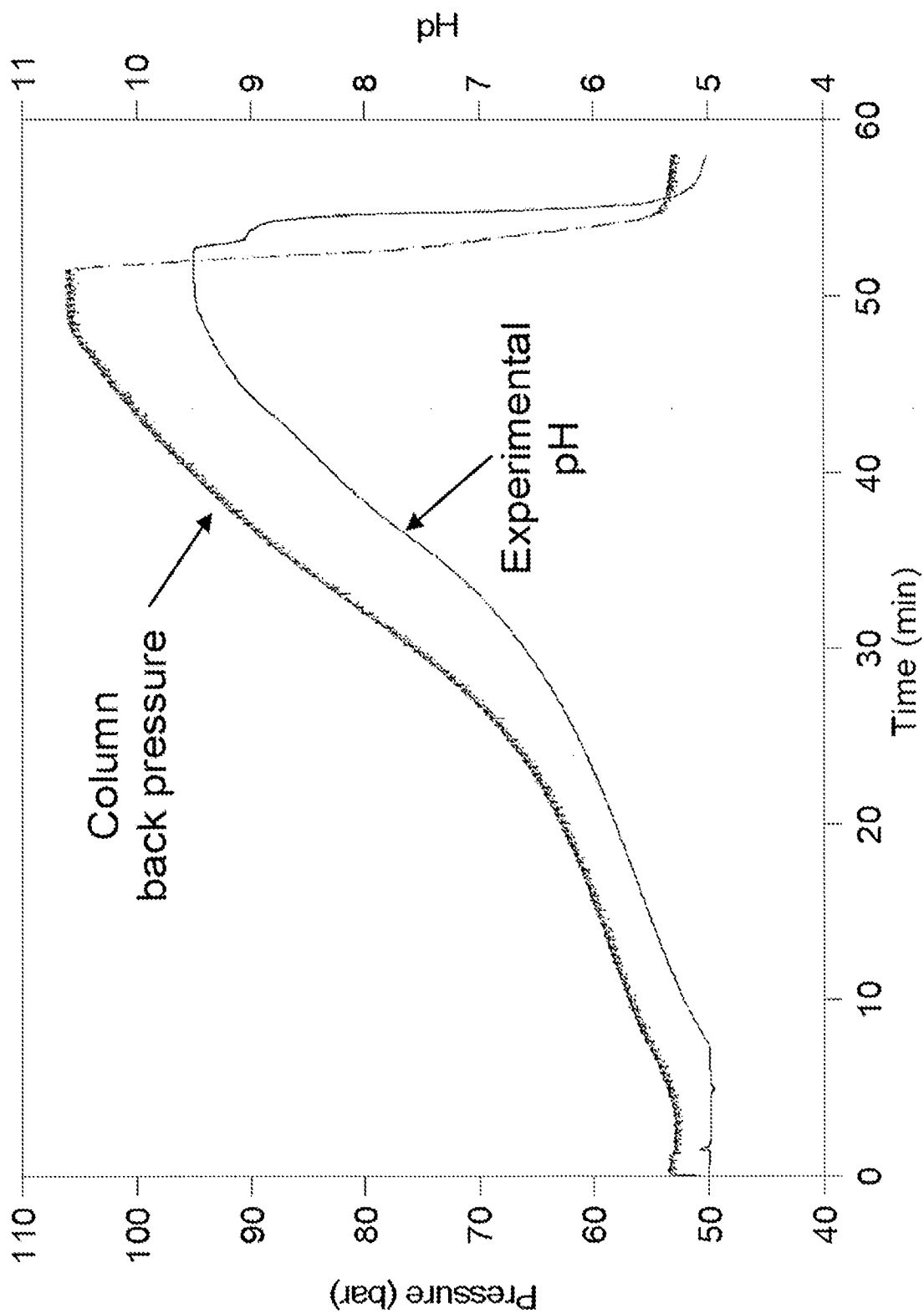


Figure 2

C. after optimization

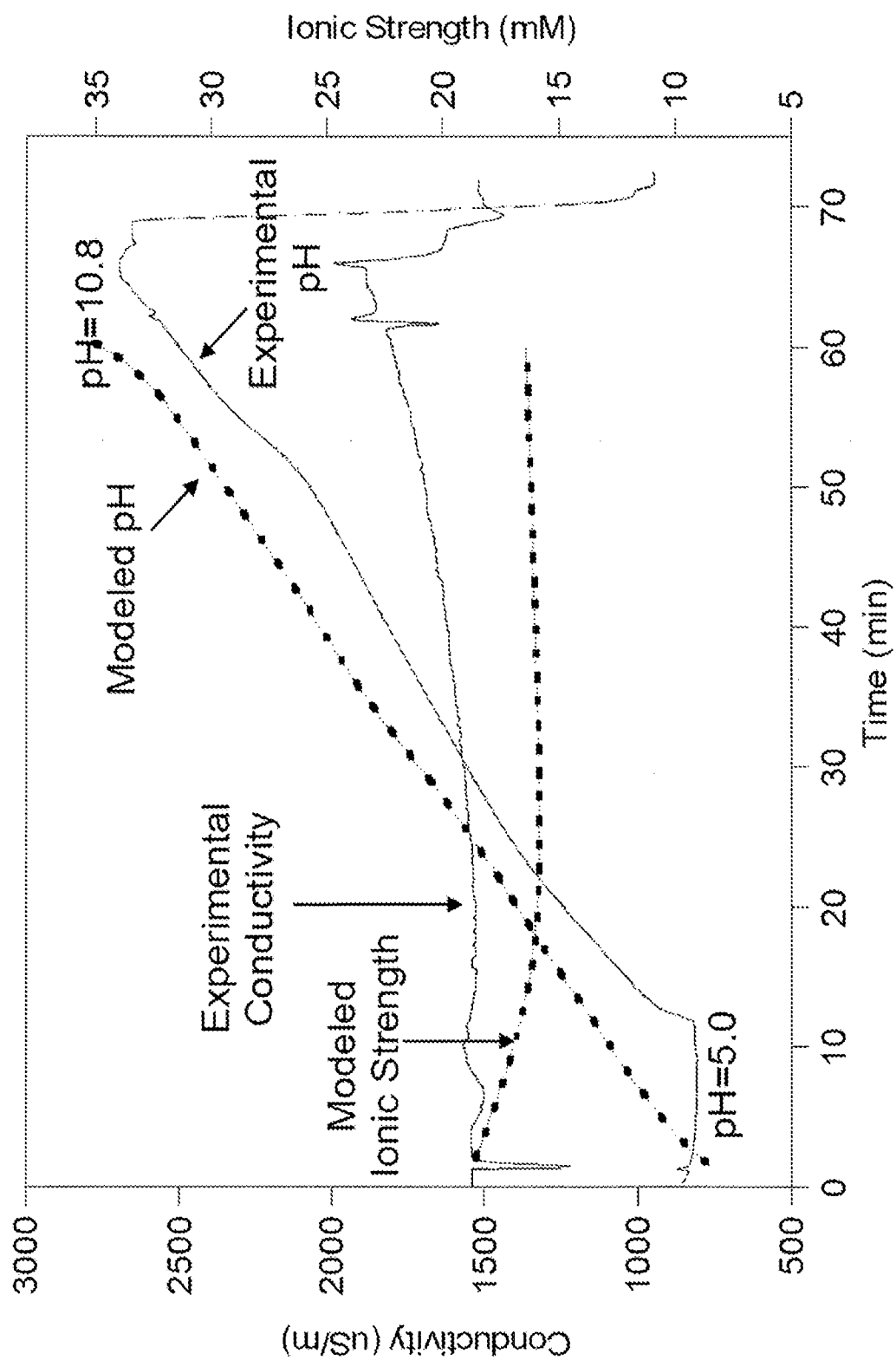


Figure 2

D. after optimization

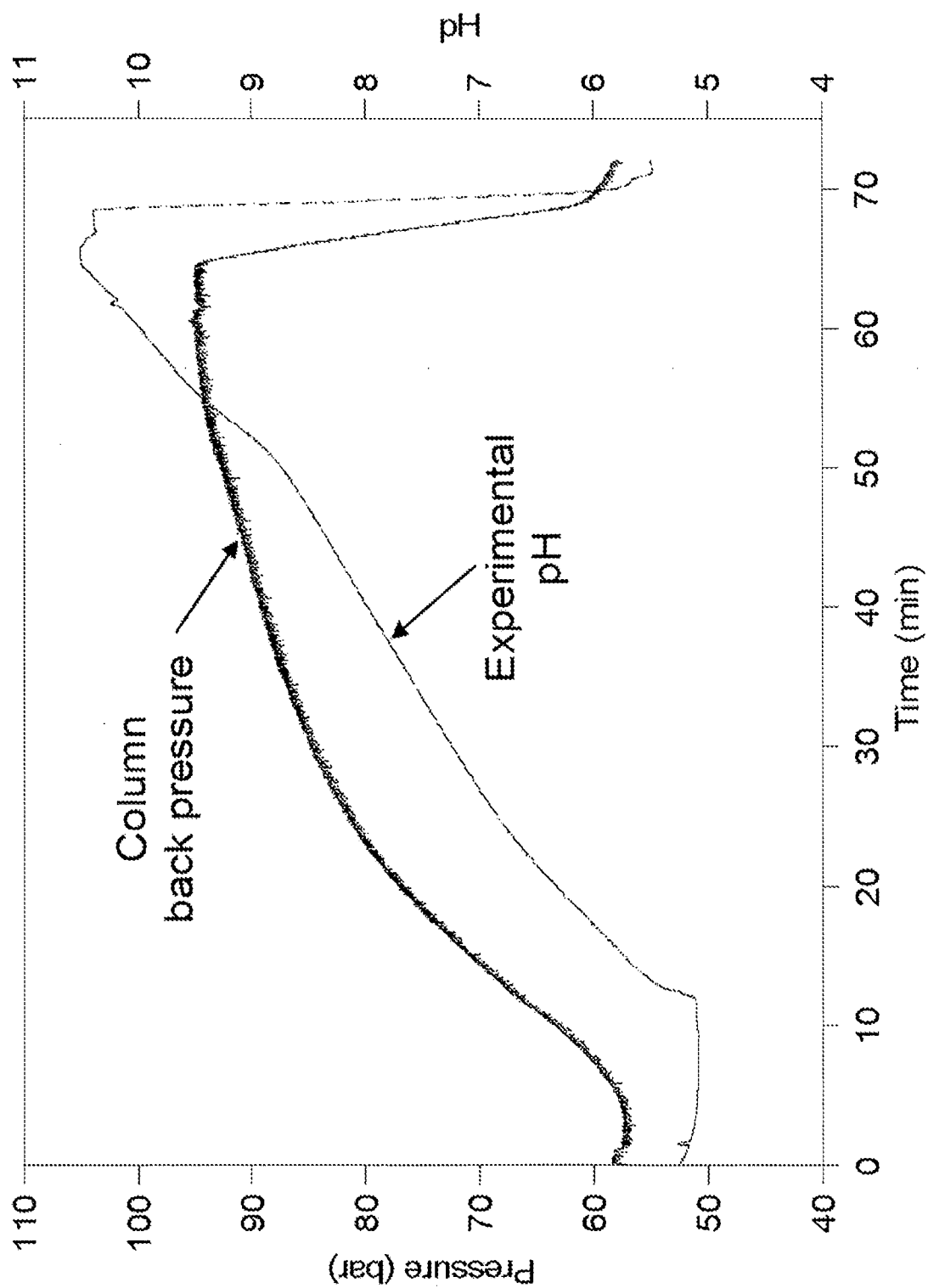
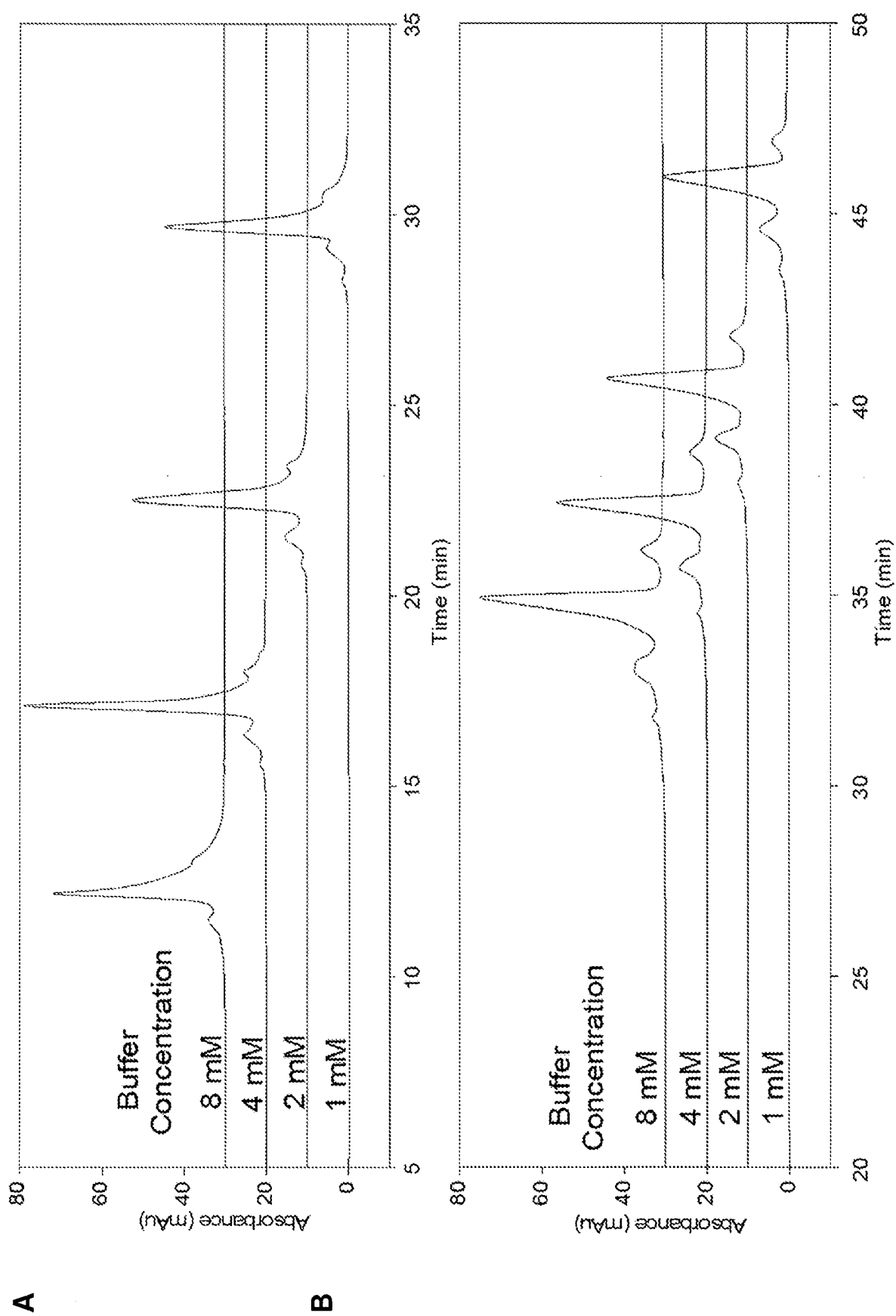
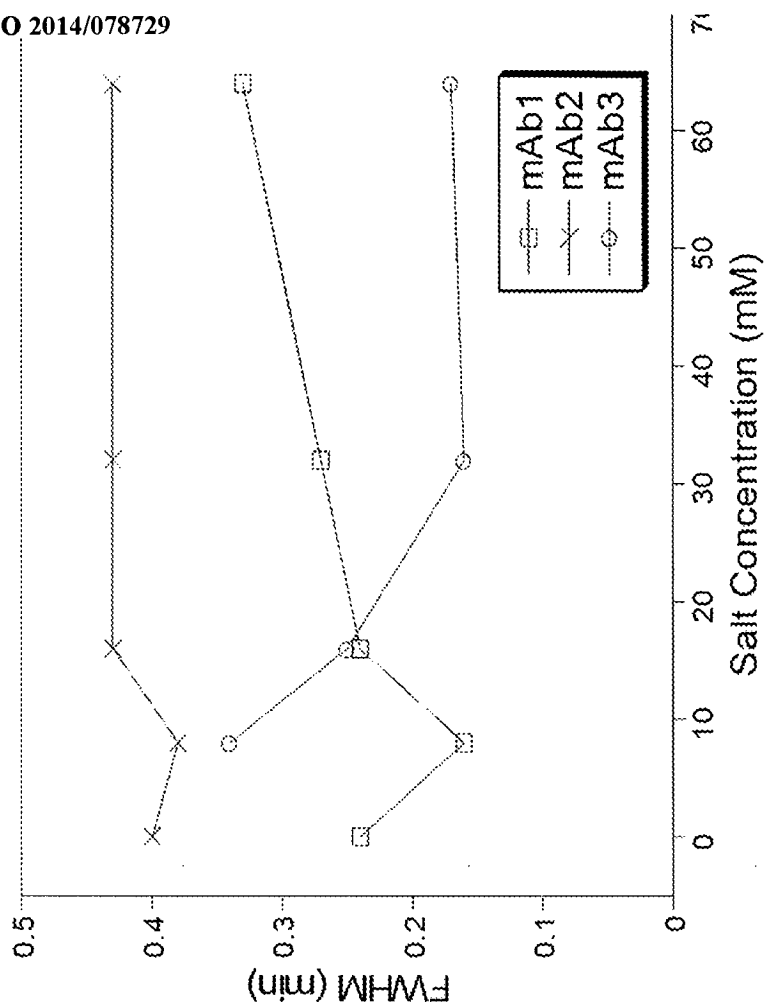


Figure 3



D



C

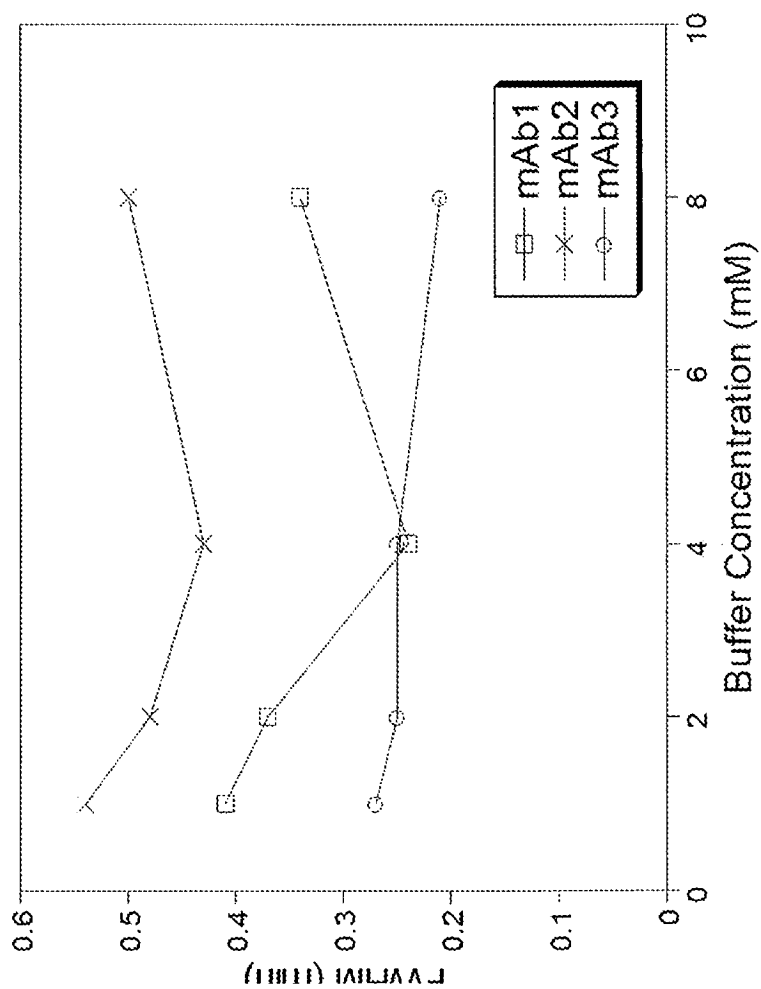
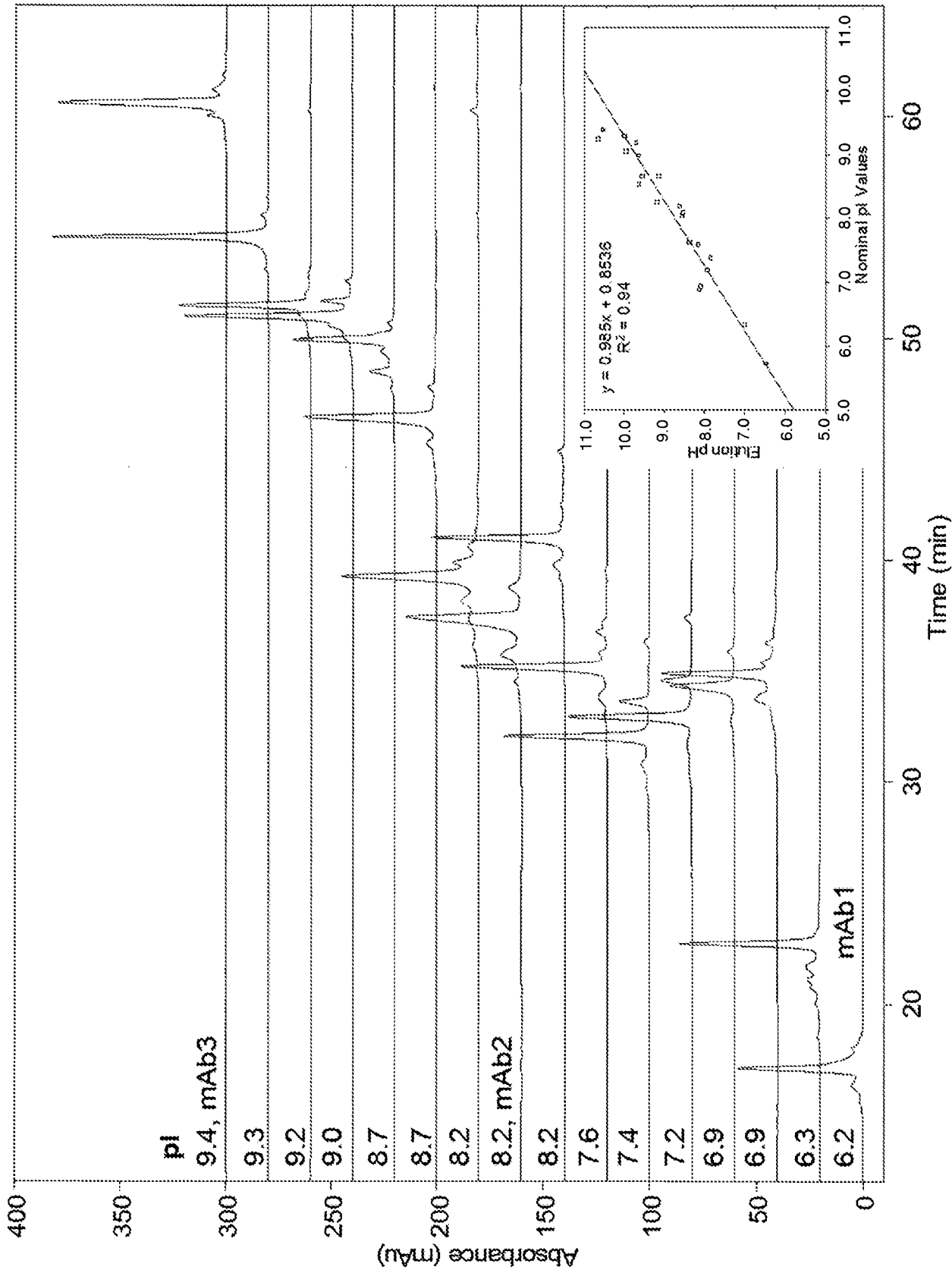


Figure 3

Figure 4



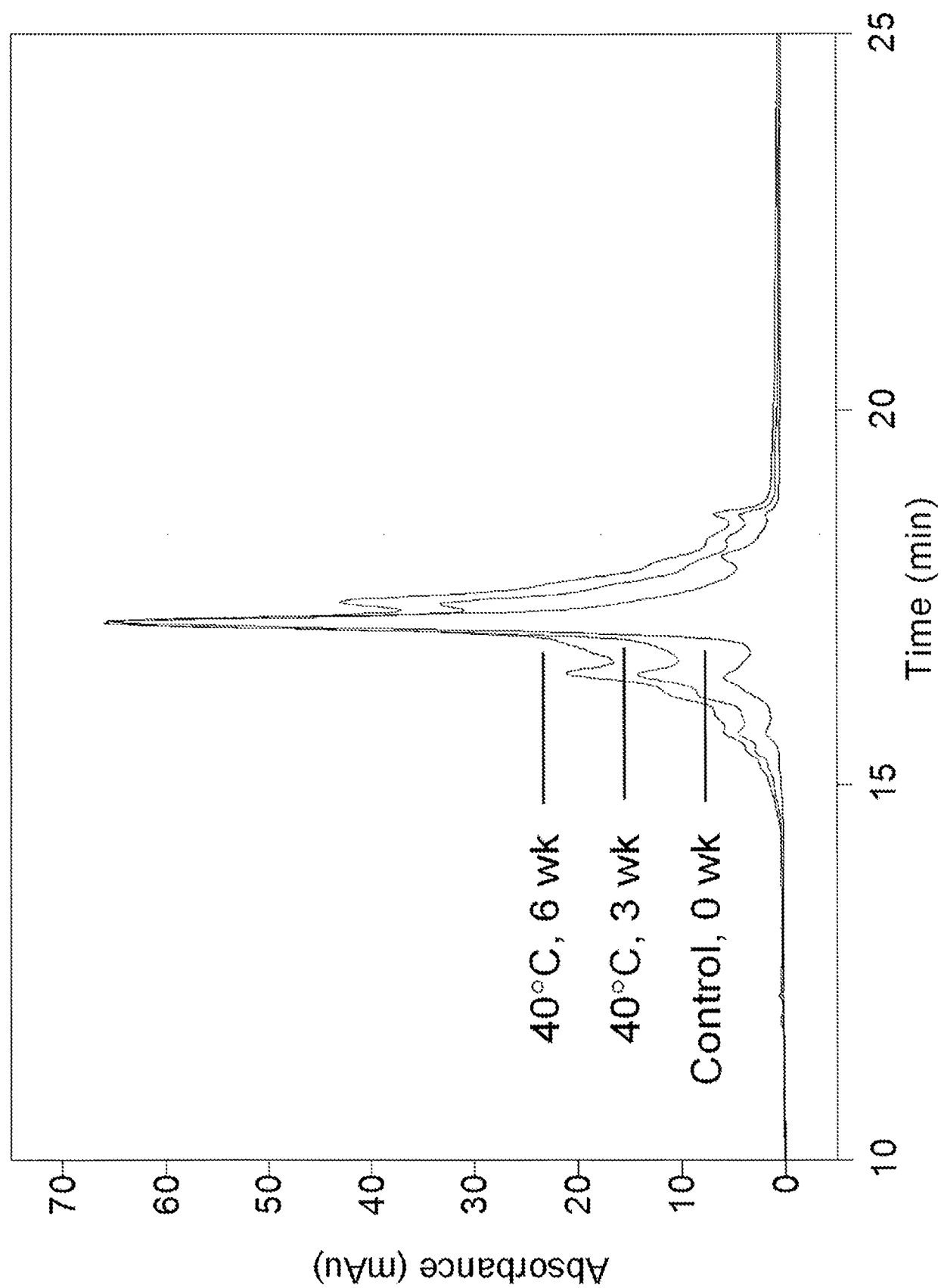


Figure 5

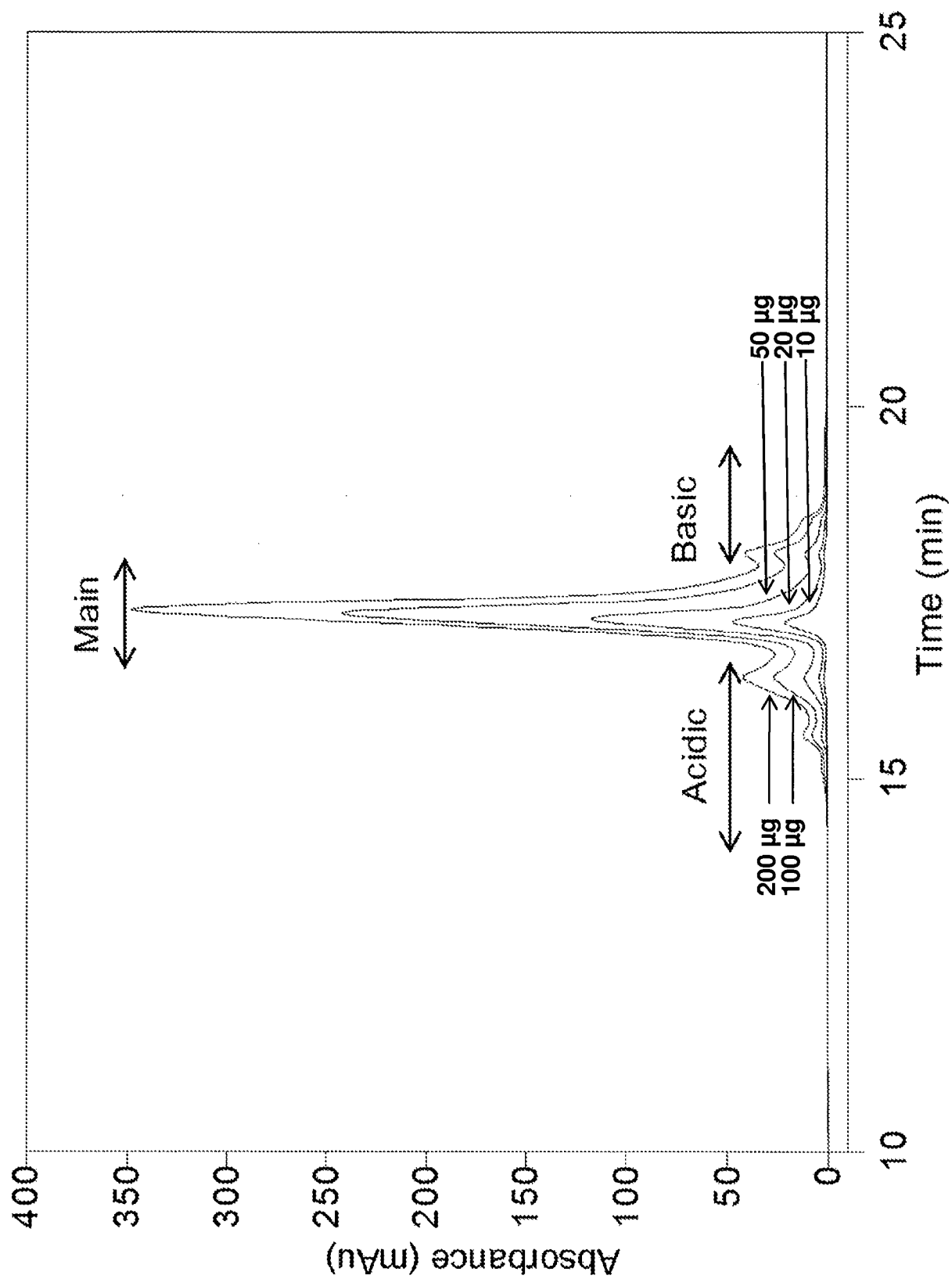
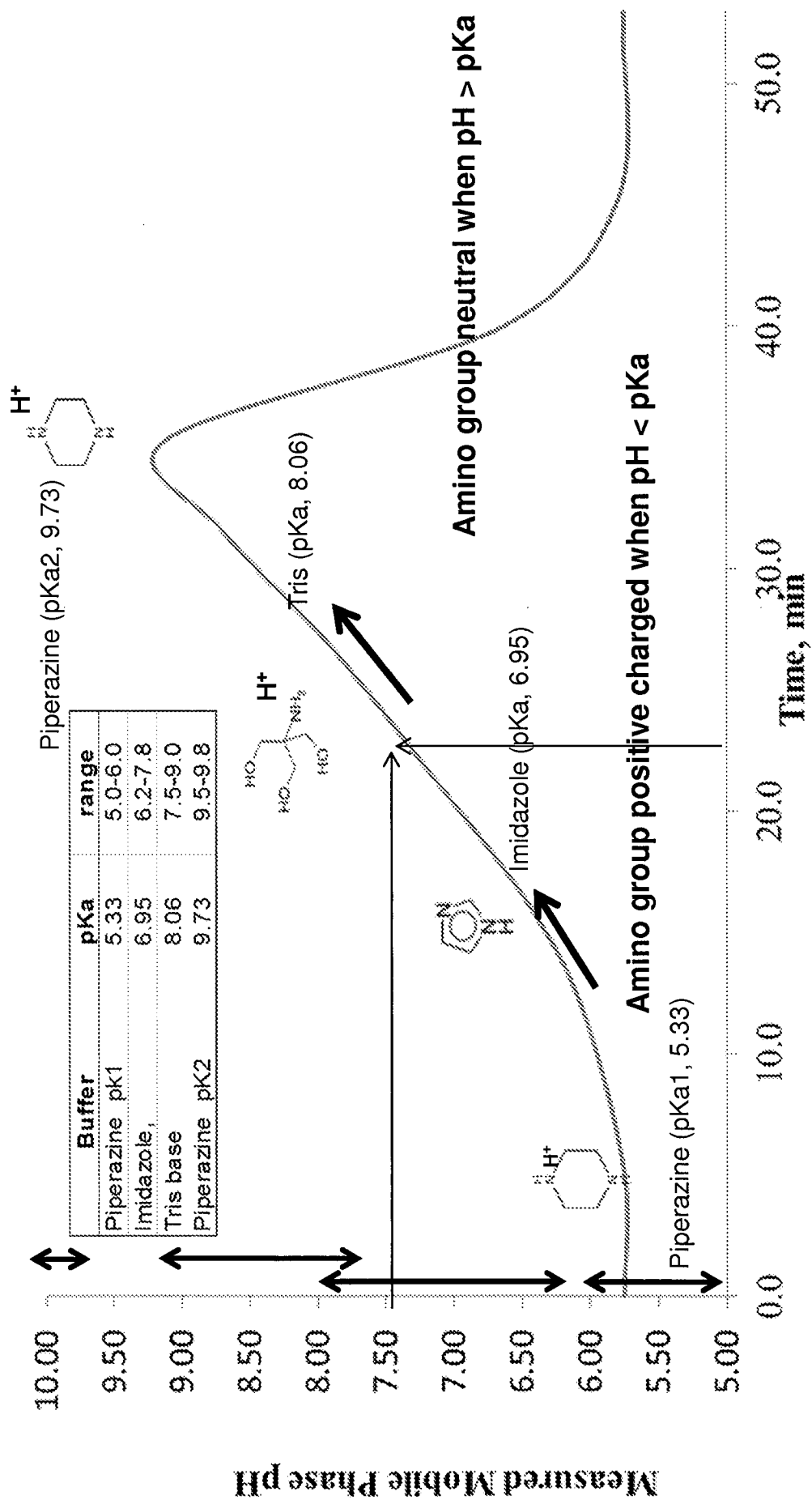


Figure 6

Figure 7



12/35

Figure 8

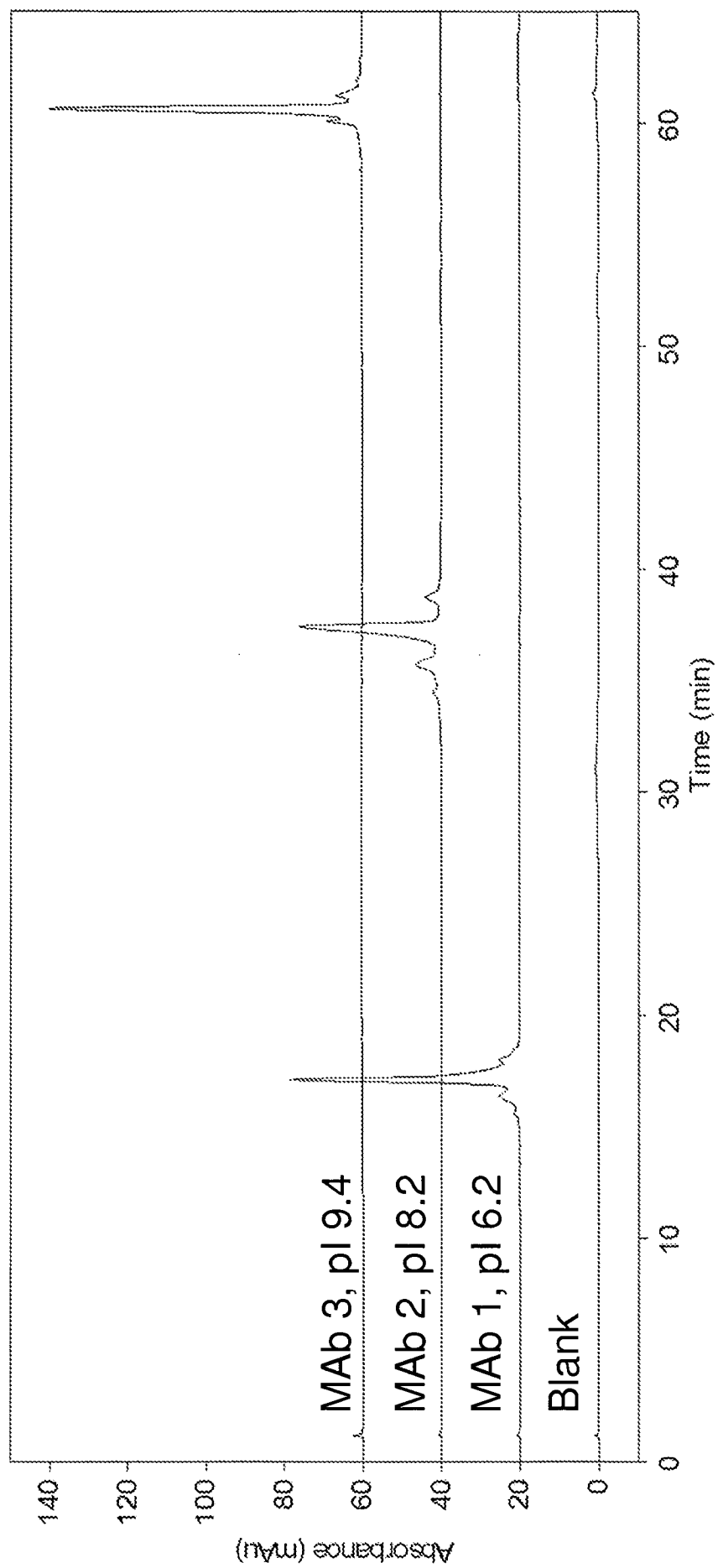


Figure 9

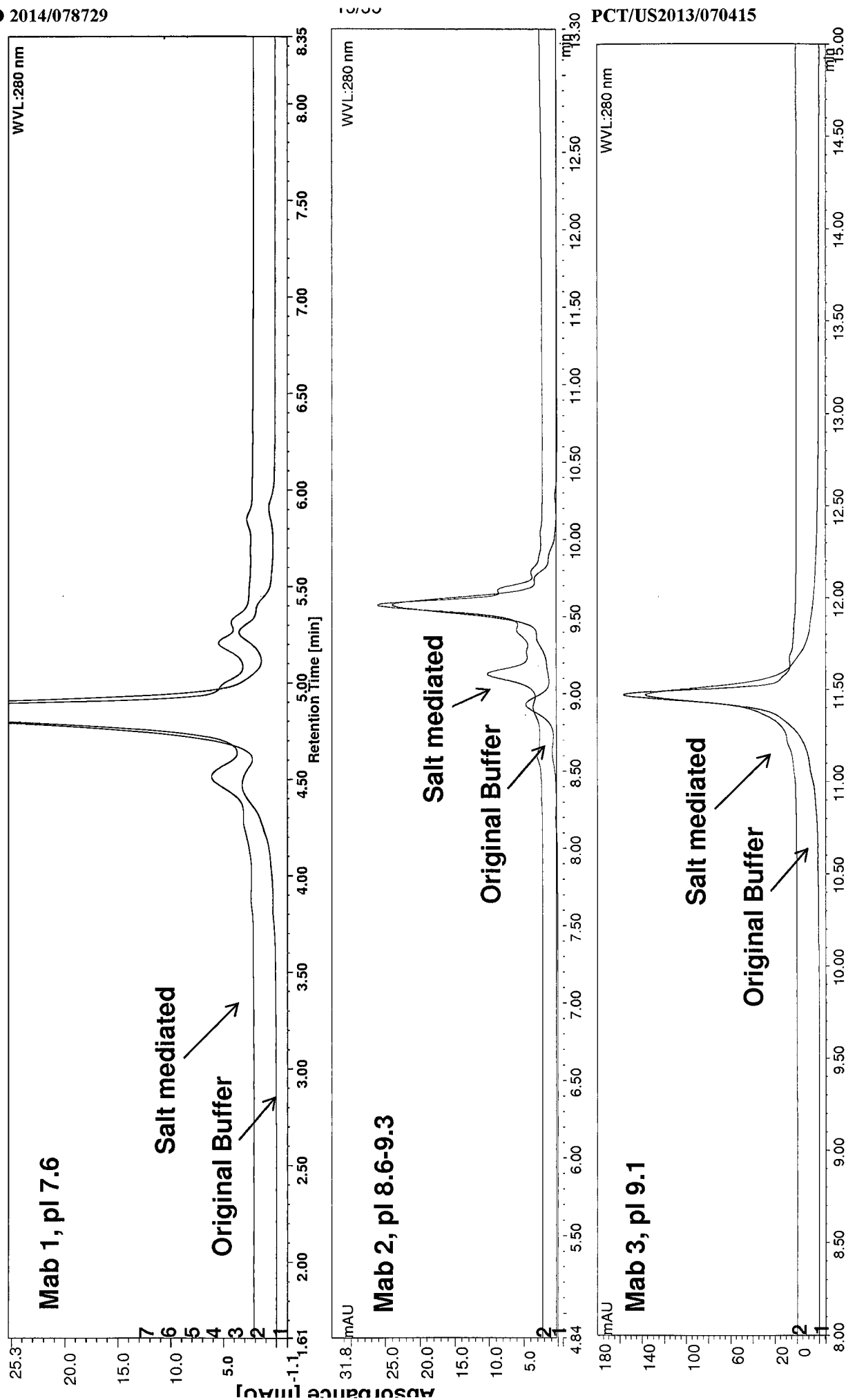
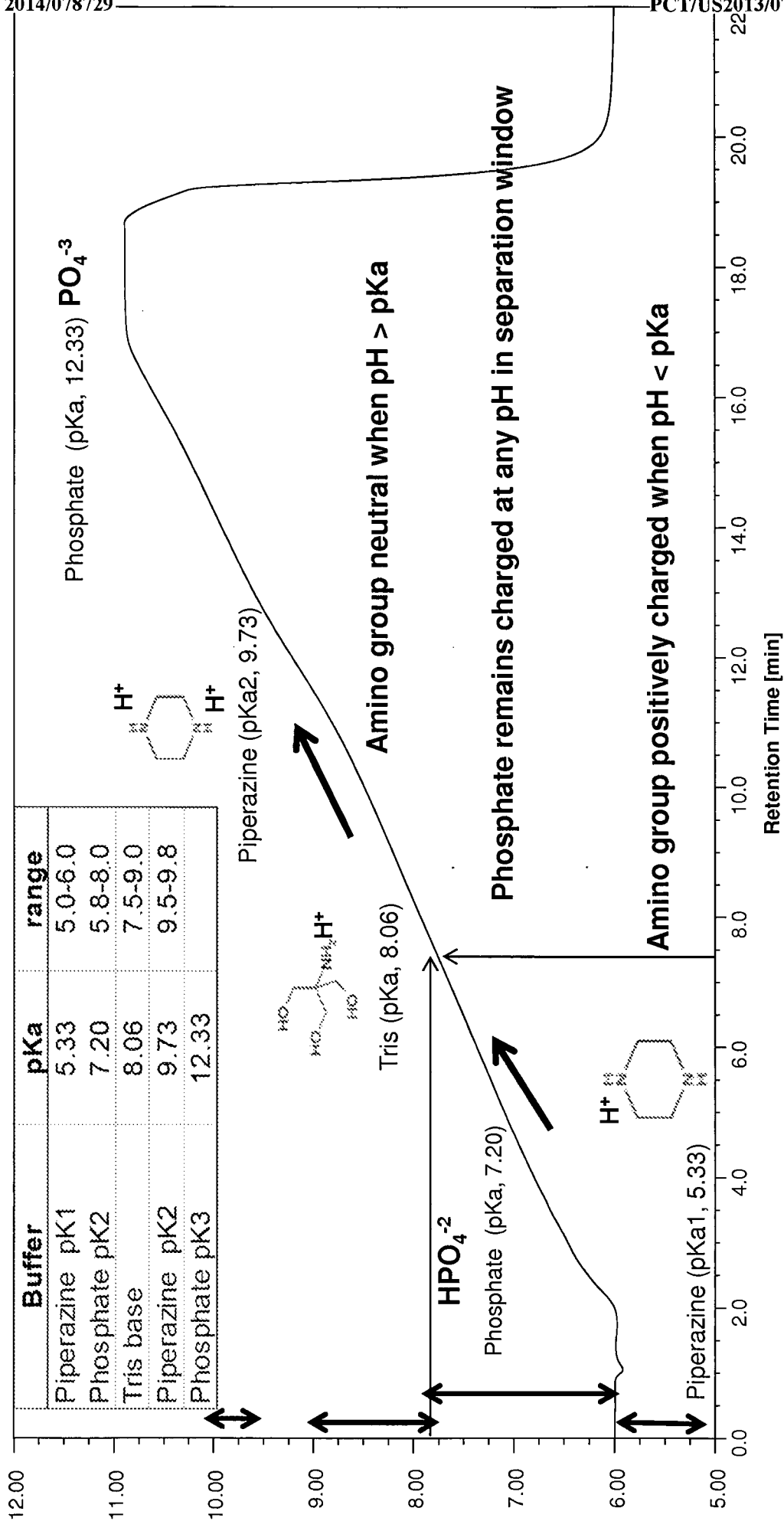


Figure 10



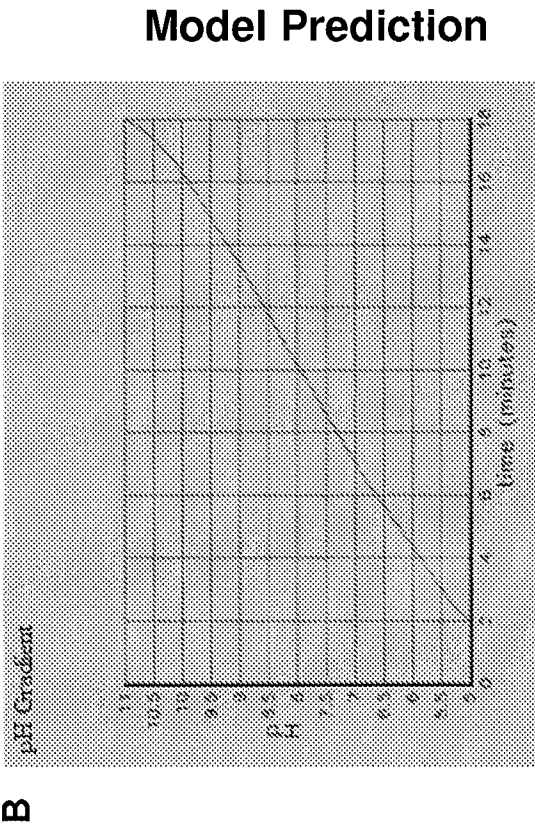
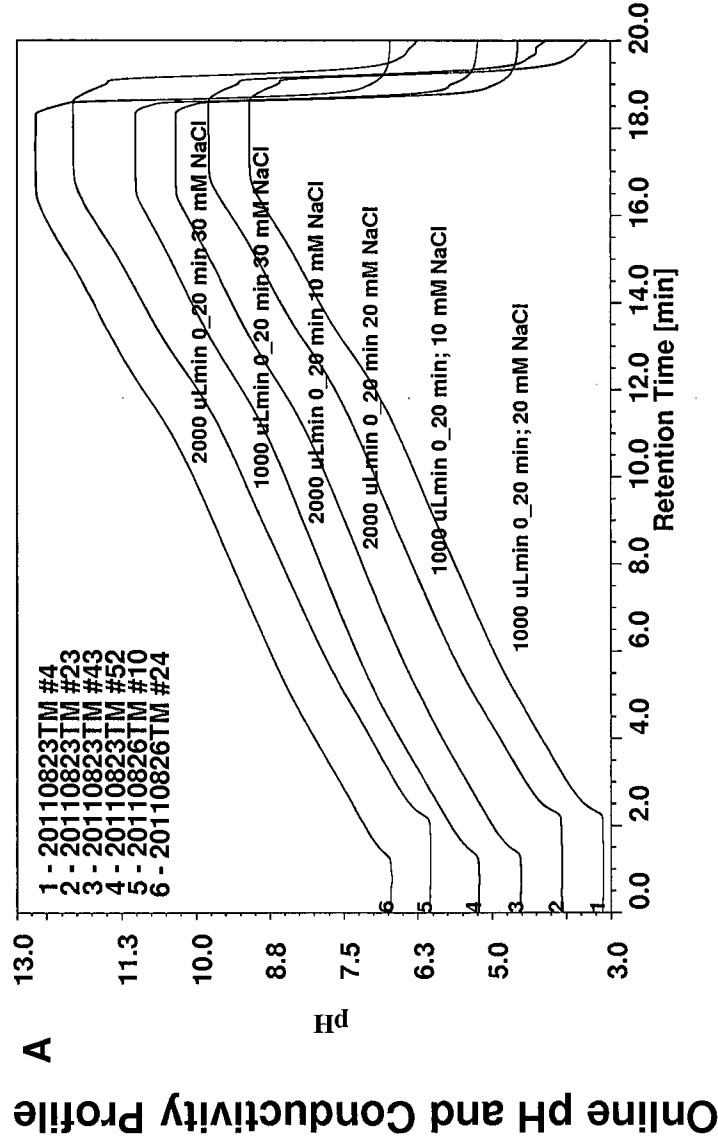
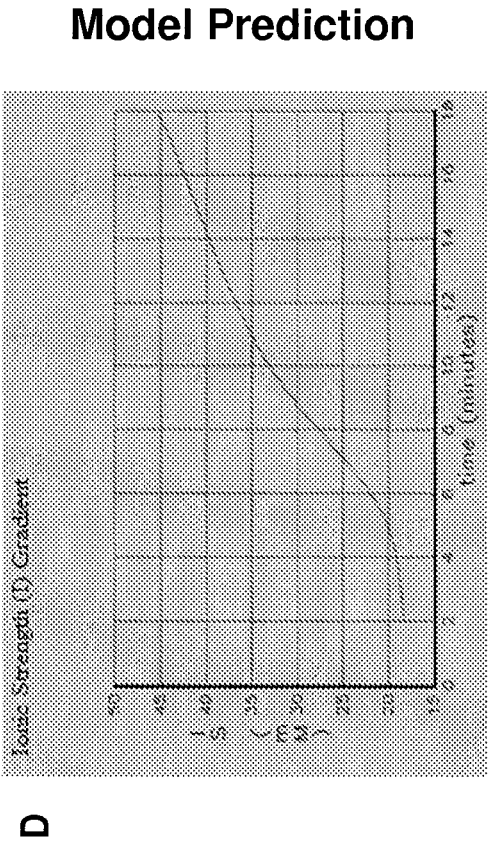
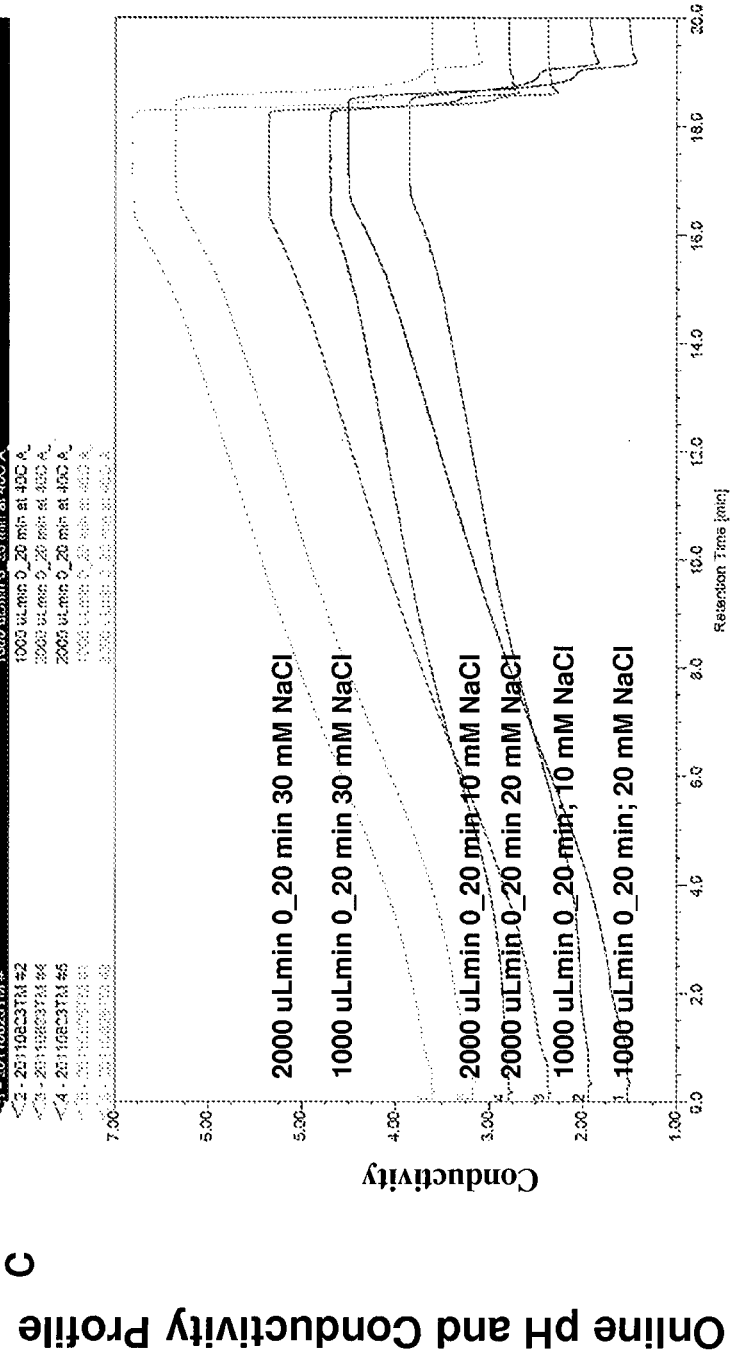


Figure 11

Figure 11



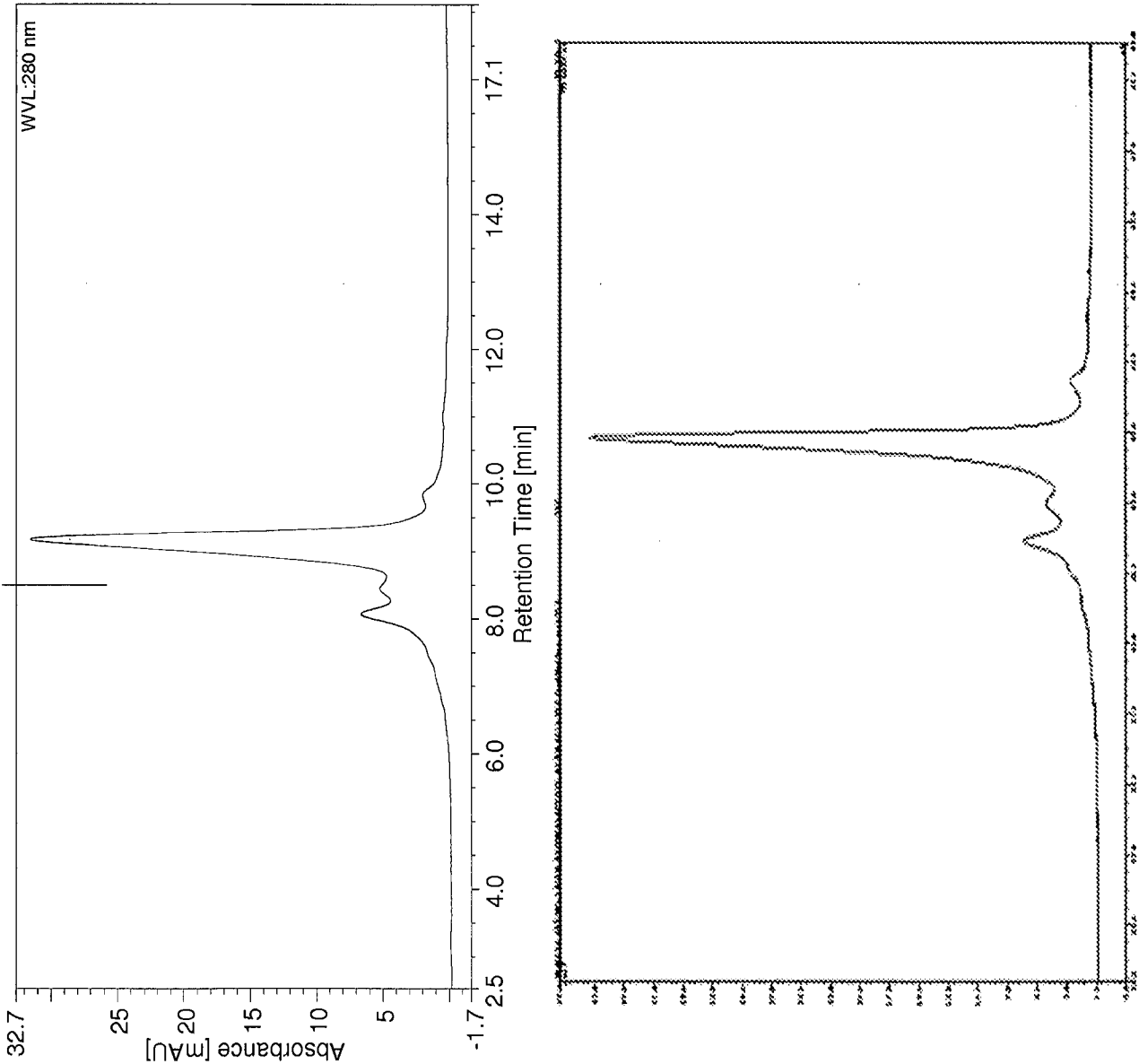


Figure 12

Figure 13

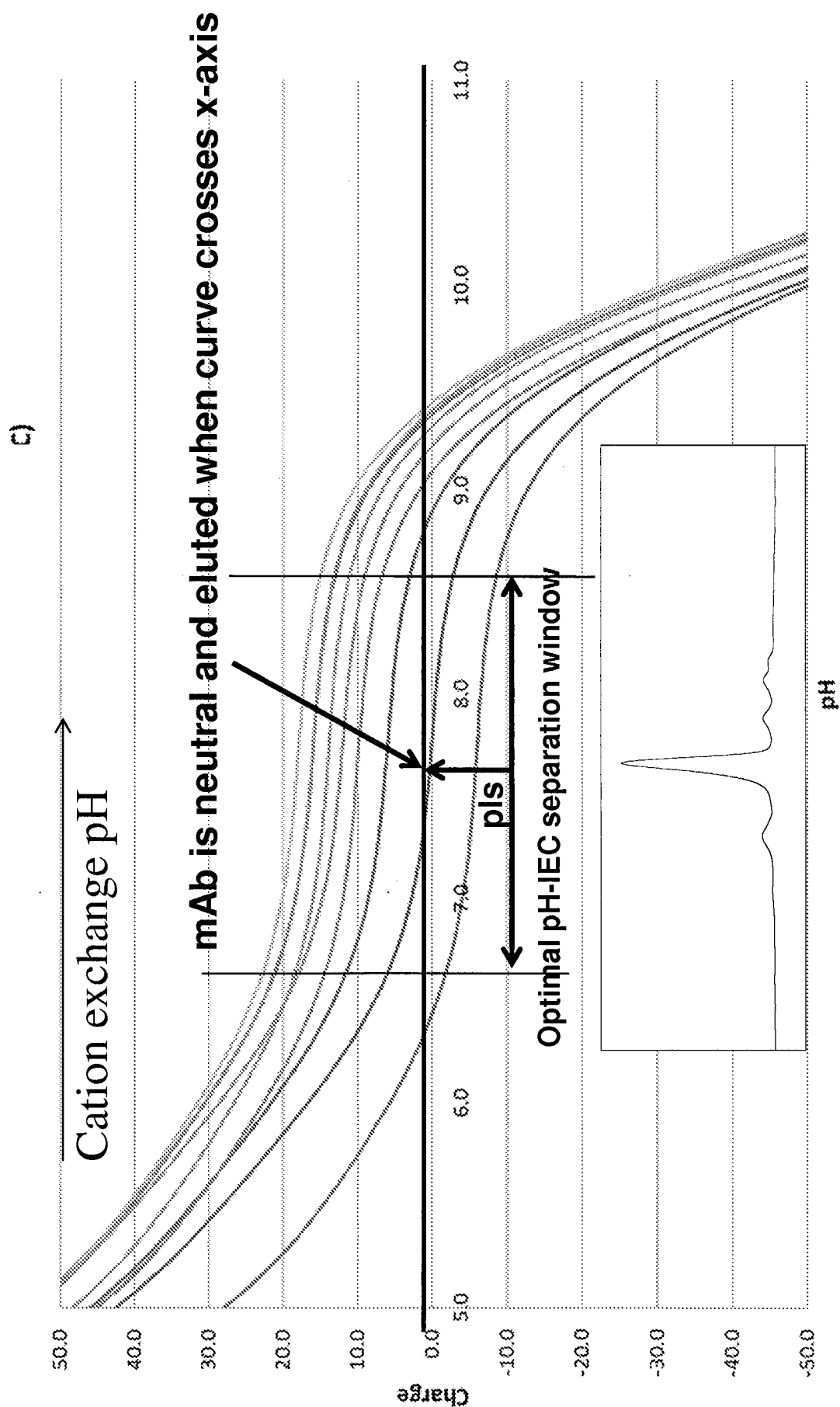


Figure 14

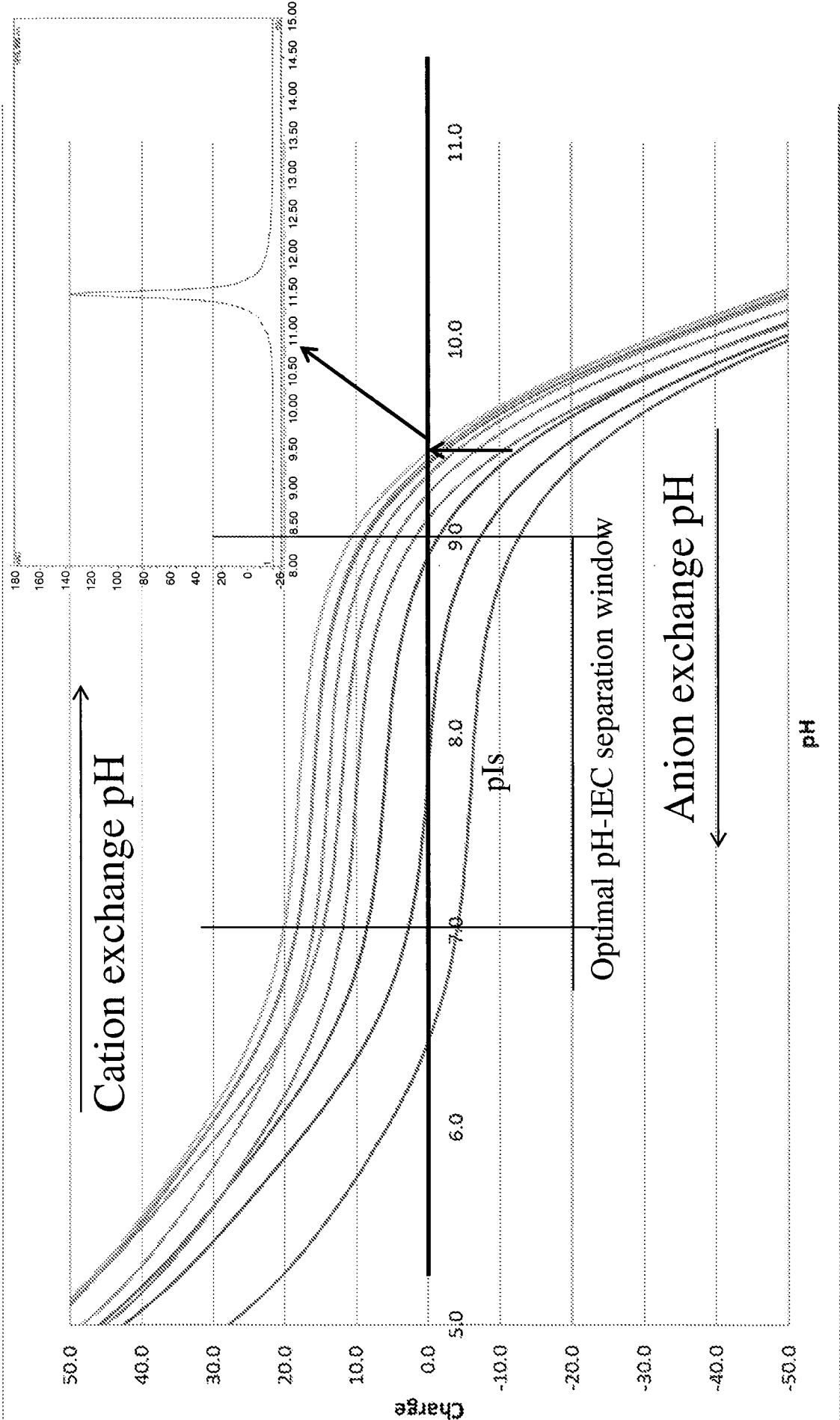


Figure 15

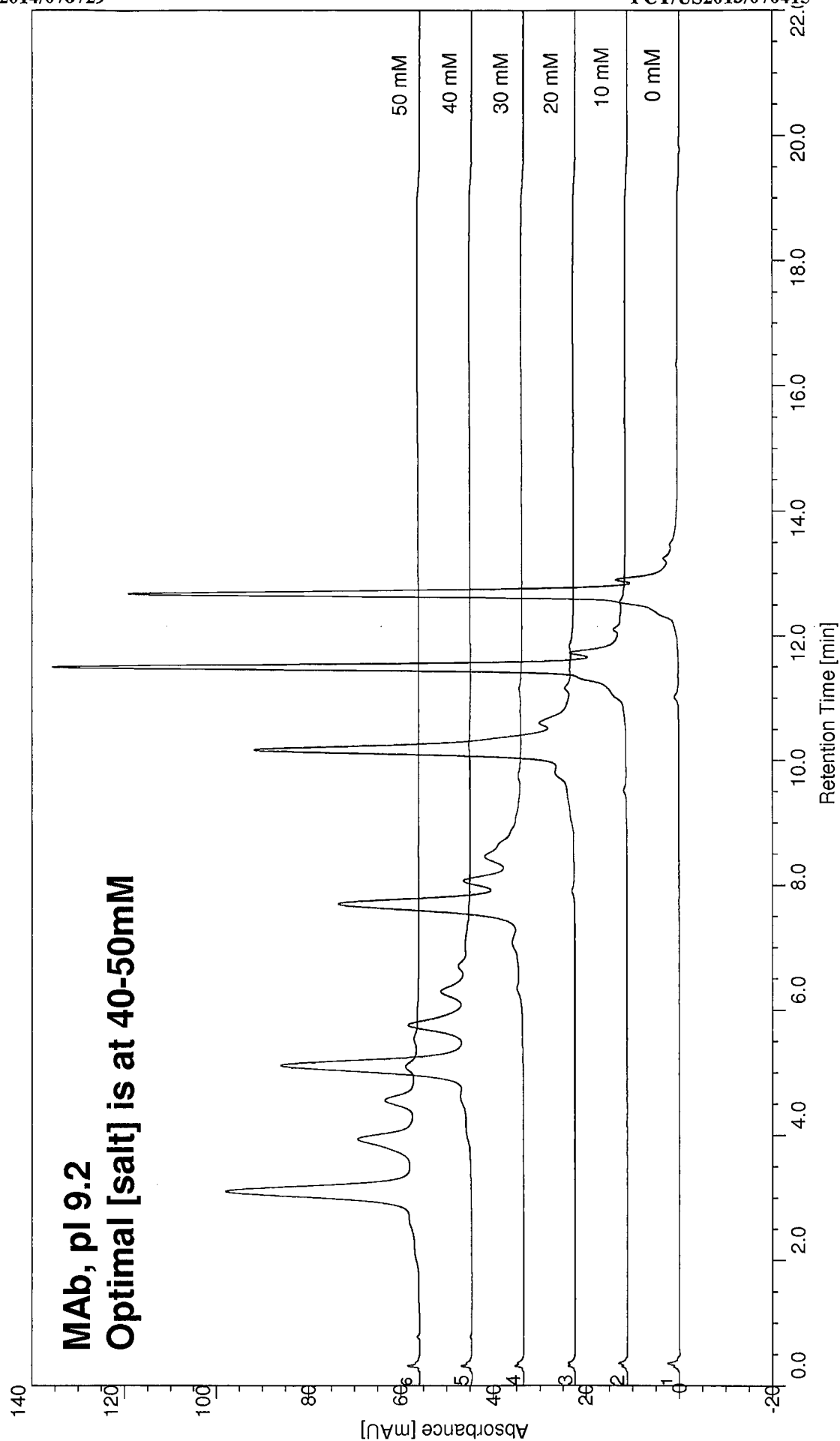


Figure 16

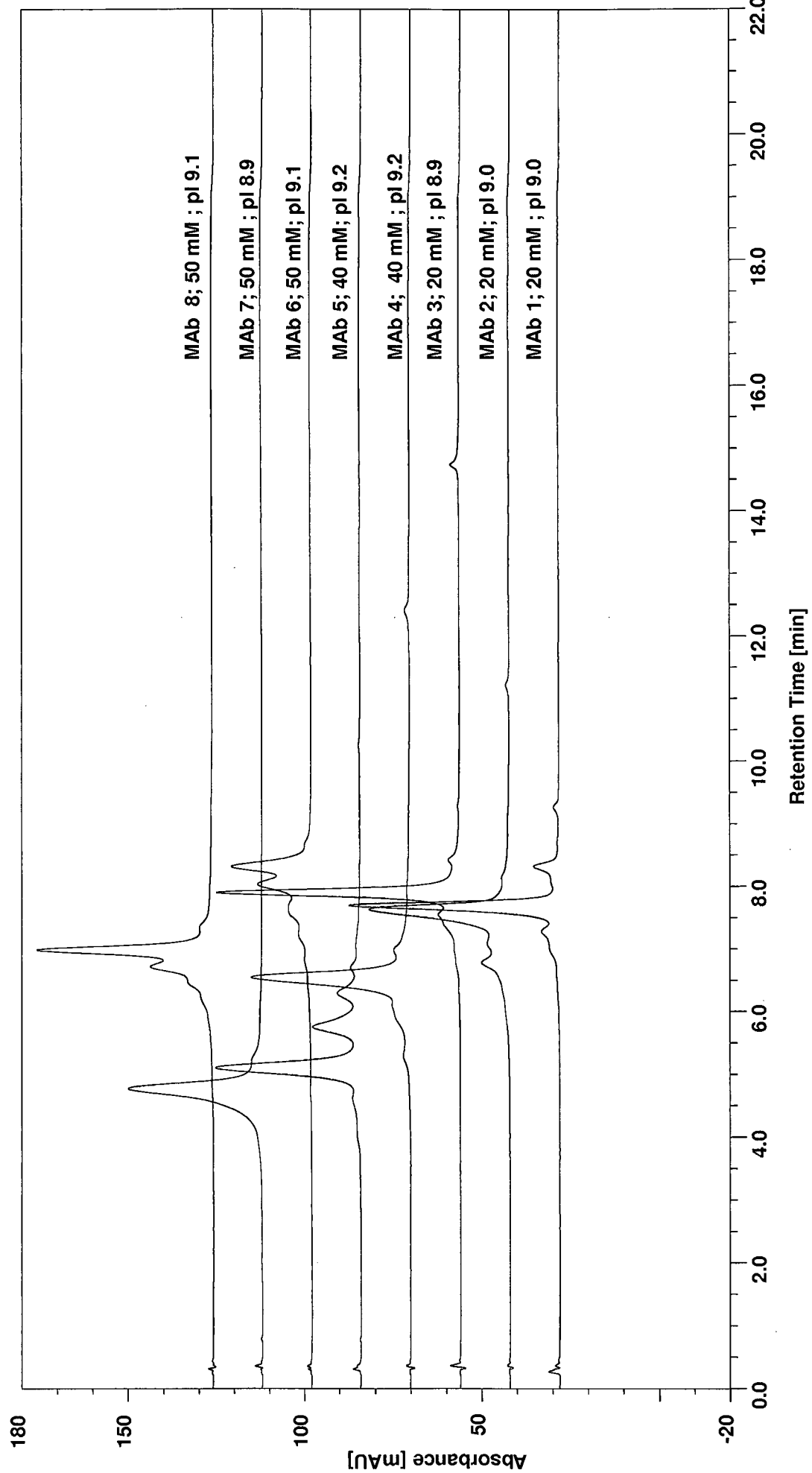


Figure 17

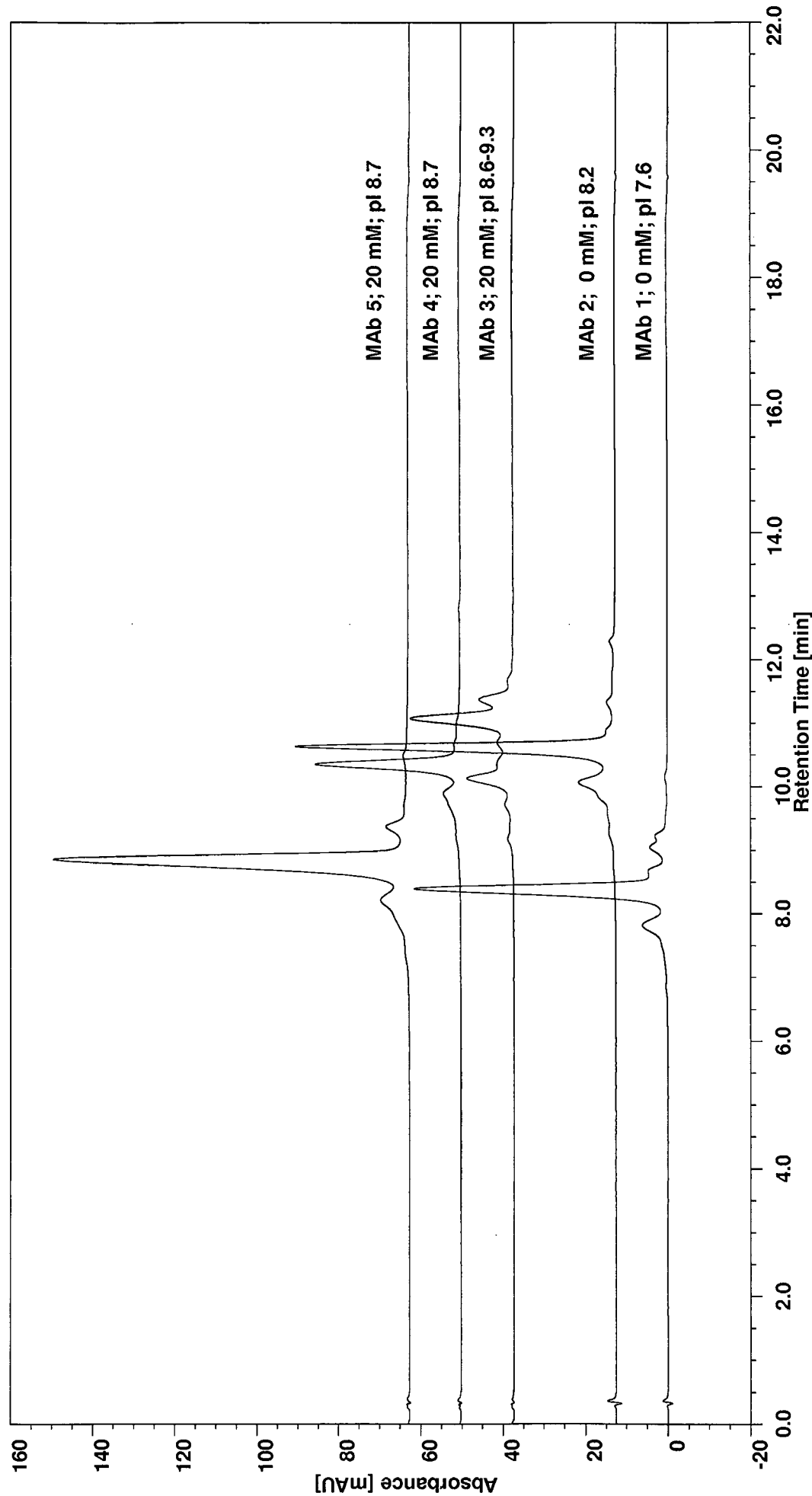


Figure 18

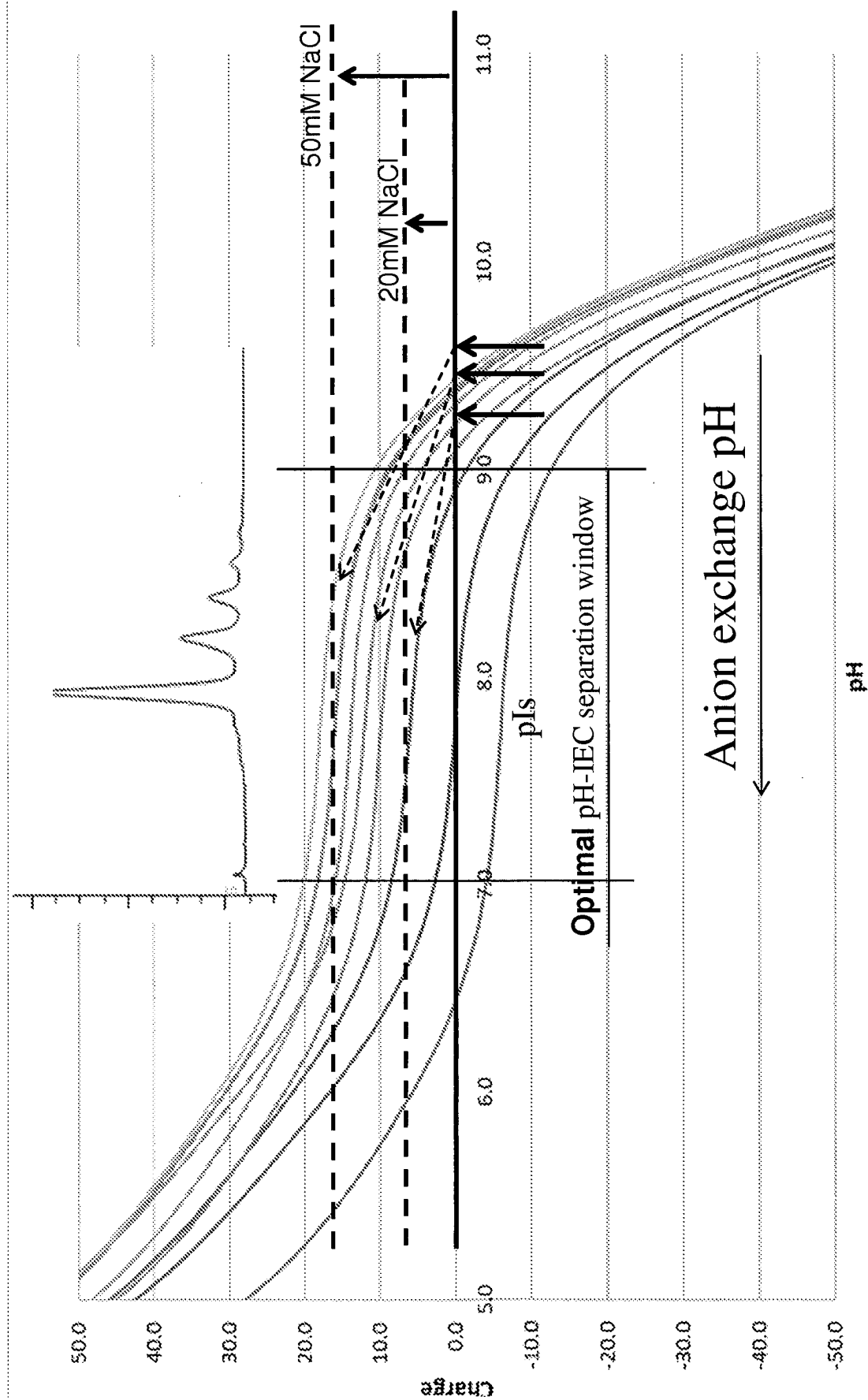


Figure 19

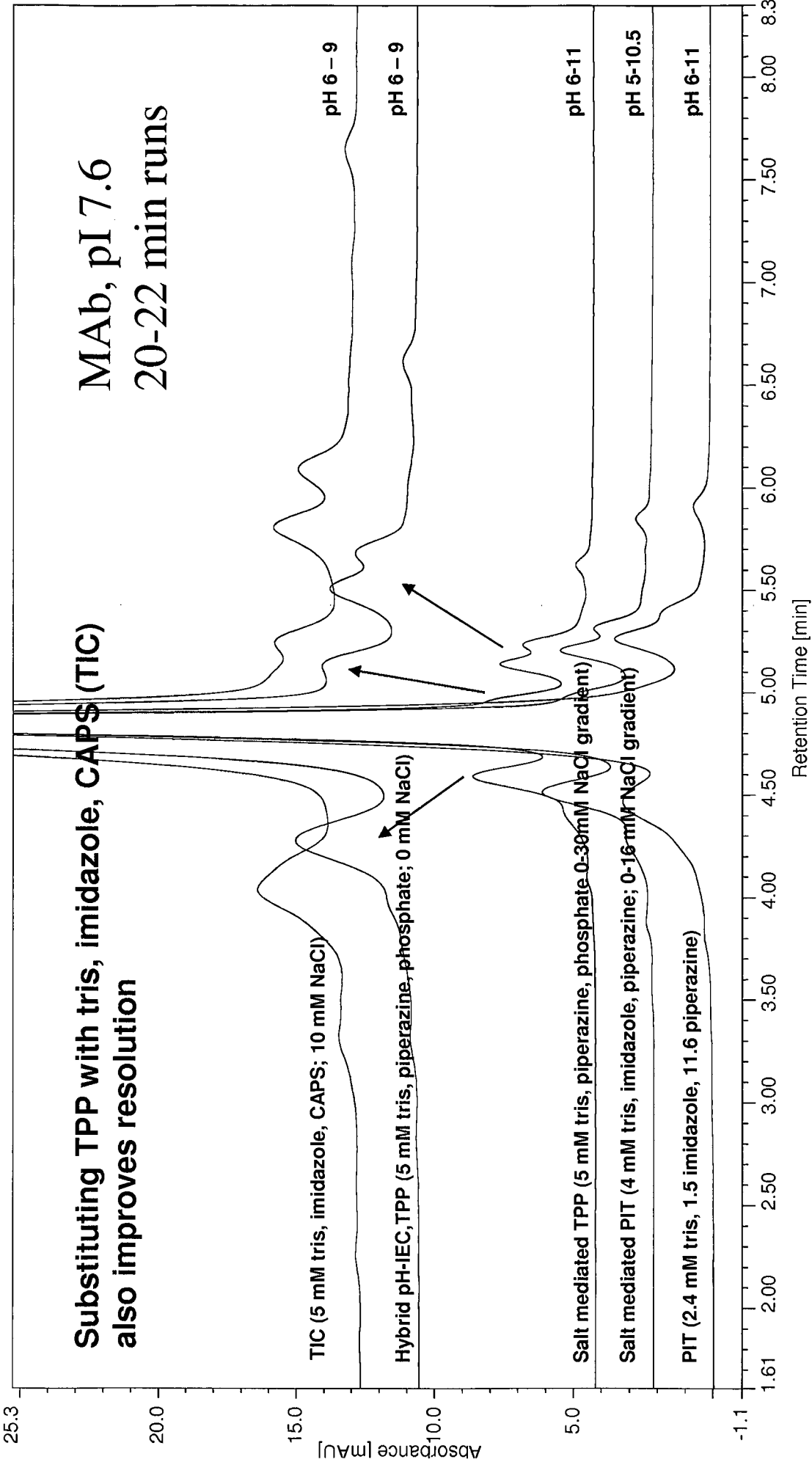


Figure 20

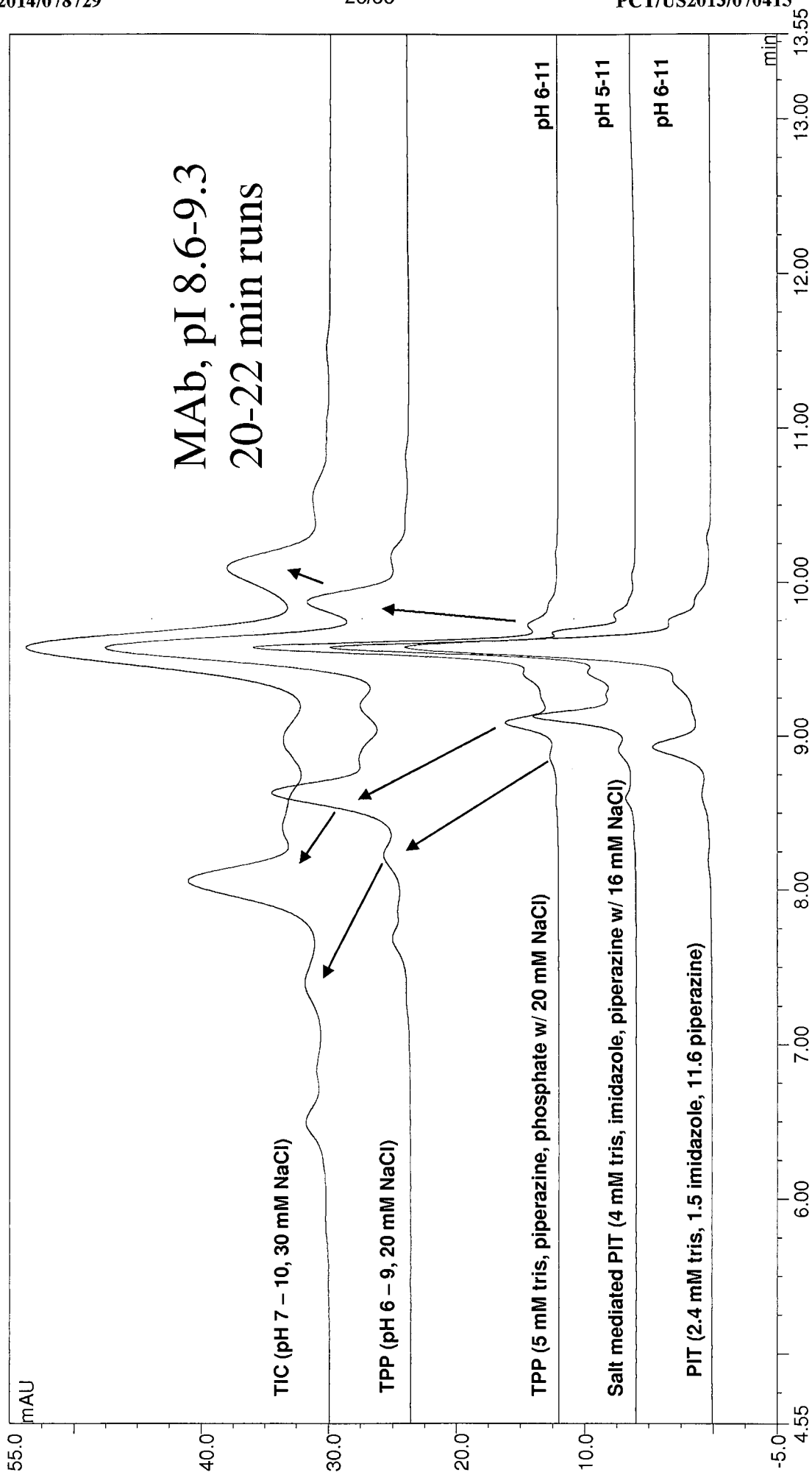


Figure 21

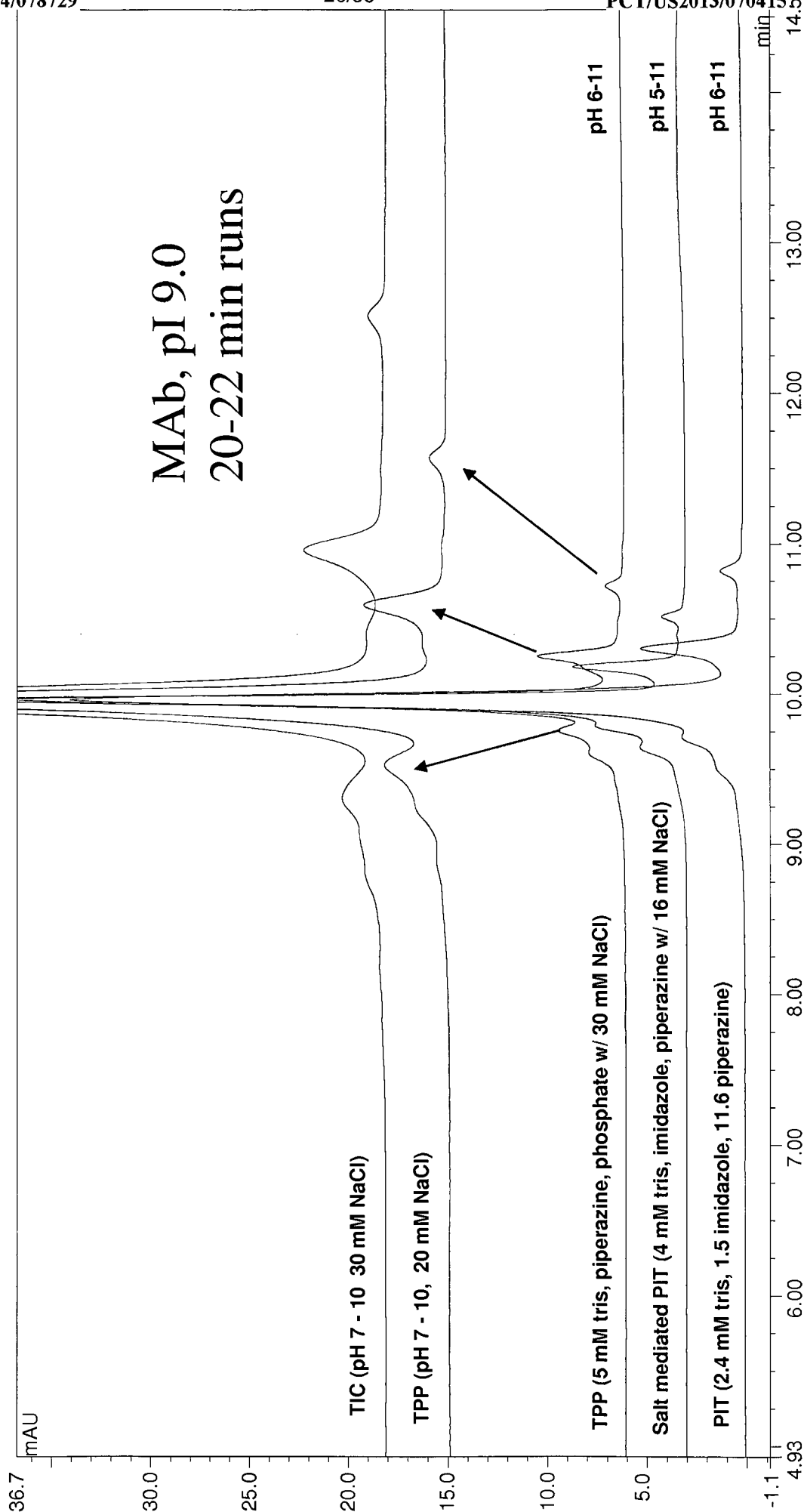


Figure 22

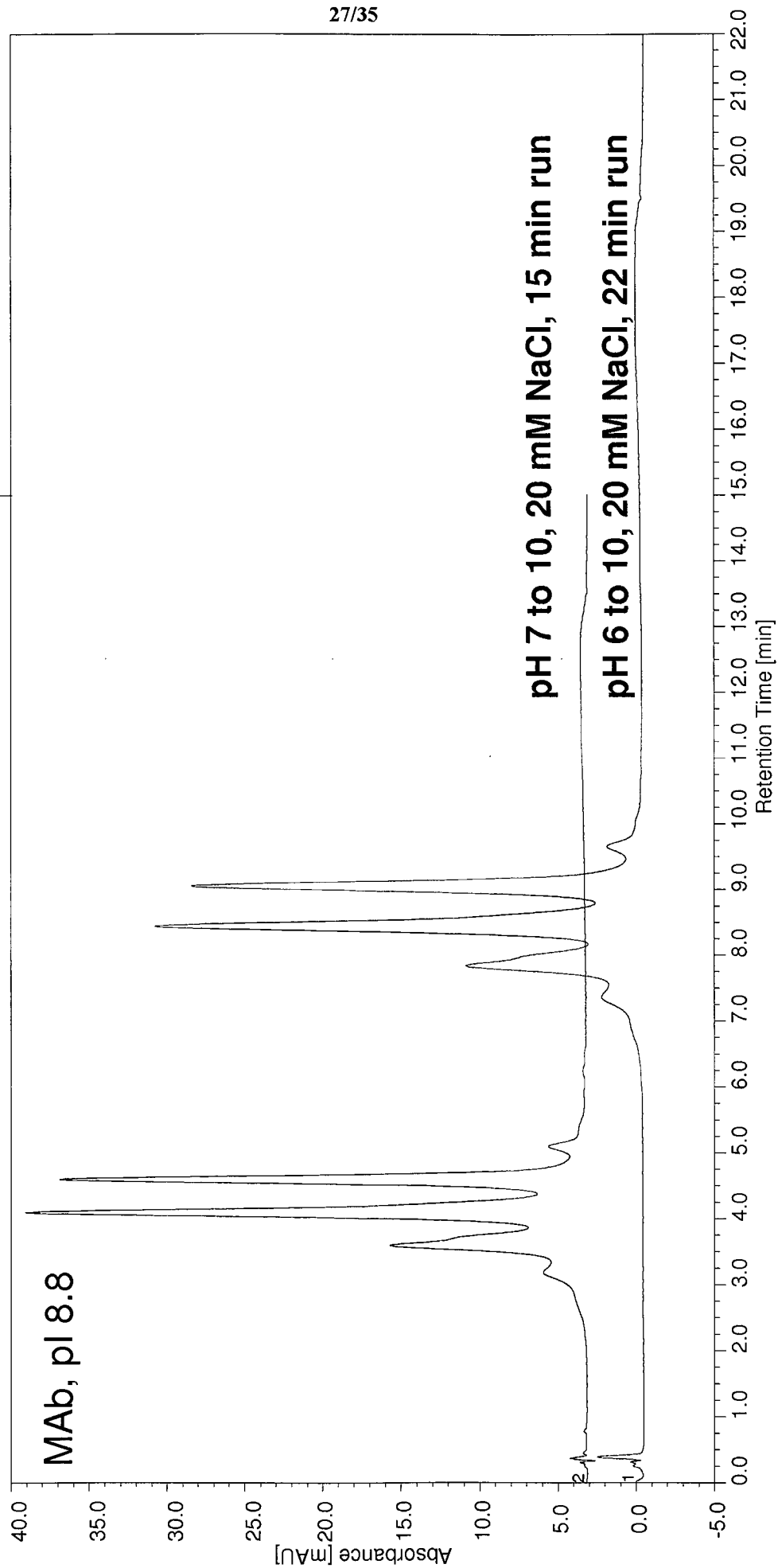


Figure 23

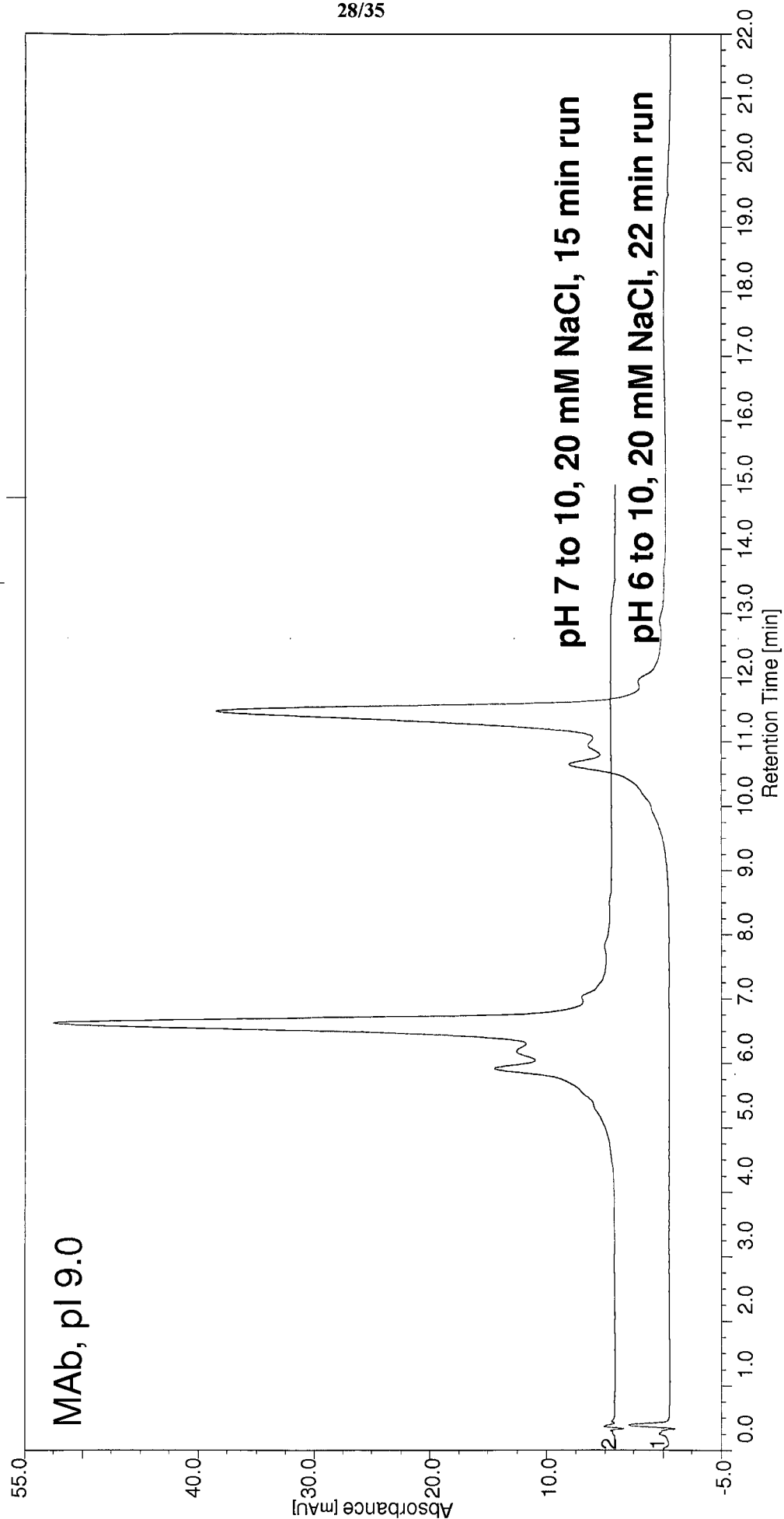


Figure 24

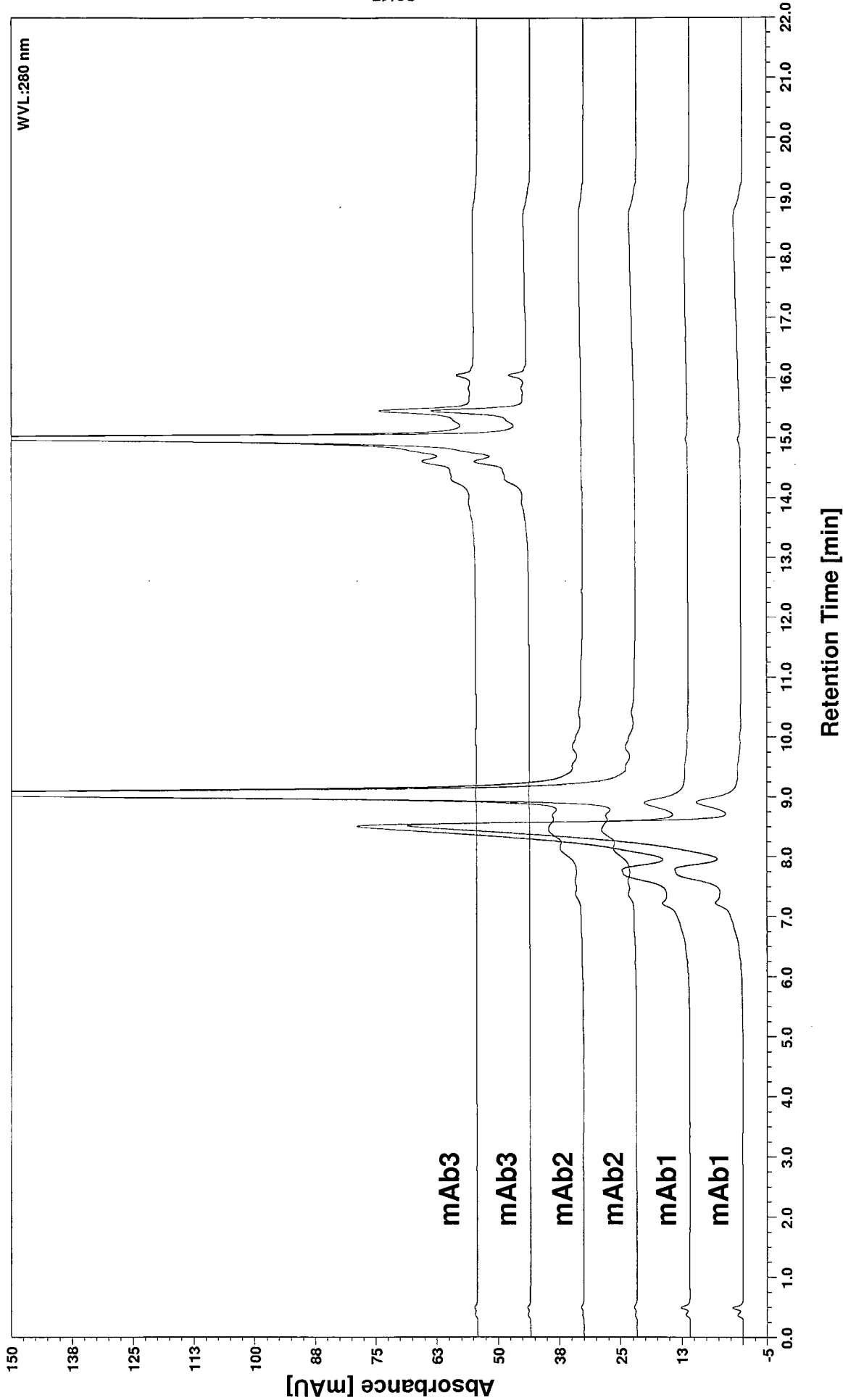


Figure 25

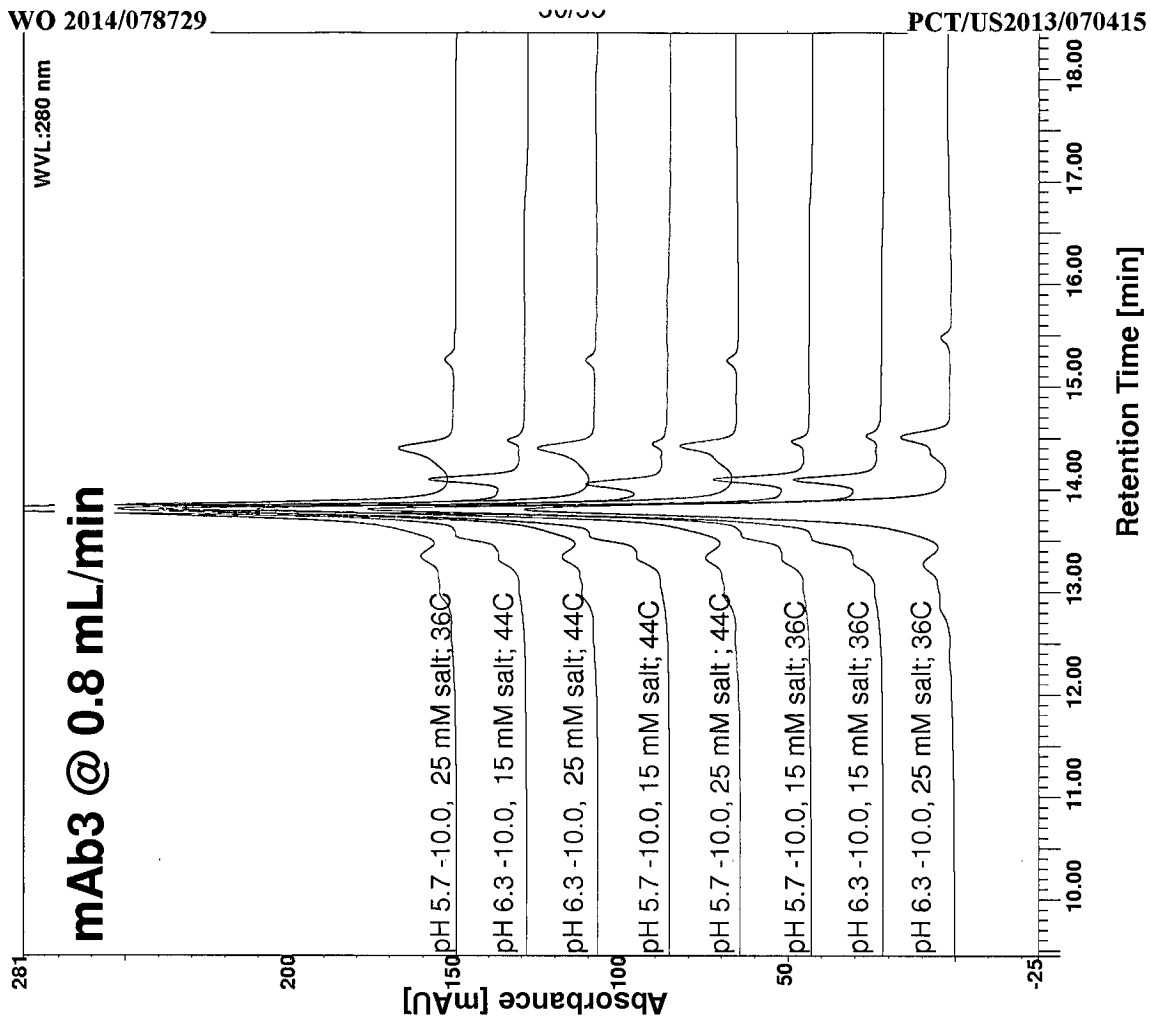
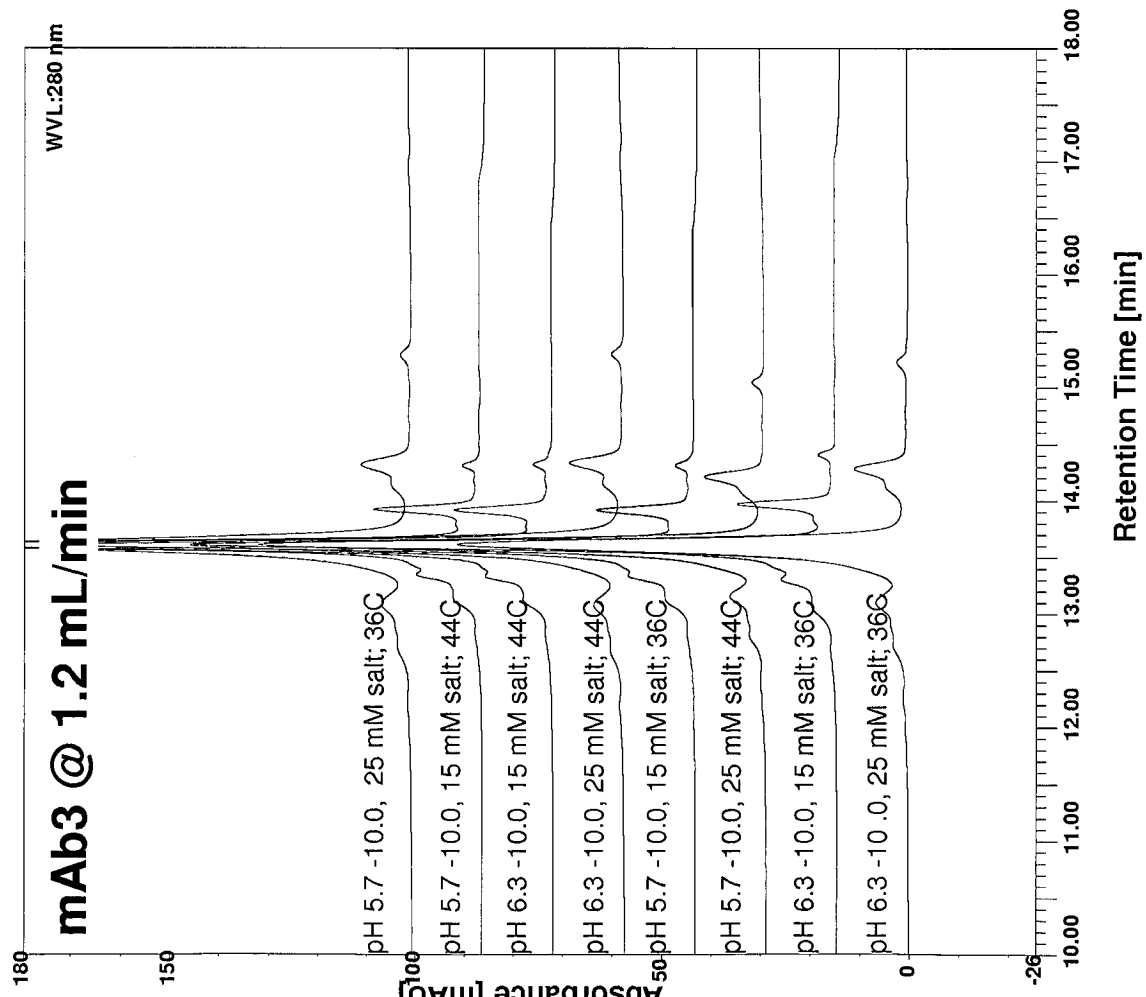


Figure 26

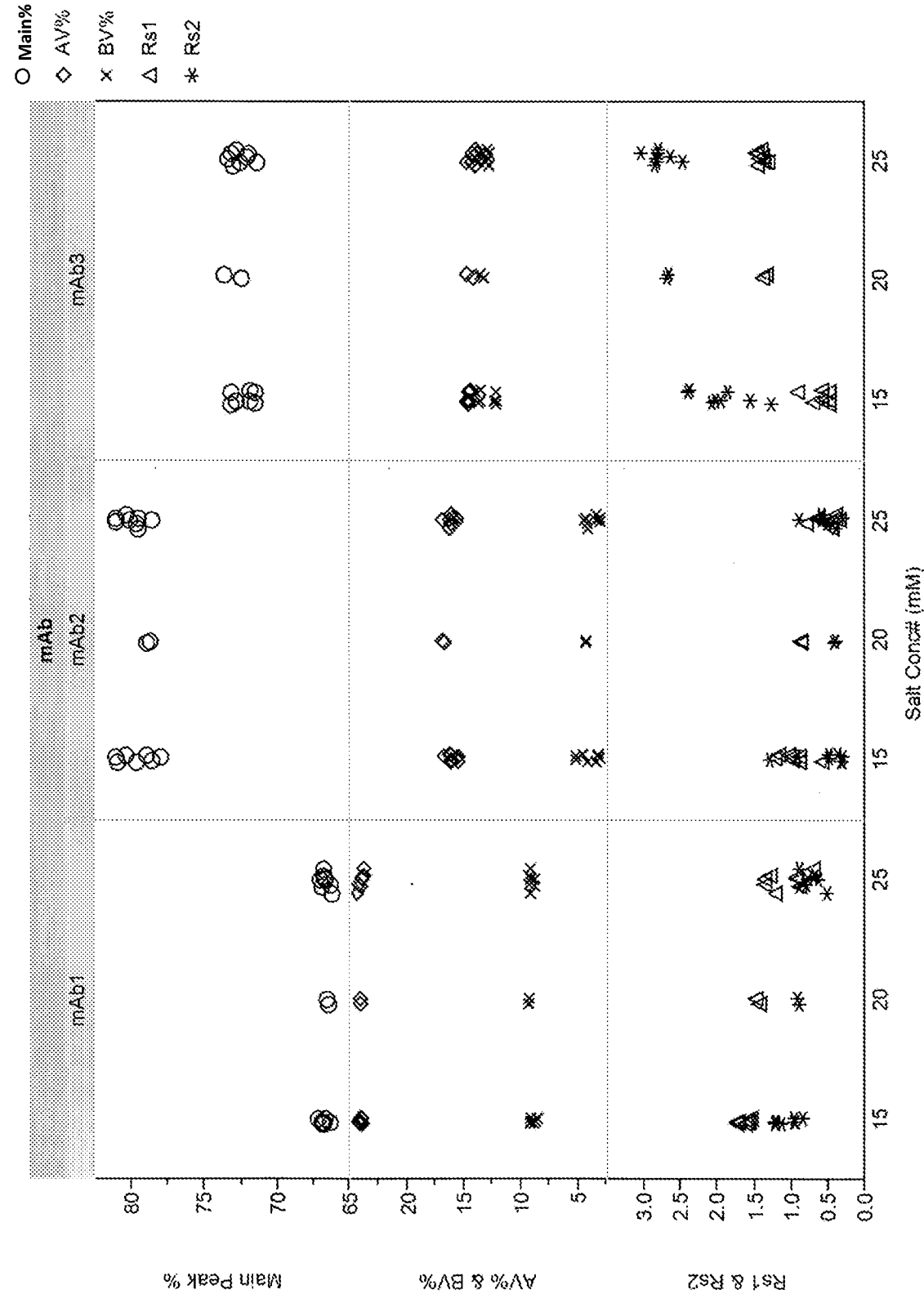
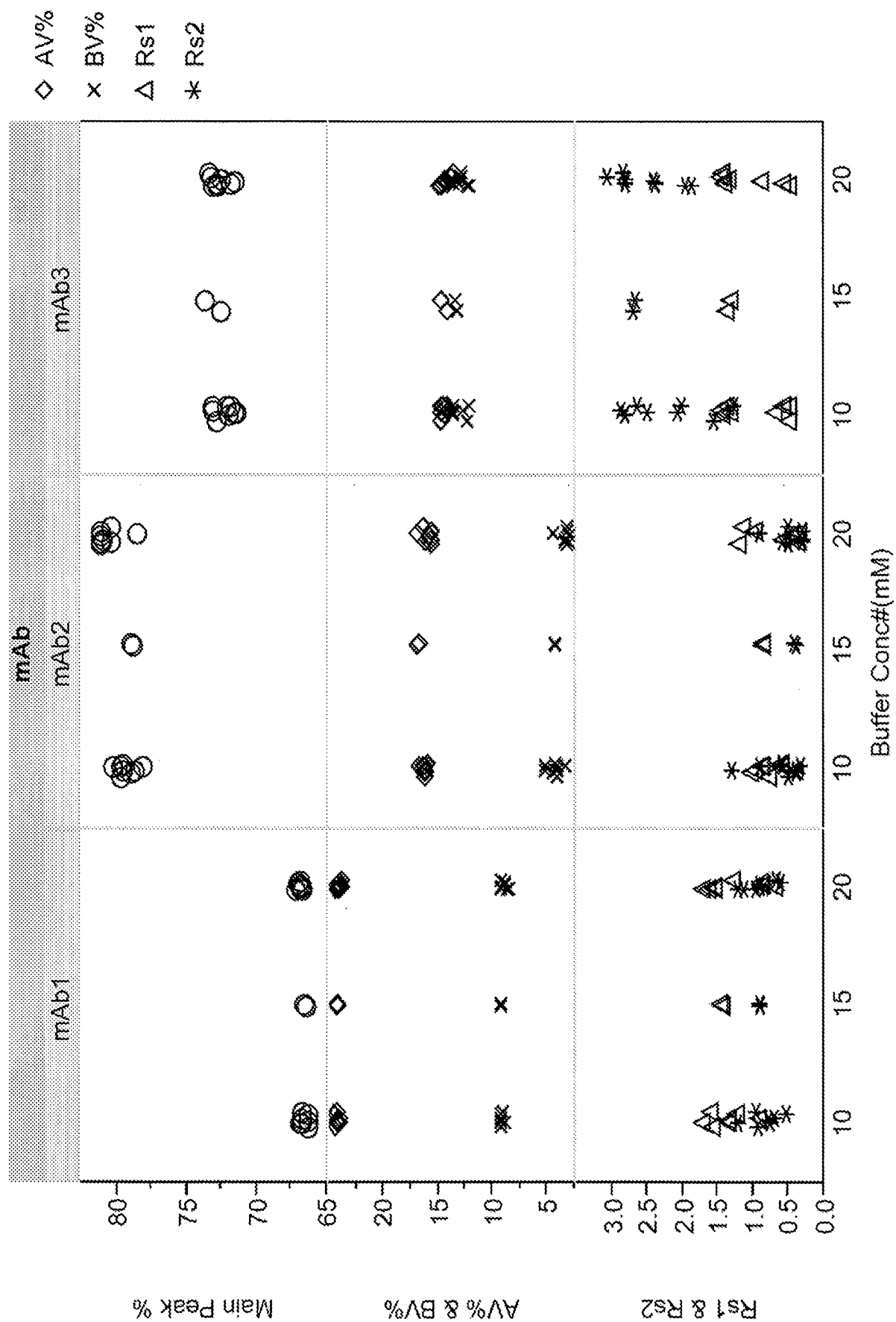


Figure 27 A



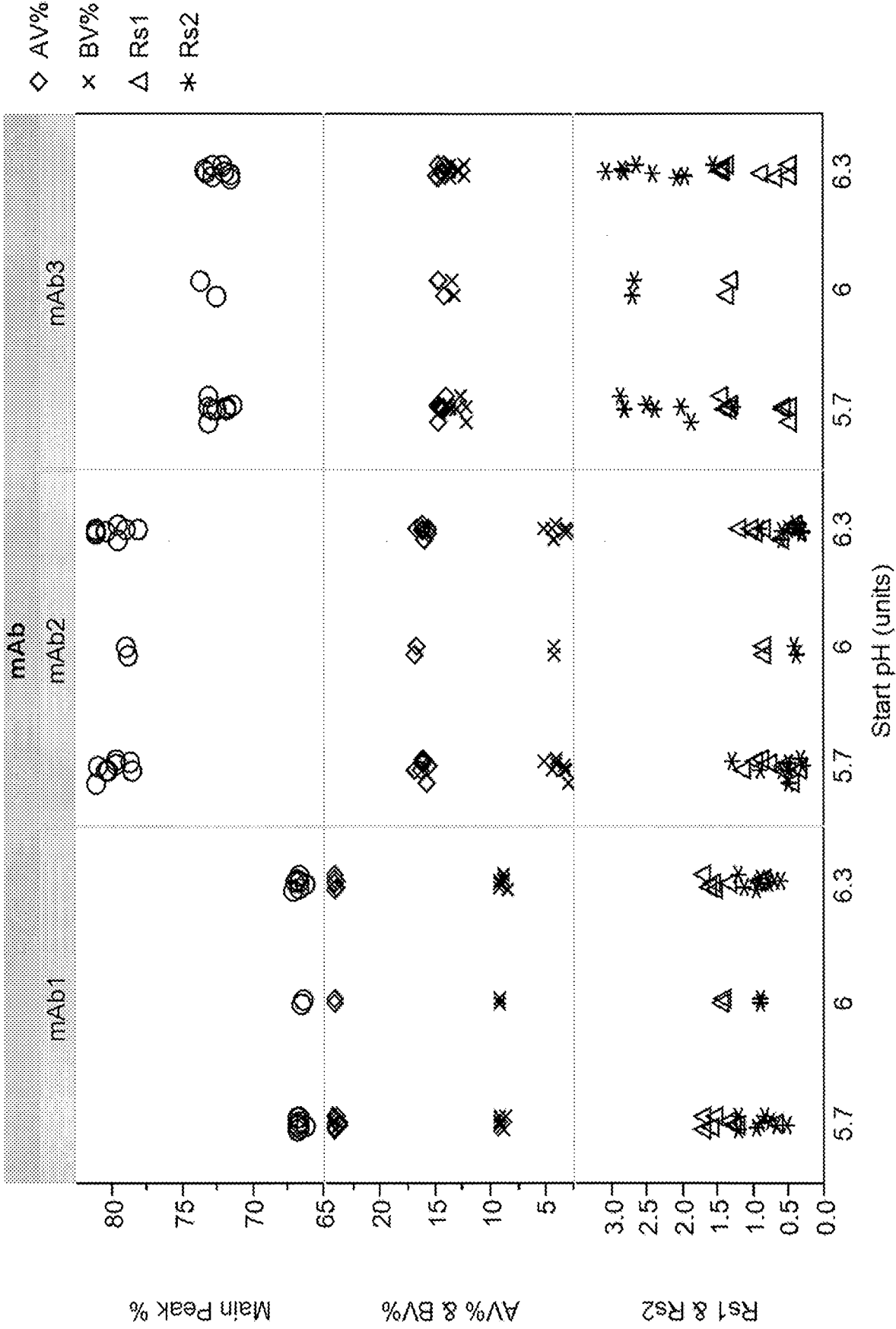
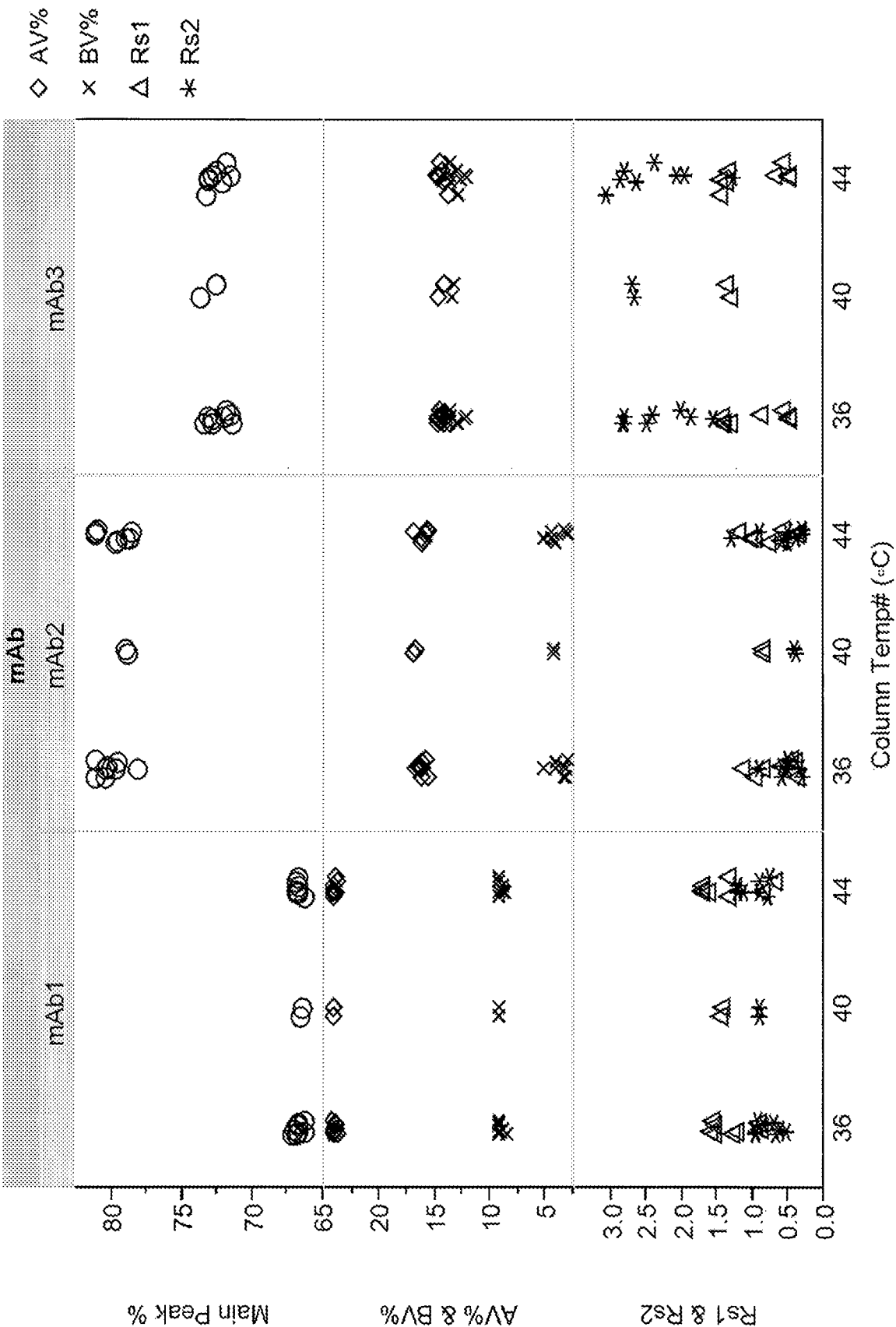


Figure 27 B

Figure 27 C



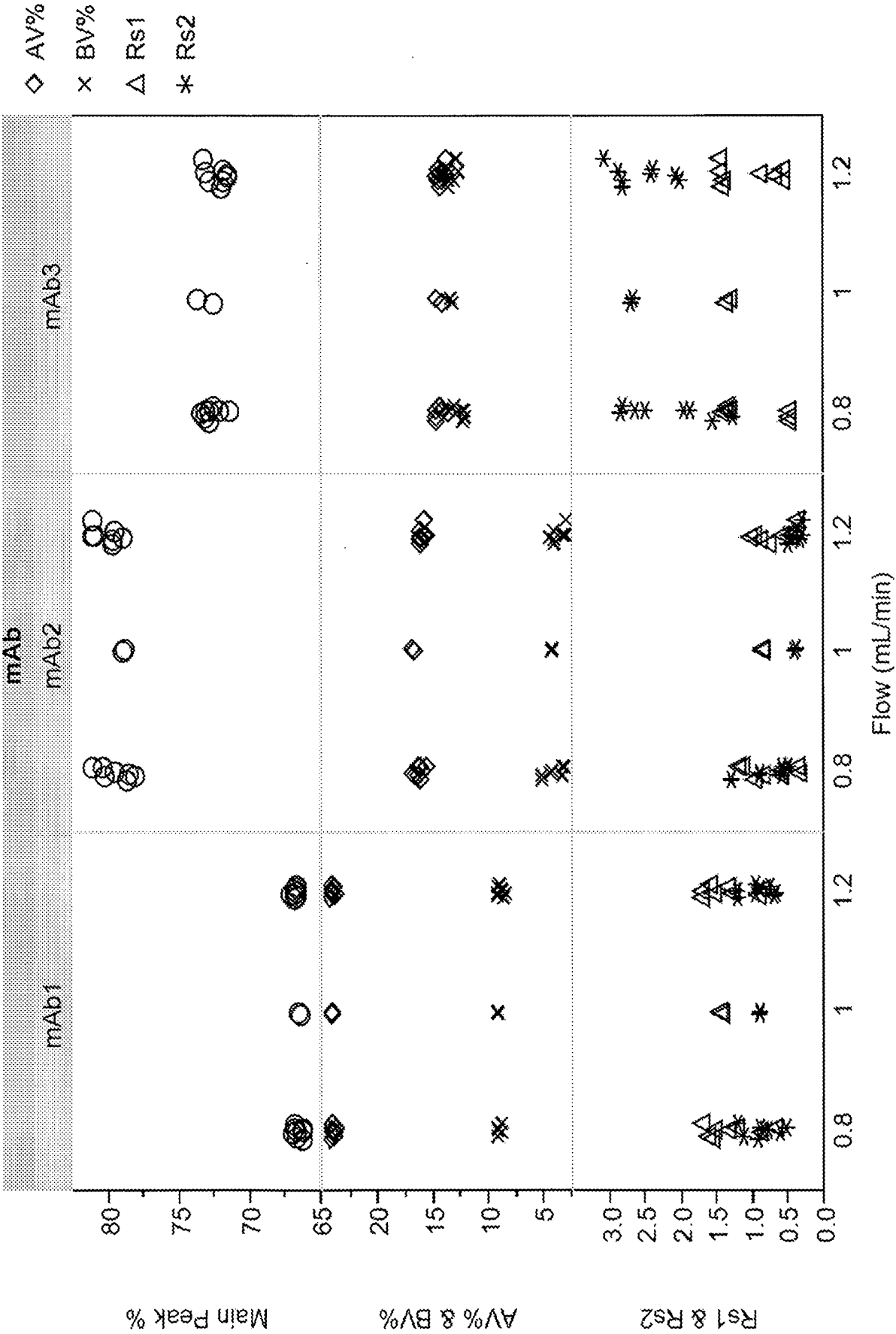


Figure 27 D

A. CLASSIFICATION OF SUBJECT MATTER

G01N 30/34 (2006.01) G01N 30/96 (2006.01) G01N 33/50 (2006.01) C07K 1/18 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, MEDLINE, HCAPLUS, BIOSIS, EMBASE & keywords: ion exchange chromatography, polypeptide, salt gradient, pH gradient, and like terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

☒ Further documents are listed in the continuation of Box C☒ See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
30 January 2014Date of mailing of the international search report
30 January 2014

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
Email address: pct@ipaustalia.gov.au
Facsimile No.: +61 2 6283 7999

Authorised officer

Richard Filmer
AUSTRALIAN PATENT OFFICE
(ISO 9001 Quality Certified Service)
Telephone No. +61 2 6283 2735

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/US2013/070415
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KALTENBRUNNER, O. et al., "Isoprotein analysis by ion-exchange chromatography using a linear pH gradient combined with a salt gradient", Journal of Chromatography, 1993, vol. 639, pages 41-49. Abstract; Results	1-33, 63, 64, 66-80
X	PODGORNIK, H. et al., "Separation of manganese peroxidase isoenzymes on strong anion-exchange monolithic column using pH-salt gradient", Journal of Chromatography B, 2004, vol. 799, pages 343-347 Abstract; 2.3; Results and Discussion	1-80
X	HWANG, H. H. et al., "Purification of ascitic fluid-derived murine monoclonal antibody isoforms by anion-exchange and size exclusion high-performance liquid chromatography", Journal of Chromatography, 1988, vol. 430, pages 329-339 Abstract; Experimental	1-33, 63, 64, 66-80
X	WINNIK, W. M., "Continuous pH/Salt Gradient and Peptide Score for Strong Cation Exchange Chromatography in 2D-Nano-LC/MS/MS Peptide Identification for Proteomics", Analytical Chemistry, 2005, vol. 77, pages 4991-4998 Abstract; Experimental Section: Chromatography and Mass Spectrometry; Table 3	1-33, 63, 64, 66-80
X	REA, J. C. et al., "Validation of a pH gradient-based ion-exchange chromatography method for high-resolution monoclonal antibody charge variant separations", Journal of Pharmaceutical and Biomedical Analysis, 2011, vol. 54, pages 317-323 Abstract; 2.1, 2.2, 2.3; Conclusions	34-63, 65-80
X	FARNAN, D. et al., "Multiproduct High-Resolution Monoclonal Antibody Charge Variant Separations by pH Gradient Ion-Exchange Chromatography", Analytical Chemistry, 2009, vol. 81, pages 8846-8857 Abstract; page 8847, column 2, paragraph 3	34-63, 65-80
X	WO 2000/055203 A1 (NOVO NORDISK, A/S) 21 September 2000 Abstract; pages 3-4, 7, 25	34-63, 65-80
P,X	ZHANG, L. et al., "Improving pH gradient cation-exchange chromatography of monoclonal antibodies by controlling ionic strength", Journal of Chromatography A, 2013, vol. 1272, pages 56-64, published online 29 November 2012. Whole document	1-33, 63, 64, 66-80

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Supplemental Box**Continuation of: Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

- Invention 1 is defined by claims 1-33 and 64 (completely) and claims 63 and 66-80 (in part). The feature of an ion exchange chromatography method comprising eluting a polypeptide with a pH gradient and an ionic strength gradient is specific to this group of claims.

- Invention 2 is defined by claims 34-62 and 65 (completely) and claims 63 and 66-80 (in part). The feature of an ion exchange chromatography method comprising eluting a polypeptide with a pH gradient at an unaltered ionic strength is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions and which provides a technical relationship among them is a method of analysing a composition comprising a polypeptide and one or more contaminants comprising the use of ion exchange chromatography wherein the polypeptide is eluted using a pH gradient. However this feature does not make a contribution over the prior art because it is disclosed in:

KALTENBRUNNER, O. et al., "Isoprotein analysis by ion-exchange chromatography using a linear pH gradient combined with a salt gradient", Journal of Chromatography, 1993, vol. 639, pages 41-49.

REA, J. C. et al., "Validation of a pH gradient-based ion-exchange chromatography method for high-resolution monoclonal antibody charge variant separations", Journal of Pharmaceutical and Biomedical Analysis, 2011, vol. 54, pages 317-323

Therefore in light of these documents this common feature cannot be a special technical feature. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/US2013/070415	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2000/055203 A1 21 Sep 2000 EP 1163268 A119 Dec 2001 EP 1163268 B111 May 2011 EP 2348044 A127 Jul 2011 JP 2002539219 A19 Nov 2002 JP 4732590 B227 Jul 2011 US 6444788 B103 Sep 2002 US 2008281078 A113 Nov 2008 US 8067554 B229 Nov 2011 US 2003027996 A106 Feb 2003 WO 0055203 A121 Sep 2000			
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			



(12) 发明专利申请

(10) 申请公布号 CN 105051528 A

(43) 申请公布日 2015. 11. 11

(21) 申请号 201380070366. X

(51) Int. Cl.

(22) 申请日 2013. 11. 15

G01N 30/34(2006. 01)

G01N 30/96(2006. 01)

(30) 优先权数据

G01N 33/50(2006. 01)

61/727, 051 2012. 11. 15 US

C07K 1/18(2006. 01)

61/780, 707 2013. 03. 13 US

(85) PCT国际申请进入国家阶段日

2015. 07. 14

(86) PCT国际申请的申请数据

PCT/US2013/070415 2013. 11. 15

(87) PCT国际申请的公布数据

WO2014/078729 EN 2014. 05. 22

(71) 申请人 弗·哈夫曼-拉罗切有限公司

地址 瑞士巴塞尔

(72) 发明人 Y·王 G·T·莫雷诺 B·张 L·张

D·法尔南 T·帕塔普夫

(74) 专利代理机构 北京市中咨律师事务所

11247

代理人 陈润杰 黄革生

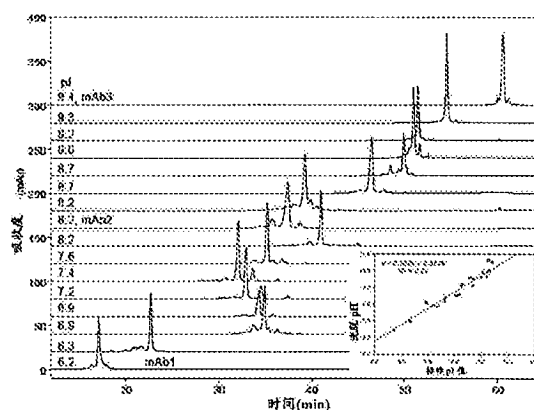
权利要求书4页 说明书53页 附图35页

(54) 发明名称

离子强度介导的 pH 梯度离子交换色谱

(57) 摘要

本发明提供了用于通过离子强度介导的 pH 梯度离子交换色谱来分析多肽如抗体组合物的方法。在一些方面,所述方法使用 pH 梯度与离子强度梯度的组合来从多肽的电荷变体分离多肽。在一些方面,所述方法使用稳定的离子强度来优化 pH 梯度分离窗从而从电荷变体分离多肽。这样的方法可用于分析 pI 大于 9 或 pI 小于 7 的多肽如抗体。在一些方面,本发明提供了用于分析具有多种 pI 的多肽的多产物方法。



1. 用于分析包含多肽以及一种或多种污染物的组合物的方法,所述方法包括
 - a) 使用加样缓冲剂、使所述多肽以及一种或多种污染物结合至离子交换色谱材料,其中所述加样缓冲剂处于第一 pH,且包含第一离子强度;
 - b) 使用洗脱缓冲剂从所述离子交换色谱材料洗脱所述多肽以及一种或多种污染物,其中所述洗脱缓冲剂的 pH 在 pH 梯度中改变,且所述洗脱缓冲剂的离子强度在离子强度梯度中改变,其中通过所述 pH 梯度和离子强度梯度的组合来分离所述多肽以及所述一种或多种污染物;以及
 - c) 检测所述多肽和所述一种或多种污染物。
2. 权利要求 1 的方法,其中所述多肽是抗体或免疫粘附素,或其片段。
3. 权利要求 1 或 2 的方法,其中所述多肽是单克隆抗体或其片段。
4. 权利要求 2 或 3 的方法,其中所述抗体是人抗体。
5. 权利要求 2 或 3 的方法,其中所述抗体是人源化抗体。
6. 权利要求 2 或 3 的方法,其中所述抗体是嵌合抗体。
7. 权利要求 2 至 6 中任一项的方法,其中所述抗体是抗体片段。
8. 权利要求 1 至 7 中任一项的方法,其中所述污染物是多肽的变体。
9. 权利要求 1 至 7 中任一项的方法,其中所述污染物是所述多肽的降解产物。
10. 权利要求 1 至 9 中任一项的方法,其中所述多肽具有大于约 9.0 的 pI。
11. 权利要求 1 至 10 中任一项的方法,其中所述色谱材料是阳离子交换色谱材料。
12. 权利要求 11 的方法,其中所述阳离子交换色谱材料是磺化的色谱材料或羧基化的色谱材料。
13. 权利要求 1 至 12 中任一项的方法,其中所述 pH 梯度是线性梯度。
14. 权利要求 1 至 12 中任一项的方法,其中所述 pH 梯度是阶跃梯度。
15. 权利要求 13 或 14 的方法,其中所述 pH 梯度包含从约 pH 5 至约 pH 11 的升高。
16. 权利要求 1 至 15 中任一项的方法,其中所述 pH 梯度通过使用一种或多种缓冲剂来产生。
17. 权利要求 16 的方法,其中所述一种或多种缓冲剂选自哌嗪、咪唑、Tris、磷酸盐或 CAPS。
18. 权利要求 1 至 17 中任一项的方法,其中所述离子强度梯度是线性梯度。
19. 权利要求 1 至 17 中任一项的方法,其中所述离子强度梯度是阶跃梯度。
20. 权利要求 18 或 19 的方法,其中所述离子强度梯度包含从约 0mM 至约 200mM 的盐浓度升高。
21. 权利要求 18 至 20 中任一项的方法,其中所述离子强度梯度是 NaCl 梯度、KCl 梯度或 Na₂SO₄ 梯度。
22. 权利要求 1 至 9 中任一项的方法,其中所述多肽具有小于约 7.0 的 pI。
23. 权利要求 22 的方法,其中所述色谱材料是阴离子交换色谱材料。
24. 权利要求 23 的方法,其中所述阴离子交换色谱材料是季胺色谱材料或叔胺色谱材料。
25. 权利要求 22 至 24 中任一项的方法,其中所述 pH 梯度是线性梯度。
26. 权利要求 22 至 24 中任一项的方法,其中所述 pH 梯度是阶跃梯度。

27. 权利要求 25 或 26 的方法, 其中所述 pH 梯度包含从约 pH 8 至约 pH 5 的降低。
28. 权利要求 22 至 27 中任一项的方法, 其中所述 pH 梯度通过使用一种或多种缓冲剂来产生。
29. 权利要求 28 的方法, 其中所述一种或多种缓冲剂选自哌嗪、咪唑或 Tris。
30. 权利要求 22 至 29 中任一项的方法, 其中所述离子强度梯度是线性梯度。
31. 权利要求 22 至 29 中任一项的方法, 其中所述离子强度梯度是阶跃梯度。
32. 权利要求 30 或 31 的方法, 其中所述离子强度梯度包含从约 0mM 至约 200mM 的盐浓度升高。
33. 权利要求 30 至 32 中任一项的方法, 其中所述离子强度梯度是 NaCl 梯度、KCl 梯度或 Na₂SO₄ 梯度。
34. 用于分析包含多肽以及一种或多种污染物的组合物的方法, 所述方法包括
 - a) 使用加样缓冲剂、使所述多肽以及一种或多种污染物结合至离子交换色谱材料, 其中所述加样缓冲剂处于初始 pH, 并且包含初始离子强度;
 - b) 使用洗脱缓冲剂从所述离子交换色谱材料洗脱所述多肽以及一种或多种污染物, 其中所述洗脱缓冲剂的 pH 在 pH 梯度中改变, 且其中所述洗脱缓冲剂的离子强度与所述加样缓冲剂的初始离子强度基本相同, 其中通过在约所述初始离子强度下的 pH 梯度来分离所述多肽以及所述一种或多种污染物; 以及
 - c) 检测所述多肽和所述一种或多种污染物。
35. 权利要求 34 的方法, 其中所述多肽是抗体或免疫粘附素, 或其片段。
36. 权利要求 34 或 35 的方法, 其中所述多肽是单克隆抗体或其片段。
37. 权利要求 35 或 36 的方法, 其中所述抗体是人抗体。
38. 权利要求 35 或 36 的方法, 其中所述抗体是人源化抗体。
39. 权利要求 35 或 36 的方法, 其中所述抗体是嵌合抗体。
40. 权利要求 35 至 39 中任一项的方法, 其中所述抗体是抗体片段。
41. 权利要求 34 至 40 中任一项的方法, 其中所述污染物是多肽的变体。
42. 权利要求 34 至 40 中任一项的方法, 其中所述污染物是所述多肽的降解产物。
43. 权利要求 34 至 42 中任一项的方法, 其中所述多肽具有大于约 9.0 的 pI。
44. 权利要求 34 至 43 中任一项的方法, 其中所述色谱材料是阳离子交换色谱材料。
45. 权利要求 44 的方法, 其中所述阳离子交换色谱材料是磺化的色谱材料或羧基化的色谱材料。
46. 权利要求 34 至 45 中任一项的方法, 其中所述 pH 梯度是线性梯度。
47. 权利要求 34 至 45 中任一项的方法, 其中所述 pH 梯度是阶跃梯度。
48. 权利要求 46 或 47 的方法, 其中所述 pH 梯度包含从约 pH 5 至约 pH 11 的升高。
49. 权利要求 34 至 48 中任一项的方法, 其中所述 pH 梯度通过使用一种或多种缓冲剂来产生。
50. 权利要求 49 的方法, 其中所述一种或多种缓冲剂选自哌嗪、咪唑、Tris、磷酸盐或 CAPS。
51. 权利要求 34 至 50 中任一项的方法, 其中所述洗脱缓冲剂的离子强度为约 0mM 至约 100mM。

52. 权利要求 51 的方法, 其中所述洗脱缓冲剂包含约 0mM NaCl 至约 100mM NaCl、约 0mM KCl 至约 100mM KCl, 或约 0mM Na₂SO₄ 至约 100mM Na₂SO₄。
53. 权利要求 34 至 42 中任一项的方法, 其中所述多肽具有小于约 7.0 的 pI。
54. 权利要求 53 的方法, 其中所述色谱材料是阴离子交换色谱材料。
55. 权利要求 54 的方法, 其中所述阴离子交换色谱材料是季胺色谱材料或叔胺色谱材料。
56. 权利要求 53 至 55 中任一项的方法, 其中所述 pH 梯度是线性梯度。
57. 权利要求 53 至 55 中任一项的方法, 其中所述 pH 梯度是阶跃梯度。
58. 权利要求 56 或 57 的方法, 其中所述 pH 梯度包含从约 pH 8 至约 pH 5 的降低。
59. 权利要求 53 至 58 中任一项的方法, 其中所述 pH 梯度通过使用一种或多种缓冲剂来产生。
60. 权利要求 59 的方法, 其中所述一种或多种缓冲剂选自哌嗪、咪唑或 Tris。
61. 权利要求 53 至 60 中任一项的方法, 其中所述洗脱缓冲剂的离子强度为约 0mM 至约 100mM。
62. 权利要求 61 的方法, 其中所述洗脱缓冲剂包含约 0mM NaCl 至约 100mM NaCl。
63. 权利要求 1 至 62 中任一项的方法, 其中所述分析通过高效液相色谱来进行。
64. 用于分析包含多肽以及一种或多种污染物的组合物中的所述多肽的方法, 其中所述方法将一种或多种污染物从所述多肽分离, 所述方法包括
- a) 使用加样缓冲剂、使所述多肽以及一种或多种污染物结合至离子交换色谱材料, 其中所述加样缓冲剂处于第一 pH, 并且包含第一离子强度;
 - b) 使用洗脱缓冲剂从所述离子交换色谱材料洗脱所述多肽以及一种或多种污染物, 其中所述洗脱缓冲剂的 pH 在 pH 梯度中改变, 且所述洗脱缓冲剂的离子强度在离子强度梯度中改变, 其中通过所述 pH 梯度和离子强度梯度的组合来分离所述多肽以及所述一种或多种污染物; 以及
 - c) 检测所述多肽和所述一种或多种污染物,
- 其中所述方法用于分析 pI 介于约 7.0 至约 9.5 范围的多肽。
65. 用于分析包含多肽以及一种或多种污染物的组合物中的多肽的方法, 所述方法包括
- a) 使用加样缓冲剂、使所述多肽以及一种或多种污染物结合至离子交换色谱材料, 其中所述加样缓冲剂处于初始 pH, 并且包含初始离子强度;
 - b) 使用洗脱缓冲剂从所述离子交换色谱材料洗脱所述多肽以及一种或多种污染物, 其中所述洗脱缓冲剂的 pH 在 pH 梯度中改变, 且其中所述洗脱缓冲剂的离子强度与所述加样缓冲剂的初始离子强度基本相同, 其中通过在约所述初始离子强度下的 pH 梯度来分离所述多肽以及所述一种或多种污染物; 以及
 - c) 检测所述多肽和所述一种或多种污染物,
- 其中所述方法用于分析 pI 介于约 7.0 至约 9.5 范围的多肽。
66. 权利要求 65 或 66 的方法, 其中所述多肽是抗体或免疫粘附素, 或其片段。
67. 权利要求 64 至 66 中任一项的方法, 其中所述多肽是单克隆抗体或其片段。
68. 权利要求 66 或 67 的方法, 其中所述抗体是人抗体。

69. 权利要求 66 或 67 的方法,其中所述抗体是人源化抗体。
70. 权利要求 66 或 67 的方法,其中所述抗体是嵌合抗体。
71. 权利要求 65 至 70 中任一项的方法,其中所述抗体是抗体片段。
72. 权利要求 64 至 71 中任一项的方法,其中所述污染物是多肽的变体。
73. 权利要求 64 至 72 中任一项的方法,其中所述污染物是所述多肽的降解产物。
74. 权利要求 64 至 73 中任一项的方法,其中所述污染物是多肽的电荷变体。
75. 权利要求 1 至 74 中任一项的方法,其中所述加样缓冲剂和 / 或所述洗脱缓冲剂中的缓冲剂浓度为约 10mM 至约 50mM。
76. 权利要求 1 至 75 中任一项的方法,其中所述第一 pH 为约 pH 5.0 至约 pH 7.0。
77. 权利要求 1 至 76 中任一项的方法,其中所述色谱材料的温度为约 20℃至约 50℃。
78. 权利要求 1 至 77 中任一项的方法,其中所述上样和洗脱在约 0.5ml/ 分钟至约 2.0ml/ 分钟的流速进行。
79. 确定组合物中多肽的纯度的方法,其包括根据权利要求 1 至 78 的方法中的任一种来分析所述组合物,并确定所述组合物中多肽与污染物的比率。
80. 确定包含多肽的组合物中所述多肽的稳定性的方法,所述方法包括:
 - a) 将所述包含多肽的组合物于 40℃孵育三周或六周,
 - b) 通过权利要求 1 至 79 的任一方法来分析步骤 a) 的组合物,以及
 - c) 确定所述组合物中变体与多肽的比率,其中,与未进行孵育的组合物相比,所述组合物中变体与多肽的比率的升高指示所述组合物多肽的降解。

离子强度介导的 pH 梯度离子交换色谱

发明领域

[0001] 本发明提供了用于使用离子强度介导的 pH 梯度离子交换色谱来分析多肽制备物的方法。

[0002] 发明背景

[0003] 在水性环境中,蛋白质如单克隆抗体 (mAb) 大多在表面上具有带电的极性氨基酸 (Barlow, DJ 和 Thornton, JM(1986)Biopolymers 25:1717)。由于分子与溶液组分的相互作用,表面残基可经历多种化学修饰和酶修饰,导致在其静电表面上具有些微差异的蛋白质变体异质混合物 (Dick, LW 等, (2009)J. Chromatogr. B 877:3841 ;Liu, HW 等, (2008) Rapid Commun. Mass Spectrom. 22:4081 ;Miller, AK 等, (2011)J. Pharm. Sci. 100:2543 ; Wang, WR 等, (2011)Mol. Immunol. 48:860)。根据 Vlasak, J 和 Ionescu, R(2008Curr. Pharm. Biotechnol. 9:468) 的最近回顾,阳离子交换色谱 (CEC) 被认为是描述蛋白治疗剂电荷异质性的金标准。一些监管机构通常要求该电荷灵敏的分离方法来确保产品制造期间的一致性,以及监测蛋白治疗剂的降解水平 (Miller, AK 等, (2011)J. Pharm. Sci. 100:2543 ; He, XPZ(2009)Electrophoresis 30:714 ;Sosic, Z 等, (2008)Electrophoresis 29:4368 ; Kim, J 等, (2010)J. Chromatogr. B 878:1973 ;Teshima, G 等, (2010)J. Chromatogr. A 1218:2091)。

[0004] 使用 pH 梯度的分析型离子交换色谱 (IEC) 法作为常规的盐梯度 IEC 的替代技术应运而生,用于描述治疗性蛋白质的电荷异质性 (Farnan, D 和 Moreno, GT Tsonev, (2009)Anal. Chem. 81:8846 ;Tsonev, LI 和 Hirsh, AG(2008)J. Chromatogr. A 1200:166 ;Nordborg, A 等, (2009)J. Sep. Sci. 32:2668 ;Rozhkova, A(2009)J. Chromatogr. A 1216:5989 ;Rea, JC 等 (2010)J. Pharm. Biomed. Anal. 54:317)。在该技术中,通过提高流动相的 pH 来洗脱通常上载于阳离子固定相的蛋白质。最近已经证实,具有 6.0 至 9.5 的较宽 pH 窗的 pH 梯度 IEC (pH-IEC) 法不仅提供了比传统盐梯度 IEC 更佳的分辨率,还通过分析 pI 为 7.3 至 9.0 的 12 种单克隆抗体 (mAb) 提供了多产物能力 (Farnan, D 和 Moreno, GT(2009) Anal. Chem. 81:8846)。该 pH-IEC 法还对具有不同离子强度 (0 至 250mM NaCl) 和 pH 值 (5.0 至 8.5) 的样品基质是高度耐受的 (Farnan, D 和 Moreno, GT(2009)Anal. Chem. 81:8846)。此外,所报道的 pH-IEC 法没有明显受到柱长度和化学成分的影响,并且可用较短的柱实现快速分离以改进蛋白变体分析的通量。根据最近的验证报告 (Rea, JC 等 (2010)J. Pharm. Biomed. Anal. 54:317),所开发的 pH-IEC 法已显示出在不同色谱条件下的优异精密性以及在不同柱负载的良好线性。因此,所报道的 pH-IEC 法适用于在生物技术产业中进行日常测试。

[0005] 虽然有许多优点,但是所报道的 pH-IEC 法主要倾向于 pI 值介于 7.3 至 9.0 的研究范围内的 mAb。mAb 的洗脱曲线可随着不同的缓冲组合物和浓度以及 mAb 洗脱时的 pH 值而不同,该事实说明,pH- 梯度 IEC 涉及组合的离子强度和 pH- 梯度洗脱机制 (Farnan, D 和 Moreno, GT(2009)Anal. Chem. 81:8846)。这也与 Anderson 和 coworkers 关于 pH- 梯度阴离子 - 交换色谱 (pH-AIEC) 的报告一致 (Anderson, DJ 和 Shan, L(2001)Clin. Chem. 47:128 ;

Shan, L 和 Anderson, DJ (2001) J. Chromatogr. A 909:191 ;Shan, L 和 Anderson, DJ (2002) Anal. Chem. 74:5641)。随着生物技术产业中研发阶段的 mAb 的数量增加,尤其是基于动物研究 (Igawa, T. (2010) Protein Eng. Des. Sel. 23:385) 显示潜在更长半衰期的更多较低 pI 的 mAb, 需要将 pH-IEC 法的可应用性扩展至更宽范围的治疗性 mAb。

[0006] 本文引用的所有文献,包括专利申请和出版物,都通过引用整体并入。

[0007] 简述

[0008] 本发明提供了用于分析包含多肽以及一种或多种污染物的组合物的方法,所述方法包括:a) 使用加样缓冲剂使所述多肽以及一种或多种污染物结合至离子交换色谱材料,其中所述加样缓冲剂处于第一 pH,并且包含第一离子强度;b) 使用洗脱缓冲剂从所述离子交换色谱材料洗脱所述多肽以及一种或多种污染物,其中所述洗脱缓冲剂的 pH 在 pH 梯度中改变,并且所述洗脱缓冲剂的离子强度在离子强度梯度中改变,其中通过所述 pH 梯度和离子强度梯度的组合来分离所述多肽以及所述一种或多种污染物;c) 检测所述多肽和所述一种或多种污染物。在本发明的一些实施方案中,可使用相同方法来分析 pI 介于 6.0 至 9.5 范围的多肽。

[0009] 在一些实施方案中,所述多肽是抗体或免疫粘附素,或其片段。在另一些实施方案中,所述多肽是单克隆抗体或其片段。在另一些实施方案中,所述抗体是人抗体。在其他实施方案中,所述抗体是人源化抗体。在其他实施方案中,所述抗体是嵌合抗体。在一些实施方案中,所述抗体是抗体片段。

[0010] 在上述实施方案的一些实施方案中,所述污染物是所述多肽的变体。在另一些实施方案中,所述污染物是所述多肽的降解产物。

[0011] 在一些实施方案中,所述多肽具有大于约 9.0 的 pI。在另一些实施方案中,所述色谱材料是阳离子交换色谱材料。在另一些实施方案中,所述阳离子交换色谱材料是磺化的色谱材料或羧基化的色谱材料。

[0012] 在任意上述实施方案的另一些实施方案中,所述 pH 梯度是线性梯度。在任意上述实施方案中另一些实施方案中,所述 pH 梯度是阶跃梯度 (step gradient)。在另一些实施方案中,所述 pH 梯度包含从约 pH 5 至约 pH 11 的升高。在上述实施方案的一些实施方案中,所述 pH 梯度通过使用一种或多种缓冲剂来产生。在另一些实施方案中,所述一种或多种缓冲剂是哌嗪、咪唑、三羟甲基氨基甲烷 (Tris)、磷酸盐或 CAPS。

[0013] 在任一实施方案的一些实施方案中,所述离子强度梯度是线性梯度。在另一些实施方案中,所述离子强度梯度是阶跃梯度。在一些实施方案中,所述离子强度梯度包含从约 0mM 至约 200mM 的盐浓度升高。在另一些实施方案中,所述离子强度梯度是 NaCl 梯度、KCl 梯度或 Na₂SO₄ 梯度。

[0014] 在上述实施方案的一些实施方案中,所述多肽具有小于约 7.0 的 pI。在另一些实施方案中,所述色谱材料是阴离子交换色谱材料。在另一些实施方案中,所述阴离子交换色谱材料是季胺色谱材料或叔胺色谱材料。在一些实施方案中,所述 pH 梯度是线性梯度。在另一些实施方案中,所述 pH 梯度是阶跃梯度。在一些实施方案中,所述 pH 梯度包含从约 pH 8 至约 pH 5 的降低。在一些实施方案中,所述 pH 梯度通过使用一种或多种缓冲剂来产生。在另一些实施方案中,所述一种或多种缓冲剂是哌嗪、咪唑或 Tris。在一些实施方案中,所述离子强度梯度是线性梯度。在另一些实施方案中,所述离子强度梯度是阶跃梯度。在再

另一些实施方案中,所述离子强度梯度包含从约 0mM 至约 200mM 的盐浓度升高。在一些实施方案中,所述离子强度梯度是 NaCl 梯度、KCl 梯度或 Na_2SO_4 梯度。

[0015] 在一些方面中,本发明提供了用于分析包含多肽以及一种或多种污染物的组合物的方法,所述方法包括 a) 使用加样缓冲剂使所述多肽以及一种或多种污染物结合至离子交换色谱材料,其中所述加样缓冲剂处于初始 pH,并且包含初始离子强度;b) 使用洗脱缓冲剂从所述离子交换色谱材料洗脱所述多肽以及一种或多种污染物,其中所述洗脱缓冲剂的 pH 在 pH 梯度中改变,并且其中所述洗脱缓冲剂的离子强度与所述加样缓冲剂的初始离子强度基本相同,其中通过在约所述初始离子强度下的 pH 梯度来分离所述多肽以及所述一种或多种污染物;c) 检测所述多肽和所述一种或多种污染物。在本发明的一些实施方案中,可使用基本相同的方法来分析 pI 介于 6.0 至 9.5 范围的多肽。

[0016] 在上述方面的一些实施方案中,所述多肽是抗体或免疫粘附素,或其片段。在另一些实施方案中,所述多肽是单克隆抗体或其片段。在实施方案的另一些实施方案中,所述抗体是人抗体。在其他实施方案中,所述抗体是人源化抗体。在再另一些实施方案中,所述抗体是嵌合抗体。在一些实施方案中,所述抗体是抗体片段。

[0017] 在上述方面的一些实施方案中,所述污染物是所述多肽的变体。在一些实施方案中,所述污染物是所述多肽的降解产物。

[0018] 在上述方面的一些实施方案中,所述多肽具有大于约 9.0 的 pI。在另一些实施方案中,所述色谱材料是阳离子交换色谱材料。在再另一些实施方案中,所述阳离子交换色谱材料是磺化的色谱材料或羧基化的色谱材料。

[0019] 在上述方面的一些实施方案中,所述 pH 梯度是线性梯度。在其他实施方案中,所述 pH 梯度是阶跃梯度。在一些实施方案中,所述 pH 梯度包含从约 pH 5 至约 pH 11 的升高。在一些实施方案中,所述 pH 梯度通过使用一种或多种缓冲剂来产生。在另一些实施方案中,所述一种或多种缓冲剂是吡啶、咪唑、tris、磷酸盐或 CAPS。

[0020] 在上述实施方案的一些实施方案中,所述洗脱缓冲剂的离子强度为约 0mM 至约 100mM。在另一些实施方案中,所述洗脱缓冲剂包含约 0mM NaCl 至约 100mM NaCl、约 0mM KCl 至约 100mM KCl,或约 0mM Na_2SO_4 至约 100mM Na_2SO_4 。

[0021] 在上述方面的一些实施方案中,所述多肽具有小于约 7.0 的 pI。在另一些实施方案中,所述色谱材料是阴离子交换色谱材料。在再另一些实施方案中,所述阴离子交换色谱材料是季胺色谱材料或叔胺色谱材料。在上述实施方案的一些实施方案中,所述 pH 梯度是线性梯度。在其他实施方案中,所述 pH 梯度是阶跃梯度。在上述实施方案的一些实施方案中,所述 pH 梯度包含从约 pH 8 至约 pH 5 的降低。在一些实施方案中,所述 pH 梯度通过使用一种或多种缓冲剂来产生。在另一些实施方案中,所述一种或多种缓冲剂是吡啶、咪唑或 Tris。在一些实施方案中,所述洗脱缓冲剂的离子强度为约 0mM 至约 100mM。在另一些实施方案中,所述洗脱缓冲剂包含约 10mM NaCl 至约 100mM NaCl。

[0022] 在任一上述实施方案的一些实施方案中,所述分析通过高效液相色谱来进行。

[0023] 在一些方面中,本发明提供了确定组合物中多肽的纯度的方法,其包括根据上述实施方案的任一种方法来分析所述组合物,并确定所述组合物中多肽与污染物的比率。

[0024] 在一些方面中,本发明提供了确定包含多肽的组合物中所述多肽的稳定性的方法,所述方法包括:a) 将所述包含多肽的组合物于 0℃ 至 40℃ 孵育一周或六周,b) 通过实施

方案 1 至 63 的任一方法来分析步骤 a) 的组合物, 以及 c) 确定所述组合物中变体与多肽的比率, 其中, 与未进行孵育的组合物相比, 所述组合物中变体与多肽的比率的升高指示所述组合物中多肽的降解。

[0025] 附图简述

[0026] 图 1 示出了用 pH 梯度离子交换色谱 (IEC) 法得到的具有不同 pI 的三种单克隆抗体 (mAb1、mAb2 和 mAb3) 的电荷异质性曲线。

[0027] 图 2 示出了优化前柱出口处的离子强度和 pH 曲线 (图 A) 以及 pH 梯度中的柱背压 (图 B), 以及优化后出口处的离子强度和 pH 曲线 (图 C) 以及 pH 梯度中的柱背压 (图 D)。图 A 和图 C 示出了模型 pH 曲线和电导率以及实验 pH 曲线和电导率。

[0028] 图 3 示出了以四种不同的缓冲剂浓度得到的 mAb1 (图 A) 和 mAb2 (图 B) 的离子强度介导的 pH IEC 色谱图。将色谱图中主峰的半峰全宽 (FWHM) 相对缓冲剂浓度 (图 C) 和盐浓度 (图 C) 作图。

[0029] 图 4 示出了对 pI 介于 6.2 至 9.4 范围的十六种单克隆抗体进行离子强度介导的 pH 梯度 IEC 而得到的电荷异质性曲线。插图示出了标称 pI 值相对洗脱 pH 绘制的图。

[0030] 图 5 示出了对天然 mAb1 (0 周) 和热应激 mAb1 (40°C 3 周和 6 周) 进行离子强度介导的 pH 梯度 IEC 而得到的色谱图。

[0031] 图 6 示出了用离子强度介导的 pH 梯度 IEC 而得到的柱负荷为 10 μ g、20 μ g、50 μ g、100 μ g 和 200 μ g 的 mAb1 的色谱图。

[0032] 图 7 示出了 pH 6 至 pH 9.5 的半线性 pH 梯度中通过哌嗪、咪唑、Tris (PIT) 缓冲溶液的氨基官能团的缓冲。

[0033] 图 8 示出了使用离子强度介导的 IEC、pI 介于 pI 6.2 至 pI 9.4 范围的 mAb 的色谱图。柱为 Propac WCX-10, 4 $\times$ 250mm。

[0034] 图 9 示出, 当使用 4 $\times$ 250mm 柱时, 在初始 pH-IEC 和离子强度介导的 pH-IEC 之间观察到相似的曲线。

[0035] 图 10 示出了使用磷酸盐的 pH 6 至 pH 11 的模型线性 pH 梯度, 以随着 pH 增加维持电导率。

[0036] 图 11 的图示出了所观察到的 pH (图 A) 和电导率 (图 C) 曲线与模型预测 (图 B 和 D) 一致。

[0037] 图 12 示出, 使用 TPP 缓冲剂可进行较短的运行。顶部的色谱图示出了使用 16 分钟梯度、4 $\times$ 50mm Propac WCX-10HT 柱的结果, 底部色谱图示出使用 58 分钟梯度、4 $\times$ 250mm 柱的结果。

[0038] 图 13 的图显示了使用造模工具、计算具有不同 pI 的单克隆抗体的电荷、在不同 pH 下的不同分子的电荷状态。当其跨越 X 轴时, 单克隆抗体上的电荷呈中性。合适的 pH-IEC 分离窗与曲线相对较平的 pH 范围相对应。

[0039] 图 14 的图示出在高 pI 下, 随着 pH 增加分子具有更短的解析时间。

[0040] 图 15 示出了确定对于 pI>9.0 抗体的最佳电荷屏蔽效应的最优盐浓度的研究结果。测试了以下盐浓度: 0mM、10mM、20mM、30mM、40mM 和 50mM。

[0041] 图 16 示出了确定对于 pI 介于范围 8.9 至 9.1 的抗体的最佳电荷屏蔽效应的最优盐浓度的研究结果。

[0042] 图 17 示出了确定对于 pI 介于范围 7.6 至 8.7 的抗体的最佳电荷屏蔽效应的最优盐浓度的研究结果。

[0043] 图 18 的图示出:为了屏蔽额外的电荷而添加盐使电荷状态移动至合适的分离窗。

[0044] 图 19 的图示出:使用浅 pH 梯度可改进使用 pI 为 7.6 的 MAb 的峰分辨率。测试的梯度如下:PIT(2.4mM Tris、1.5mM 咪唑、11.6mM 哌嗪、pH 6 至 11);盐介导的 PIT(4mM Tris、4mM 咪唑、4mM 哌嗪、pH 6 至 11、0mM 至 16mM NaCl 梯度);盐介导的 TPP(5mM Tris、5mM 哌嗪、5mM 磷酸盐、pH 6 至 11、0mM 至 30mM NaCl 梯度);混合 pH IEC、TPP(5mM Tris、5mM 哌嗪、5mM 磷酸盐、pH 6 至 9、0mM NaCl);TIC(5mM Tris、5mM 哌嗪、5mM CAPS、pH 6 至 9、10mM NaCl)。运行 20 至 22 分钟。

[0045] 图 20 的图示出:使用浅的 pH 梯度可改进使用 pI 为 8.6 至 9.3 的 MAb 的峰分辨率。测试的梯度如下:PIT(2.4mM Tris、1.5mM 咪唑、11.6mM 哌嗪、pH 6 至 11);盐介导的 PIT(4mM Tris、4mM 咪唑、4mM 哌嗪、pH 5 至 11、16mM NaCl);盐介导的 TPP(5mM Tris、5mM 哌嗪、5mM 磷酸盐、pH 6 至 11、20mM NaCl 梯度);TPP(5mM Tris、5mM 哌嗪、5mM 磷酸盐、pH 6 至 9、20mM NaCl);TIC(5mM Tris、5mM 哌嗪、5mM CAPS、pH 7 至 10、30mM NaCl)。运行 20 至 22 分钟。

[0046] 图 21 的图示出:使用浅的 pH 梯度可改进使用 pI 为 9.0 的 MAb 的峰分辨率。测试的梯度如下:PIT(2.4mM Tris、1.5mM 咪唑、11.6mM 哌嗪、pH 6 至 11);盐介导的 PIT(4mM Tris、4mM 咪唑、4mM 哌嗪、pH 5 至 11、16mM NaCl);TPP(5mM Tris、5mM 哌嗪、5mM 磷酸盐、pH 6 至 11、30mM NaCl 梯度);TPP(5mM Tris、5mM 哌嗪、5mM 磷酸盐、pH 7 至 10、20mM NaCl);TIC(5mM Tris、5mM 哌嗪、5mM CAPS、pH 7 至 10、30mM NaCl)。运行 20 至 22 分钟。

[0047] 图 22 的图示出十五分钟运行时间是可行的。Mab 具有 8.8 的 pI。示出了两个色谱图,一个为 6 至 10 的 pH 梯度,于 20mM NaCl 中 22 分钟;一个为 7 至 10 的 pH 梯度,于 20mM NaCl 中 15 分钟。

[0048] 图 23 的图示出十五分钟运行时间是可行的。Mab 具有 9.0 的 pI。示出了两个色谱图,一个为 6 至 10 的 pH 梯度,于 20mM NaCl 中 22 分钟;一个为 7 至 10 的 pH 梯度,于 20mM NaCl 中 15 分钟。

[0049] 图 24 的图示出在靶条件下对三种单克隆抗体进行重复分析的重叠色谱图。

[0050] 图 25 的图示出不同色谱条件下 MAb3 的重叠色谱图。将主峰对齐。

[0051] 图 26 的分布图示出盐浓度对色谱性能的影响。圈表示主峰的百分率,菱形表示酸性变体百分率,× 表示碱性变体百分率,三角形表示分辨率 1,且 * 表示分辨率 2。

[0052] 图 27 的分布图示出另一些参数对色谱性能的影响。左上图示出缓冲剂浓度 (mM) 的影响。右上图示出起始 pH 的影响。左下图示出以 °C 计的柱温的影响。右下图示出流量 (ml/分钟) 的影响。

[0053] 发明详述

[0054] 本发明提供了分析包含多肽以及一种或多种污染物、例如多肽变体的组合物的方法,其包括使用具有初始 pH 和初始离子强度的加样缓冲剂 (loading buffer) 使所述多肽以及一种或多种污染物结合至离子交换色谱材料,使用洗脱缓冲剂从所述离子交换柱洗脱所述多肽以及一种或多种污染物,其中通过 pH 梯度改变所述洗脱缓冲剂的 pH,并且通过离子强度梯度改变所述洗脱缓冲剂的离子强度,从而使所述多肽和所述一种或多种污染物作为不同的分离实体从色谱材料洗脱。本发明的方法可用于分析等电点不处于中性 pH 范围

的多肽。在一些实施方案中,所述方法可用于有效地从污染物分离 pI 大于 9 的多肽。在其他实施方案中,所述方法可用于有效地从污染物分离 pI 小于 7 的多肽。在一些实施方案中,所述方法有效地从多肽分离一种或多种污染物,其中所述多肽具有范围介于约 7.0 至约 12 的 pI。在一些实施方案中,所述方法可用于有效地从多肽分离一种或多种污染物,其中所述多肽具有范围介于约 7.0 至约 12 的 pI。多肽的例子包括但不限于抗体和抗体片段。污染物的例子包括但不限于抗体变体,例如抗体电荷变体。

[0055] 在其他方面,本发明提供了分析包含多肽以及一种或多种污染物、例如多肽变体的组合物的方法,其包括使用具有初始 pH 和初始离子强度的加样缓冲剂使所述多肽以及一种或多种污染物结合至离子交换色谱材料,使用洗脱缓冲剂从所述离子交换柱洗脱所述多肽以及一种或多种污染物,其中通过 pH 梯度改变所述洗脱缓冲剂的 pH,并且所述离子强度基本保持相同,从而使所述多肽和所述一种或多种污染物作为不同的分离实体从色谱材料洗脱。本发明的方法可用于分析等电点不处于中性 pH 范围的多肽。在一些实施方案中,所述方法可用于有效地从污染物分离 pI 大于 9 的多肽。在其他实施方案中,所述方法可用于有效地从污染物分离 pI 小于 7 的多肽。多肽的例子包括但不限于抗体和抗体片段。污染物的例子包括但不限于抗体变体,例如抗体电荷变体。

[0056] 在一些方面中,本发明提供了分析包含多肽以及一种或多种污染物、例如多肽变体的组合物的方法,其包括使用具有初始 pH 和初始离子强度的加样缓冲剂使所述多肽以及一种或多种污染物结合至离子交换色谱材料,使溶液中多肽的电荷状态落在最优 pH 梯度离子交换分离窗内。然后将所述多肽和所述一种或多种污染物作为不同的分离实体从色谱材料洗脱。

[0057] 在本发明的一些实施方案中,所述污染物是多肽电荷变体,包括酸性变体,即阳离子交换模式中保留时间小于主峰的变体。酸性变体的例子包括但不限于其中一个或多个谷氨酰胺和 / 或天冬酰胺残基已经被脱酰胺化的多肽。在本发明的一些实施方案中,所述污染物是多肽电荷变体,包括碱性变体,即阳离子交换模式中保留时间大于主峰的变体。例子包括但不限于其中天冬氨酸残基已被修饰成琥珀酰亚胺部分的变体。

[0058] 在一些实施方案中,本发明提供了分析包含多肽以及一种或多种污染物的组合物的方法,其中基本相同的方法可用于分析具有不同 pI 的多肽。例如,所述方法可用于分析 pI 介于 6.0 至 9.5 范围的多肽。在一些实施方案中,所述多肽是抗体或其片段。在一些实施方案中,所述污染物是抗体变体或抗体片段的变体。在一些实施方案中,所述污染物是抗体电荷变体或抗体片段的电荷变体。在一些实施方案中,本发明提供了用于分析抗体或抗体片段的组合物中电荷变体(例如酸性变体和 / 或碱性变体)的存在的方法,其中所述方法可用于分析包含抗体的不同组合物,其中所述抗体具有范围介于 6.0 至 9.5 的 pI。

[0059] I. 定义

[0060] 术语“多肽”或“蛋白质”在本文中可交换用于表示任何长度的氨基酸聚合物。所述聚合物可为直链的或支化的,其可包含修饰的氨基酸,并且其可由非氨基酸中断。该术语还涵盖经过天然修饰或通过干涉修饰的氨基酸聚合物;所述修饰例如二硫键形成、糖基化、脂化、乙酰化、磷酸化或任何其他操作或修饰,例如用标记性组分缀合。还包含于该定义内的是例如包含一个或多个氨基酸类似物(包括例如非天然氨基酸等)以及本领域已知的其他修饰的多肽。本文所用术语“多肽”和“蛋白质”特别涵盖抗体。

[0061] 本文所用术语“多肽电荷变体”是指从其天然状态修饰成使多肽的电荷发生改变的多肽。在一些例子中,电荷变体比母多肽更具酸性;即具有比母多肽更低的pI。在其他例子中,电荷变体比母多肽更具碱性;即具有比母多肽更高的pI。这样的修饰可为工程化的,或者是天然过程的结果,例如氧化、脱酰胺化、赖氨酸残基C端加工、N端焦谷氨酸形成和糖基化。在一些例子中,多肽电荷变体是糖蛋白,其中附接至蛋白质的多糖被修饰成该糖蛋白的电荷与母糖蛋白相比发生改变,例如,通过添加唾液酸或其衍生物。本文所用的“抗体电荷变体”是抗体或其片段,其中所述抗体或其片段已从其天然状态修饰成所述抗体或其片段的电荷发生改变。

[0062] “纯化的”多肽(例如抗体或免疫粘附素)意为多肽的纯度升高,使其以比其存在于天然环境中或/或比其当在实验室条件下初始合成和/或扩增时更纯的形式存在。纯度是相对性的术语,并且不一定是指绝对纯度。

[0063] 术语“拮抗剂”以最广含义使用,并且包括部分或完全阻断、抑制或中和天然多肽的生物活性的任何分子。以相似的方式,术语“激动剂”以最广含义使用,并且包括任何模拟天然多肽的生物活性的任何分子。合适的激动剂或拮抗剂分子具体包括天然多肽的激动剂或拮抗剂抗体或抗体片段、片段或氨基酸序列变体等。用于鉴定多肽的激动剂或拮抗剂的方法可包括使多肽与候选激动剂或拮抗剂分子接触,并测量通常与所述多肽相关的一种或多种生物学活性的可检测变化。

[0064] “结合”感兴趣抗原、例如肿瘤相关多肽抗原标靶的多肽是这样一种多肽,其以充分亲和力结合抗原,使得该多肽可用作靶向表达所述抗原的细胞或组织的诊断剂或治疗剂,并且不显著地与其他多肽交叉反应。在这样的实施方案中,通过荧光活化细胞分选(FACS)分析或放射免疫沉淀(RIA)确定,多肽结合“非标靶”多肽的程度将小于该多肽结合其特定靶多肽的约10%。

[0065] 关于多肽与靶分子的结合,术语“特异性结合”或“特异性结合至”或“特异于”特定多肽或特定多肽标靶上的表位,意为可测量地不同于非特异性相互作用的结合。例如,特异性结合可通过确定分子的结合与对照分子的结合的比较来测量,所述对照分子一般是不具有结合活性的相似结构的分子。例如,特异性结合可通过与和标靶相似的对照分子、例如过量的非标记标靶进行竞争来确定。在这种情况下,如果标记的标靶对探针的结合被过量的未标记标靶竞争性抑制,则表示存在特异性结合。

[0066] 本文的术语“抗体”以最广的含义使用,并且具体涵盖单克隆抗体、多克隆抗体、由至少两个完整抗体形成的多特异性抗体(例如双特异性抗体),以及抗体片段,只要表现出期望生物学活性即可。术语“免疫球蛋白”(Ig)在本文中可与抗体互换使用。

[0067] 抗体是天然存在的免疫球蛋白分子,其具有多种结构,所有结构都基于免疫球蛋白折叠。例如,IgG抗体具有两条“重”链和两条“轻”链,其通过二硫键形成有功能的抗体。每条重链和轻链本身都包含“恒定”(C)区和“可变”(V)区。V区确定抗体的抗原结合特异性,而C区提供结构支撑以及与免疫效应子的非抗原特异性相互作用中的功能。抗体或抗体的抗原结合片段的抗原结合特异性是抗体特异性结合特定抗原的能力。

[0068] 抗体的抗原结合特异性通过V区的结构特征来确定。变异性在可变结构域的110个氨基酸范围之间不是平均分布的。相反,V区由被各为9至12个氨基酸长的称为“高变区”的极具变异性的较短区域分隔的、15至30个氨基酸的相对不变的延伸(称为构架区,

FR) 组成。天然的重链和轻链的可变结构域各自包含四个 FR, 大多采取 β -叠片构型, 由三个高变区连接, 高变区形成环, 连接 β -叠片结构且在一些情况下形成 β -叠片结构的部分。各链中的高变区被 FR 维持紧邻在一起, 并且与来自其他链的高变区一起促进形成抗体的抗原结合位点 (参见 Kabat 等, *Sequences of Proteins of Immunological Interest*, 第 5 版, Public Health Service, National Institutes of Health, Bethesda, Md. (1991))。恒定结构域不直接涉及使抗体结合至抗原, 但表现出多种效应子功能, 例如抗体参与抗体依赖的细胞介导的细胞毒性 (ADCC)。

[0069] 每个 V 区通常包含三个互补决定区 (“CDR”, 每一个都包含“高变环”), 以及四个构架区。抗体结合位点——以高亲和力结合至特定抗原所需的最小结构单元——因此将通常包含三个 CDR 和至少三个、优选四个穿插其间从而将以适当构象维持和呈递 CDR 的构架区。典型的四链抗体具有由 VH 和 VL 结构域协同限定的抗原结合位点。某些抗体如骆驼抗体或鲨鱼抗体, 没有轻链, 并且依靠由仅重链形成的结合位点。可制备单一结构域工程化免疫球蛋白, 其中结合位点由仅重链或轻链形成, 没有 VH 和 VL 之间的协同作用。

[0070] 术语“可变”是指这样的事实, 即可变结构域某些部分的序列在抗体之间广泛不同, 并且用于各特定抗体针对其特定抗原的结合和特异性。然而, 该变异性不是平均遍布于抗体的可变结构域。无论是在轻链可变结构域还是在重链可变结构域中, 其都浓缩于三个被称为高变区的区段中。可变结构域的更高保守的部分称为构架区 (FR)。天然重链和轻链可变结构域各自包含四个 FR, 大多采取 β -叠片构型, 由三个高变区连接, 高变区形成环, 连接 β -叠片结构且在一些情况下形成 β -叠片结构的部分。各链中的高变区被 FR 维持紧邻在一起, 并且与来自其他链的高变区一起促进形成抗体的抗原结合位点 (参见 Kabat 等, *Sequences of Proteins of Immunological Interest*, 第 5 版, Public Health Service, National Institutes of Health, Bethesda, MD. (1991))。恒定结构域不直接涉及使抗体结合至抗原, 但表现出多种效应子功能, 例如抗体参与抗体依赖的细胞介导的细胞毒性 (ADCC)。

[0071] 用于本文时, 术语“高变区”是指抗体的负责抗原结合的氨基酸残基。高变区可包含来自“互补决定区”或“CDR” (例如 VL 中的大约第 24 至 34 位 (L1)、第 50 至 56 位 (L2) 和第 89 至 97 位 (L3) 残基, 以及 VH 中的大约第 31 至 35B 位 (H1)、第 50 至 65 位 (H2) 和第 95 至 102 位 (H3) 残基 (Kabat 等, *Sequences of Proteins of Immunological Interest*, 第 5 版, Public Health Service, National Institutes of Health, Bethesda, Md. (1991))), 和 / 或来自“高变环”的那些残基 (例如 VL 中的第 26 至 32 位 (L1)、第 50 至 52 位 (L2) 和第 91 至 96 位 (L3) 残基, 以 VH 中的第 26 至 32 位 (H1)、第 52A 至 55 位 (H2) 和第 96 至 101 位 (H3) 残基 (Chothia 和 Lesk J. Mol. Biol. 196:901-917(1987)))。

[0072] 如本文定义, “骨架”或“FR”残基是除了高变区残基外的可变结构域残基。

[0073] “抗体片段”包含完整抗体的一部分, 优选包含其抗原结合区。抗体片段例子包括 Fab、Fab'、F(ab')₂ 和 Fv 片段; 双抗体; 串联双抗体 (taDb)、线性抗体 (例如美国专利 No. 5, 641, 870, 实施例 2; Zapata 等, *Protein Eng.* 8(10):1057-1062(1995)); 单臂抗体、单一可变结构域抗体、微型抗体、单链抗体分子; 由抗体片段形成的多特异性抗体 (例如包括但不限于 Db-Fc、taDb-Fc、taDb-CH₃、(scFV)₄-Fc、二-scFv、bi-scFv 或串联 (双, 三)-scFv); 以及双特异性 T 细胞衔接物 (BiTE)。

[0074] 抗体木瓜蛋白酶消化产生两个相同的、被称为“Fab”片段的抗原结合片段（每个片段具有单一的抗原结合位点），以及残余的“Fc”片段（其名称反映了其易于结晶的能力）。胃蛋白酶处理产生了具有两个抗原结合位点并且仍然能够交联抗原的 $F(ab')_2$ 片段。

[0075] “Fv”是包含完全抗原识别和抗原结合位点的最小抗体片段。该区域由一条重链和一条轻链可变结构域以紧密的非共价结合形成的二聚物组成。正是该构型使各可变结构域的三个高变区相互作用从而限定 V_H - V_L 二聚物表面上的抗原结合位点。这六个高变区共同赋予抗体以抗原结合特异性。然而，即使是单一的可变结构域（或Fv的包含仅三个对抗原具有特异性的高变区的一半）亦具有识别和结合抗原的能力，尽管比全结合位点具有更低的亲和力。

[0076] Fab片段还包含轻链的恒定结构域和重链的第一恒定结构域（CH1）。Fab'片段与Fab片段的不同之处在于，添加了处于重链CH1结构域羧基末端的一些残基，包括来自抗体铰链区的一个或多个半胱氨酸。Fab'-SH是本文对Fab'的定义，其中恒定结构域的半胱氨酸残基（多个）带有至少一个游离巯基。 $F(ab')_2$ 抗体片段初始产生为其间具有铰链半胱氨酸的Fab'片段对。抗体片段的其他化学耦合也是已知的。

[0077] 来自任何脊椎动物种的抗体（免疫球蛋白）的“轻链”可基于其恒定结构域的氨基酸序列分为两种明显不同的类型中的一种，分别称为kappa(κ)和lambda(λ)。

[0078] 根据其重链的恒定结构域的氨基酸序列，抗体可分为不同类型。存在五个完整抗体的主要类型：IgA、IgD、IgE、IgG和IgM，并且其中一些可进一步分为亚类（亚型），例如IgG1、IgG2、IgG3、IgG4、IgA和IgA2。与这些不同抗体类型相对应的重链恒定结构域分别称为 α 、 δ 、 ϵ 、 γ 和 μ 。不同类型的免疫球蛋白的亚基结构和三维构型是公知的。

[0079] “单链Fv”或“scFv”抗体片段包含抗体的 V_H 和 V_L 结构域，其中这些结构域存在于单一的多肽链中。在一些实施方案中，Fv多肽还包含 V_H 结构域与 V_L 结构域之间的多肽接头，这使scFv能够形成用于抗原结合的期望结构。scFv的综述参见Plückthun in The Pharmacology of Monoclonal Antibodies, 第113卷, Rosenberg和Moore编, Springer-Verlag, New York, 第269-315页(1994)。

[0080] 术语“双抗体”是指具有两个抗原结合位点的小抗体片段，所述片段包含在相同多肽链（ V_H - V_L ）中连接至轻链可变结构域（ V_L ）的重链可变结构域（ V_H ）。通过使用短至不能使相同链上的两个结构域配对的接头来迫使结构域与另一链上的互补结构域配对，并创造出两个抗原结合位点。在例如以下文献中更完整地描述了双抗体：EP 404,097；WO 93/11161；以及Hollinger等, Proc. Natl. Acad. Sci. USA, 90:6444-6448(1993)。

[0081] 术语“多特异性抗体”以最广含义使用，并且特别涵盖具有多表位特异性的抗体。这样的多特异性抗体包括但不限于：包含重链可变结构域（ V_H ）和轻链可变结构域（ V_L ）、其中所述 V_H V_L 单元具有多表位特异性的抗体；具有两个或多个 V_L 和 V_H 结构域、各 V_H V_L 单元结合不同表位的抗体；具有两个或多个单一可变结构域、各单一可变结构域结合不同表位的抗体；全长抗体；抗体片段如Fab、Fv、dsFv、scFv、双抗体、双特异性双抗体、三抗体、三功能抗体，已经被共价或非共价连接的抗体片段。“多表位特异性”是指特异性结合相同或不同靶（多种）上的两个或更多个不同表位的能力。“单特异性”是指结合仅一个表位的能力。根据一个实施方案，所述多特异抗体是以 $5\mu M$ 至 $0.001pM$ 、 $3\mu M$ 至 $0.001pM$ 、 $1\mu M$ 至 $0.001pM$ 、 $0.5\mu M$ 至 $0.001pM$ 或 $0.1\mu M$ 至 $0.001pM$ 的亲和力结合各表位的IgG抗体。

[0082] 表述“单一结构域抗体”(sdAb)或“单一可变结构域(SVD)抗体”一般是指其中单一可变结构域(VH或VL)可赋予抗原结合的抗体。换言之,单一可变结构域不需要为了识别靶抗原而与另一可变结构域相互作用。单一结构域抗体的例子包含来源于骆驼科(camelids)(驼羊(lamas)和骆驼)和软骨鱼(例如护士鲨)的那些和通过重组方法衍生自人抗体和小鼠抗体的那些(Nature(1989)341:544-546; Dev Comp Immunol(2006)30:43-56;Trend Biochem Sci(2001)26:230-235; Trends Biotechnol(2003):21:484-490;WO 2005/035572;WO 03/035694;Febs Lett(1994)339:285-290;W000/29004;WO 02/051870)。

[0083] 本文所用术语“单克隆抗体”是指得自基本同质的抗体群的抗体,即组成群体的单个抗体是相同的和/或结合相同表位,除非在单克隆抗体的生产期间可产生可能的变体,这样的变体一般以少量存在。与通常包含针对不同决定子(表位)的不同抗体的多克隆抗体相比,每种单克隆抗体都针对抗原上单一的决定子。除了其特异性外,单克隆抗体的优点还在于,它们未被其他免疫球蛋白污染。修饰语“单克隆”表示这样的抗体特征,即得自基本同质的抗体群,并且其不应解释为需要通过任何特定的方法来产生该抗体。例如,根据本文提供的方法使用的单克隆抗体可通过由Kohler等Nature 256:495(1975)首次描述的杂交瘤法来制成,或者可通过重组DNA法来制成(参见例如美国专利No. 4,816,567)。“单克隆抗体”还可通过使用例如Clackson等Nature 352:624-628(1991)和Marks等J. Mol. Biol. 222:581-597(1991)描述的技术分离自噬菌体抗体文库。

[0084] 在本文中,单克隆抗体特别包括“嵌合”抗体(免疫球蛋白),其中重链和/或轻链的部分与来源于特定种的或者属于特定抗体类型或亚类型的抗体的对应序列相同或同源,而一条或多条链的其余部分与来源于另一种或者属于另一抗体类型或亚类型的抗体的对应序列相同或同源,以及这样的抗体的片段,只有它们表现出期望的生物学活性即可(美国专利No. 4,816,567;Morrison等,Proc. Natl. Acad. Sci. USA 81:6851-6855(1984))。本文中感兴趣的嵌合抗体包括包含来源于非人灵长类(例如旧大陆猴(Old World Monkey),如狒狒、恒河猴或食蟹猴)的可变结构域抗原结合序列和人恒定区序列的“灵长类化的(primatized)”抗体(US Pat No. 5,693,780)。

[0085] 非人(例如鼠科)抗体的“人源化”形式是包含来源于非人免疫球蛋白的最小序列的嵌合抗体。大多数情况下,人源化抗体是人免疫球蛋白(受体抗体),其中受体高变区的残基被来自非人物种(供体抗体)如小鼠、大鼠、兔子或非人灵长类、具有期望特异性、亲和力和能力的高变区的残基替换。在一些例子中,人免疫球蛋白的构架区(FR)残基被对应的非人残基替换。此外,人源化抗体可包括未在受体抗体或供体抗体中发现的残基。进行这些改造是为了进一步改善抗体性能。一般而言,人源化抗体将包含几乎所有的至少一个(通常两个)可变结构域,其中所有或几乎所有高变环与非人免疫球蛋白的高变环对应,并且除了上述FR取代之外,所有或几乎所有FR是人免疫球蛋白序列的FR。人源化抗体还将任选包含免疫球蛋白恒定区的至少部分,通常为人免疫球蛋白恒定区。另一些细节参见Jones等,Nature 321:522-525(1986);Riechmann等Nature 332:323-329(1988);以及Presta, Curr. Op. Struct. Biol. 2:593-596(1992)。

[0086] 出于本文的目的,“完整抗体”是包含重链和轻链可变结构域以及Fc区的抗体。恒定结构域可以是天然序列恒定结构域(例如人天然序列恒定结构域)或其氨基酸序列变

体。优选地,完整抗体可具有一种或多种效应子功能。

[0087] “天然抗体”通常是约 150,000 道尔顿的异四聚体糖蛋白,由两条相同的轻(L)链和两条相同的重(H)链组成。每条轻链都通过一个共价二硫键连接至重链,而不同的免疫球蛋白亚型的重链之间,二硫键连接的数量不同。每条重链和轻链还都有规律地间隔着链内二硫桥。每条重链在其一端具有可变结构域(V_H)继之以一些恒定结构域。每条轻链的一端具有可变结构域(V_L)并且另一端具有恒定结构域;轻链的恒定结构域与重链的第一恒定结构域对齐,并且轻链可变结构域与重链的可变结构域对齐。特定的氨基酸残基被认为形成处于轻链与重链可变结构域之间的界面。

[0088] “裸抗体”是(如本文所定义)未缀合至例如细胞毒性部分或放射性标记等异源分子的抗体。

[0089] 在一些实施方案中,抗体“效应子功能”是指可归因于抗体的 Fc 区(天然序列 Fc 区或氨基酸序列变体 Fc 区)的那些生物学活性,并且随着抗体亚型而变化。抗体效应子功能的例子包括: C1q 结合和补体依赖的细胞毒性; Fc 受体结合; 抗体依赖的细胞介导的细胞毒性(ADCC); 吞噬作用; 细胞表面受体的下调。

[0090] “抗体依赖的细胞介导的细胞毒性”和“ADCC”是指细胞介导的反应,其中表达 Fc 受体(FcR)的非特异性细胞毒性细胞(例如天然杀伤(NK)细胞、嗜中性粒细胞和巨噬细胞)识别靶细胞上结合的抗体并随后引起靶细胞裂解。用于介导 ADCC 的主要细胞是 NK 细胞,其仅表达 $Fc\gamma RIII$,而单核细胞表达 $Fc\gamma RI$ 、 $Fc\gamma RII$ 和 $Fc\gamma RIII$ 。Ravetch 和 Kinet, *Annu. Rev. Immunol* 9:457-92(1991) 第 464 页的表 3 概述了造血细胞上的 FcR 表达。为了评价感兴趣分子的 ADCC 活性,进行了例如在美国专利 No. 5,500,362 或 5,821,337 中描述的体外 ADCC 测定。用于这样的测定的可用效应细胞包括外周血单核细胞(PBMC)和天然杀伤(NK)细胞。作为另一选择或此外,可在体内评估感兴趣分子的 ADCC 活性,例如如 Clynes 等 *Proc. Natl. Acad. Sci. (USA)* 95:652-656(1998) 公开的在动物模型内进行。

[0091] “人效应细胞”是表达一种或多种 FcR 并执行效应子功能的白细胞。在一些实施方案中,所述细胞表达至少 $Fc\gamma RIII$,并执行 ADCC 效应子功能。介导 ADCC 的人白细胞的例子包括外周血单核细胞(PBMC)、天然杀伤(NK)细胞、单核细胞、细胞毒性 T 细胞和嗜中性粒细胞;其中 PBMC 和 NK 细胞是优选的。

[0092] “补体依赖的细胞毒性”或“CDC”是指分子在补体的存在下裂解标靶的能力。补体活化途径由补体系统的第一组分(C1q)结合与同源抗原复合的分子(例如多肽(例如抗体))而引发。为了评价补体活化,可进行 CDC 测定,例如如 Gazzano-Santoro 等, *J. Immunol. Methods* 202:163(1996) 中所描述。

[0093] 术语“Fc 受体”或“FcR”用于描述结合抗体 Fc 区的受体。在一些实施方案中, FcR 是天然序列人 FcR。此外,优选 FcR 是结合 IgG 抗体(γ 受体)的 FcR,并且包括 $Fc\gamma RI$ 、 $Fc\gamma RII$ 和 $Fc\gamma RIII$ 亚类的受体,包括等位基因变体以及这些受体的可选剪切形式。 $Fc\gamma RII$ 受体包括 $Fc\gamma RIIA$ (“活化性受体”)和 $Fc\gamma RIIB$ (“抑制性受体”),其具有相似的氨基酸序列,区别主要在其胞质结构域。活化性受体 $Fc\gamma RIIA$ 在其胞质结构域中包含免疫受体酪氨酸基活化基序(ITAM)。抑制性受体 $Fc\gamma RIIB$ 在其胞质结构域中包含免疫受体酪氨酸基抑制基序(ITIM)。(参见 Daëron, *Annu. Rev. Immunol.* 15:203-234(1997))。FcR 在以下文献中有综述, Ravetch 和 Kinet, *Annu. Rev. Immunol* 9:457-92(1991); Capel 等,

Immunomethods 4:25-34(1994) ; 以及 de Haas 等, J. Lab. Clin. Med. 126:330-41(1995)。本文的术语“FcR”涵盖其他 FcR, 包括未来将鉴定的 FcR 在内。该术语还包括新生儿受体 FcRn, 该受体负责将母体 IgG 传递至胎儿 (Guyer 等, J. Immunol. 117:587(1976), 以及 Kim 等, J. Immunol. 24:249(1994))。

[0094] “污染物”是指与期望多肽产物不同的材料。在本发明的一些实施方案中, 污染物包括多肽的电荷变体。在本发明的一些实施方案中, 污染物包括抗体或抗体片段的电荷变体。在本发明的另一些实施方案中, 污染物包括而限于: 宿主细胞材料, 如 CHOP ; 浸沥的 (leached) 蛋白 A ; 核酸 ; 期望多肽的变体、片段、聚集物或衍生物 ; 另一种多肽 ; 内毒素 ; 病毒污染物 ; 细胞培养基组分等。在一些例子中, 污染物可为来自例如但不限于细菌细胞 (例如大肠杆菌细胞)、昆虫细胞、原核细胞、真核细胞、酵母菌细胞、哺乳动物细胞、鸟类细胞、真菌细胞的宿主细胞蛋白 (HCP)。

[0095] 本文所用术语“免疫粘附素”表示组合了异源多肽的结合特异性与免疫球蛋白恒定结构域的效应子功能的类抗体分子。从结构上而言, 免疫粘附素包含氨基酸序列与免疫球蛋白恒定结构域序列的融合物, 所述氨基酸序列具有期望的结合特异性, 其不同于抗体的抗原识别和结合位点 (即是“异源的”)。免疫粘附素分子的粘附素部分通常是至少包含受体或配体的结合位点的连续的氨基酸序列。免疫粘附素中的免疫球蛋白恒定结构域序列可得自任何免疫球蛋白, 例如 IgG-1、IgG-2、IgG-3 或 IgG-4 亚型、IgA (包括 IgA-1 和 IgA-2)、IgE、IgD 或 IgM。

[0096] 本文所用的“基本相同”表示, 值或参数未以显著效果变化。例如, 如果离子强度未显著变化, 则柱出口处的色谱流动相的离子强度与流动相的初始离子强度基本相同。例如, 柱出口处的离子强度处于初始离子强度的 10%、5% 或 1% 以内, 其与初始离子强度基本相同。

[0097] 关于“约”, 本文的值或参数包括 (或描述) 针对该值或参数本身的变体。例如, 关于“约 X”的描述包括“X”的描述。

[0098] 除非上下文另有清楚指明, 否则本文以及所附权利要求中所用的单数形式“一个”、“一种”和“该 / 所述”包括复数引用。应理解, 本文中描述的本发明方面和变体包括“由”和 / 或“基本由”方面和变体“组成”。

[0099] II. 色谱方法

[0100] 在一些方面中, 本发明提供了分析包含多肽以及一种或多种污染物如多肽电荷变体的组合物的方法, 其包括使用具有初始 pH 和初始离子强度的加样缓冲剂使所述多肽以及一种或多种污染物结合至离子交换色谱材料, 使用洗脱缓冲剂从所述离子交换柱洗脱所述多肽以及一种或多种污染物, 其中通过 pH 梯度改变所述洗脱缓冲剂的 pH, 并且通过离子强度梯度改变所述洗脱缓冲剂的离子强度, 从而使所述多肽和所述一种或多种污染物作为不同的分离实体从色谱材料洗脱。

[0101] 在其他方面, 本发明提供了分析包含多肽以及一种或多种污染物如多肽变体的组合物的方法, 其包括使用具有初始 pH 和初始离子强度的加样缓冲剂使所述多肽以及一种或多种污染物结合至离子交换色谱材料, 使用洗脱缓冲剂从所述离子交换柱洗脱所述多肽以及一种或多种污染物, 其中通过 pH 梯度改变所述洗脱缓冲剂的 pH, 并且通过离子强度梯度改变所述洗脱缓冲剂的离子强度, 从而使所述多肽和所述一种或多种污染物作为不同的

分离实体从色谱材料洗脱。

[0102] 在本文描述的任何方法的一些实施方案中,所述色谱材料是阳离子交换材料。在一些实施方案中,所述阳离子交换材料是带负电的固相,并且具有自由阳离子用于与跨过或通过所述固相的水性溶液中的阳离子交换。在本文描述的任何方法的一些实施方案中,所述阳离子交换材料可为膜、整料(monolith)或树脂。在一些实施方案中,所述阳离子交换材料可为树脂。所述阳离子交换材料可包含羧酸官能团或磺酸官能团,例如但不限于磺酸根、羧酸、羧甲基磺酸、磺基异丁基、磺基乙基、羧基、磺基丙基、磺酰基、次硫酸乙基(sulphoxyethyl)或正磷酸根。在上文的一些实施方案中,所述阳离子交换色谱材料是阳离子交换色谱柱。在一些实施方案中,阳离子交换色谱材料用于 pI 大于约 9 的多肽,例如抗体或其片段。例如,抗体或其片段可具有 9 至 10、9 至 11、10 至 11、9 至 12、10 至 12 或 11 至 12 的 pI。

[0103] 在本文描述的任何方法的一些实施方案中,所述色谱材料是阴离子交换材料。在一些实施方案中,所述阴离子交换色谱材料是带正电的固相,并且具有自由阴离子用于与跨过或通过所述固相的水性溶液中的阴离子交换。在本文描述的任何方法的一些实施方案中,所述阴离子交换材料可为膜、整料或树脂。在一个实施方案中,所述阴离子交换材料可为树脂。在一些实施方案中,所述阴离子交换材料可包含伯胺、仲胺、叔胺或季铵离子官能团、多胺官能团或二乙基氨基乙基官能团。在上文的一些实施方案中,所述阴离子交换色谱材料是阴离子交换色谱柱。在一些实施方案中,阴离子交换色谱材料用于具有小于约 7 的 pI 的多肽,例如抗体或其片段。例如,抗体或其片段可具有 6 至 7、5 至 7、5 至 6、4 至 7、4 至 6 或 4 至 5 的 pI。

[0104] 在本文描述的任何方法的一些实施方案中,所述离子交换材料可利用常规色谱材料或对流色谱材料。所述常规色谱材料包括例如灌注性材料(例如聚(苯乙烯-二乙烯基苯)树脂)和扩散性材料(例如交联的琼脂糖树脂)。在一些实施方案中,所述聚(苯乙烯-二乙烯基苯)树脂可为 Poros 树脂。在一些实施方案中,所述交联的琼脂糖树脂可为磺基丙基-琼脂糖快流(sulphopropyl-Sepharose Fast Flow,“SPSFF”)树脂。所述对流色谱材料可为膜(例如聚醚砜)或整体式材料(例如交联聚合物)。所述聚醚砜膜可为 Mustang。所述交联的聚合物整体式材料可为交联的聚(缩水甘油基甲基丙烯酸酯-共-乙烯二甲基丙烯酸酯)。

[0105] 在本发明的方法的一些实施方案中,所述色谱材料处于色谱柱中;例如阳离子交换色谱柱或阴离子交换色谱柱。在一些实施方案中,所述色谱柱用于液相色谱。在一些实施方案中,所述色谱柱用于高效液相色谱(HPLC)。在一些实施方案中,所述色谱柱是 HPLC 色谱柱;例如,阳离子交换 HPLC 柱或阴离子交换 HPLC 柱。

[0106] 阳离子交换材料的例子是本领域已知的,包括但不限于:Mustang S、Sartobind S、S03 Monolith、S Ceramic HyperD、Poros XS、Poros HS50、Poros HS20、SPSFF、SP-Sepharose XL(SPXL)、CM Sepharose Fast Flow、Capto S、Fractogel Se HiCap、Fractogel S03 或 Fractogel C00。在本文描述的任何方法的一些实施方案中,所述阳离子交换材料是 Poros HS50。在一些实施方案中,所述 Poros HS 树脂可为 Poros HS 50 μm 或 Poros HS 20 μm 颗粒。用于本发明方法的阳离子交换色谱柱的例子包括但不限于 ProPac WCX-10 和 ProPac WCX-10HT。

[0107] 阴离子交换材料的例子是本领域已知,并且包括但不限于 Poros HQ 50、Poros PI 50、Poros D、Mustang Q、Q Sepharose FF 和 DEAE Sepharose。用于本发明方法的阴离子交换色谱柱的例子包括但不限于 Dionex ProPac 10SAX 和 Tosoh GSKgel Q STAT 7 μ M WAX。

[0108] 一种可用于本发明方法的示例性 HPLC 方法如下;但是,本发明的方法不应解释为受这些方法的限制。将样品添加至自动进样器,并冷藏 ($5\pm 3^{\circ}\text{C}$)。将柱放置于柱室中,并在分析期间可采用温度控制特征来使柱室温度处于设定值的窄范围 ($\pm 1^{\circ}\text{C}$) 内。在 280nm 监测柱流出物。

[0109] 用流动相将样品稀释至约 1mg/mL 至 2mg/mL 的目标多肽浓度。在一些实施方案中,可用以 1:100 (w/w) 的比率添加的羧肽酶 B (CpB) 消化多肽,并于 37°C 孵育 20 分钟。样品可在 5°C 储存直至分析。

[0110] 仪器可包括低压四元梯度泵、具有温度自动控制能力的快速分离自动进样器、热控柱室和二极管阵列 UV 检测器。在检测器的出口处,可连接 PCM-3000pH 和电导率监测器,以实时收集 pH 和电导率数据。可进行仪器控制、数据获取和数据分析;例如通过使用 Thermo Scientific Dionex Chromeleon 软件,6.8 版来进行。

[0111] 流动相制备物的非限制性例子如下。tris 和咪唑的单独的储备缓冲剂溶液制备为 1.0M, CAPS 溶液制备为 0.1M 浓度,不调节 pH 值,并储存于室温。使用去离子水将单独的组分稀释至约 90% 目标体积中的 10mM 终浓度,并使其混合。当溶液稀释 (quench) 至最终体积后,将混合物分成两等份。

[0112] 将缓冲剂的 pH 值用盐酸调节至 6.0 (缓冲剂 A),以及用氢氧化钠调节至 11.0 (缓冲剂 B)。用去离子水将氯化钠制备为 0.1M 溶液 (缓冲剂 C)。将 MilliQ 水 (18M Ω cm) 分配于单独的容器中 (缓冲剂 D)。然后,在使用前通过 0.2 μ m 尼龙过滤器单独过滤所有流动相。

[0113] 阳离子交换色谱的一个非限制性例子如下。用缓冲剂 A 与 D 的 1:1 混合物将单克隆抗体样品稀释至 1mg/mL,并于 $5\pm 3^{\circ}\text{C}$ 保存于自动进样器中。将 Propac WCX-10HT, $4\times 50\text{mm}$ 柱放置于温度设置为 $40\pm 1^{\circ}\text{C}$ 的柱室中。每次色谱运行注射 20 μ L 蛋白 (20 μ g)。流动相流速为 1.0mL/分钟。通过 280nm 的紫外 (UV) 吸光度来检测蛋白质。

[0114] 在一些例子中,通过使用缓冲剂 A、B、C 和 D、使用四元泵上形成的四元梯度来建立混合 pH 梯度。该布置提供了调节以下的灵活性:1) 起始 pH 和终止 pH,使用缓冲剂 A 和 B 调节,以及 2) 用于各梯度的盐的量,使用缓冲剂 C 和 D 调节。例如,通过将缓冲剂 B 从 0 增加至 40%、同时保持缓冲剂 C 和 D 分别为 10% 和 40% 来建立 6 至 10 的 pH 梯度以及恒定的 10mM 盐浓度。下面实施例的表 2 提供了混合梯度的例子。

[0115] 可根据例如期望的缓冲剂 pH、期望的缓冲剂电导率、感兴趣的蛋白质的性质以及分析方法来采用多种缓冲剂。

[0116] 本文所用的洗脱是从色谱材料移除产物,例如多肽和 / 或污染物。洗脱缓冲剂是从色谱材料洗脱多肽或其他感兴趣的产物所使用的缓冲剂。在一些实施方案中,所述洗脱缓冲剂是色谱流动相的部分。在一些实施方案中,将包含多肽和污染物的组合物作为流动相的部分施加至色谱材料。然后改变流动相,以随着多肽和污染物从色谱材料洗脱而使多肽从污染物分离。在许多情况下,洗脱缓冲剂具有与加样缓冲剂不同的物理特征。例如,洗脱缓冲剂可具有与加样缓冲剂不同的 pH 和 / 或与加样缓冲剂不同的离子强度。在一些实

施方案中,通过改变洗脱缓冲剂的 pH 和离子强度来从色谱材料洗脱多肽和污染物。在一些实施方案中,在洗脱过程中,相对于加样缓冲剂,提高洗脱缓冲剂的 pH 和洗脱缓冲剂的离子强度。在一些实施方案中,在洗脱过程中相对于加样缓冲剂提高洗脱缓冲剂的 pH,并使洗脱缓冲剂的离子强度保持基本相同。

[0117] 在本发明的一些实施方案中,将 $pI > 9$ 的多肽施用至阳离子交换色谱材料,并且通过提高色谱流动相的 pH 和离子强度来从阳离子交换色谱材料洗脱多肽。在本发明的一些实施方案中,将 $pI > 9$ 的多肽施用至阳离子交换色谱材料,并且通过提高色谱流动相的 pH 同时维持流动相的离子强度来从阳离子交换色谱材料洗脱多肽。

[0118] 在本发明的一些实施方案中,将 $pI < 7$ 的多肽施用至阴离子交换色谱材料,并且通过降低色谱流动相的 pH 并且提高色谱流动相的离子强度来从阴离子交换色谱材料洗脱多肽。在本发明的一些实施方案中,将 $pI < 7$ 的多肽施用至阴离子交换色谱材料,并且通过降低色谱流动相的 pH 同时维持色谱流动相的离子强度来从阴离子交换色谱材料洗脱多肽。

[0119] 在任意上述实施方案的一些方面中,通过线性梯度或阶跃梯度将洗脱缓冲剂的 pH 相对于加样缓冲剂改变。

[0120] 在本发明的一些实施方案中,通过提高流动相中洗脱缓冲剂的 pH 来从色谱材料洗脱多肽。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 5 升高至约 pH 11。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 6 升高至约 pH 9。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 6 升高至约 pH 10。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 6 升高至约 pH 11。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 7 升高至约 pH 9。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 7 升高至约 pH 10。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 7 升高至约 pH 11。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 8 升高至约 pH 9。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 8 升高至约 pH 10。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 8 升高至约 pH 11。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 9 升高至约 pH 11。在任何上述实施方案中,将洗脱缓冲剂的 pH 升高与洗脱缓冲剂的离子强度升高组合。在任何上述实施方案的其他实施方案中,提高洗脱缓冲剂的 pH,但是洗脱缓冲剂的离子强度保持基本相同。在任何上述实施方案中,在多于约 10 分钟、15 分钟、20 分钟、25 分钟、30 分钟、35 分钟、40 分钟、45 分钟、50 分钟、55 分钟或 60 分钟内建立 pH 梯度。在任何上述实施方案中,所述色谱材料是阳离子交换色谱材料。在任何上述实施方案中,所述多肽的 $pI > 9$ 。在任何上述实施方案中,所述多肽是抗体或其片段。

[0121] 在本发明的一些实施方案中,通过降低流动相中洗脱缓冲剂的 pH 来从色谱材料洗脱多肽。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 9 降低至约 pH 5。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 9 降低至约 pH 6。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 9 降低至约 pH 7。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 8 降低至约 pH 5。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 8 降低至约 pH 6。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 8 降低至约 pH 7。在一些实施方案中,洗脱缓冲剂的 pH 从约 pH 7 降低至约 pH 5。在任何上述实施方案中,将洗脱缓冲剂的 pH 降低与洗脱缓冲剂的离子强度升高组合。在任何上述实施方案的其他实施方案中,降低洗脱缓冲剂的 pH,但是洗脱缓冲剂的离子强度保持基本相同。在任何上述实施方案中,在多于约 10 分钟、15 分钟、20 分钟、25 分钟、30 分钟、35 分钟、40 分钟、45 分钟、50 分钟、55 分钟或 60 分钟内建立 pH 梯度。在任

何上述实施方案中,所述色谱材料是阴离子交换色谱材料。在任何上述实施方案中,所述多肽的 $pI < 7$ 。在任何上述实施方案中,所述多肽是抗体或其片段。

[0122] 可使用本领域已知的缓冲剂制备具有特定 pH 的洗脱缓冲剂。缓冲剂的例子包括但不限于哌嗪、咪唑、Tris、磷酸盐和 N- 环己基 -3- 氨基丙烷磺酸 (CAPS)。在一些实施方案中,缓冲剂的 pH 可通过例如添加 HCl 使缓冲剂更酸性或添加 NaOH 使缓冲剂更碱性来调节。在一些实施方案中,洗脱缓冲剂包含缓冲剂的组合。在一些实施方案中,洗脱缓冲剂包含哌嗪、咪唑和 Tris 的组合 (例如 PIT 缓冲剂)。在一些实施方案中,洗脱缓冲剂包含 11.6mM 哌嗪、1.5mM 咪唑和 2.4mM Tris。在一些实施方案中,洗脱缓冲剂包含 4mM 哌嗪、4mM 咪唑和 4mM Tris。在本发明的一些实施方案中,包含哌嗪、咪唑和 Tris 的组的洗脱缓冲剂用于多肽的阳离子交换色谱的流动相中。在本发明的一些实施方案中,包含哌嗪、咪唑和 Tris 的组的洗脱缓冲剂用于 $pI > 9$ 的多肽的阳离子交换色谱的流动相中。在本发明的一些实施方案中,包含哌嗪、咪唑和 Tris 的组的洗脱缓冲剂用于抗体的阳离子交换色谱的流动相中。在本发明的一些实施方案中,包含哌嗪、咪唑和 Tris 的组的洗脱缓冲剂用于 $pI > 9$ 的抗体的阳离子交换色谱的流动相中。在本发明的一些实施方案中,包含哌嗪、咪唑和 Tris 的组的洗脱缓冲剂用于多肽的阴离子交换色谱的流动相中。在本发明的一些实施方案中,包含哌嗪、咪唑和 Tris 的组的洗脱缓冲剂用于 $pI < 7$ 的多肽的阴离子交换色谱的流动相中。在本发明的一些实施方案中,包含哌嗪、咪唑和 Tris 的组的洗脱缓冲剂用于抗体的阴离子交换色谱的流动相中。在本发明的一些实施方案中,包含哌嗪、咪唑和 Tris 的组的洗脱缓冲剂用于 $pI < 7$ 的抗体的阴离子交换色谱的流动相中。在一些实施方案中,洗脱缓冲剂包含 Tris、哌嗪和磷酸盐的组合 (例如 TPP 缓冲剂)。在一些实施方案中,洗脱缓冲剂包含 5mM Tris、5mM 哌嗪和 5mM 磷酸盐。在一些实施方案中,洗脱缓冲剂包含 10mM Tris、10mM 哌嗪和 10mM 磷酸盐。在本发明的一些实施方案中,包含 Tris、哌嗪和磷酸盐的组的洗脱缓冲剂用于多肽的阳离子交换色谱的流动相中。在本发明的一些实施方案中,包含 Tris、哌嗪和磷酸盐的组的洗脱缓冲剂用于 $pI > 9$ 的多肽的阳离子交换色谱的流动相中。在本发明的一些实施方案中,包含 Tris、哌嗪和磷酸盐的组的洗脱缓冲剂用于抗体的阳离子交换色谱的流动相中。在本发明的一些实施方案中,包含 Tris、哌嗪和磷酸盐的组的洗脱缓冲剂用于 $pI > 9$ 的抗体的阳离子交换色谱的流动相中。在一些实施方案中,洗脱缓冲剂包含 Tris、咪唑和 CAPS 的组合 (例如 TIC 缓冲剂)。在一些实施方案中,洗脱缓冲剂包含 5mM Tris、5mM 咪唑和 5mM CAPS。在本发明的一些实施方案中,包含 Tris、咪唑和 CAPS 的组的洗脱缓冲剂用于多肽的阳离子交换色谱的流动相中。在本发明的一些实施方案中,包含 Tris、咪唑和 CAPS 的组的洗脱缓冲剂用于 $pI > 9$ 的多肽的阳离子交换色谱的流动相中。在本发明的一些实施方案中,包含 Tris、咪唑和 CAPS 的组的洗脱缓冲剂用于抗体的阳离子交换色谱的流动相中。在本发明的一些实施方案中,包含 Tris、咪唑和 CAPS 的组的洗脱缓冲剂用于 $pI > 9$ 的抗体的阳离子交换色谱的流动相中。

[0123] 在本发明的一些实施方案中,通过如上文所述的提高流动相中洗脱缓冲剂的 pH 并且通过提高流动相中洗脱缓冲液的离子强度来形成 pH 梯度和离子强度梯度,从而从色谱材料洗脱多肽。在一些实施方案中,通过提高色谱流动相的浓度来提高洗脱缓冲剂的离子强度。在本发明的其他实施方案中,通过提高洗脱缓冲剂的 pH 来从色谱材料洗脱多肽,在所述洗脱缓冲液中,通过在洗脱过程中维持盐浓度而使洗脱缓冲剂的离子强度在洗脱过

程中维持基本相同。在一些实施方案中,所述盐浓度选择为使多肽的电荷状态提供最优的 pH 梯度离子交换色谱分离窗。可通过本领域已知的造模方法来确定多肽的电荷状态。盐的例子包括但不限于 NaCl、KCl 和 Na₂SO₄。

[0124] 在一些实施方案中,所述离子强度梯度是盐梯度。在一些实施方案中,所述盐梯度是约 0mM 盐至约 200mM 盐的梯度。在一些实施方案中,所述盐梯度是以下任意梯度:约 0mM 至约 100mM、0mM 至约 60mM、0mM 至约 50mM、0mM 至约 40mM、0mM 至约 30mM、0mM 至约 20mM、0mM 至约 10mM、10mM 至约 200mM、10mM 至约 100mM、10mM 至约 50mM、10mM 至约 40mM、10mM 至约 30mM、10mM 至约 20mM、20mM 至约 200mM、20mM 至约 100mM、20mM 至约 50mM、20mM 至约 30mM、30mM 至约 200mM、30mM 至约 100mM,以及 30mM 至约 50mM。

[0125] 在上述实施方案的一些实施方案中,通过 pH 梯度和离子强度梯度的组合来从色谱材料洗脱多肽和 / 或一种或多种污染物。在一些实施方案中,pH 梯度为 pH 5 至 pH 10.8,且离子强度梯度为 0mM 盐至 16mM 盐。在一些实施方案中,pH 梯度为 pH 6 至 pH 11,且离子强度梯度为 0mM 盐至 60mM 盐。在一些实施方案中,pH 梯度为 pH 5 至 pH 9.5,且离子强度梯度为 0mM 盐至 16mM 盐。在一些实施方案中,pH 梯度为 pH 5 至 pH 9.5,且离子强度梯度为 0mM 盐至 30mM 盐。在任何上述实施方案中,所述盐是 NaCl、KCl 或 Na₂SO₄。在任何上述实施方案中,所述色谱材料是阳离子交换色谱材料。在任何上述实施方案中,所述多肽的 pI>9。在任何上述实施方案中,所述多肽是 pI>9 的抗体。

[0126] 在本发明的一些实施方案中,通过离子交换色谱来分析多肽和 / 或一种或多种污染物,其中通过 pH 梯度和离子强度梯度的组合来从色谱材料洗脱多肽和 / 或一种或多种污染物。在一些实施方案中,pH 梯度为 pH 7 至 pH 5,且离子强度梯度为 0mM 盐至 25mM 盐。在一些实施方案中,pH 梯度为 pH 7 至 pH 5,且离子强度梯度为 0mM 盐至 100mM 盐。在一些实施方案中,pH 梯度为 pH 7 至 pH 5,且离子强度梯度为 0mM 盐至 200mM 盐。在一些实施方案中,pH 梯度为 pH 7 至 pH 5,且离子强度梯度为 0mM 盐至 300mM 盐。在任何上述实施方案中,所述盐是 NaCl、KCl 或 Na₂SO₄。在任何上述实施方案中,所述色谱材料是阴离子交换色谱材料。在任何上述实施方案中,所述多肽的 pI<7。在任何上述实施方案中,所述多肽是 pI<7 的抗体。

[0127] 在本发明的一些实施方案中,通过离子交换色谱来分析多肽和 / 或一种或多种污染物,其中通过在洗脱期间流动相的离子强度保持基本相同的情况下的 pH 梯度来从色谱材料洗脱多肽和 / 或一种或多种污染物。在一些实施方案中,pH 梯度为 pH 6 至 pH 11,且流动相的离子强度为约 10mM 盐。在一些实施方案中,pH 梯度为 pH 6 至 pH 11,且流动相的离子强度为约 20mM 盐。在一些实施方案中,pH 梯度为 pH 6 至 pH 11,且流动相的离子强度为约 30mM 盐。在一些实施方案中,pH 梯度为 pH 6 至 pH 11,且流动相的离子强度为约 40mM 盐。在一些实施方案中,pH 梯度为 pH 6 至 pH 11,且流动相的离子强度为约 50mM 盐。在一些实施方案中,pH 梯度为 pH 6 至 pH 10,且流动相的离子强度为约 10mM 盐。在一些实施方案中,pH 梯度为 pH 6 至 pH 10,且流动相的离子强度为约 20mM 盐。在一些实施方案中,pH 梯度为 pH 6 至 pH 10,且流动相的离子强度为约 30mM 盐。在一些实施方案中,pH 梯度为 pH 6 至 pH 10,且流动相的离子强度为约 40mM 盐。在一些实施方案中,pH 梯度为 pH 6 至 pH 10,且流动相的离子强度为约 50mM 盐。在一些实施方案中,pH 梯度为 pH 6 至 pH 9,且流动相的离子强度为约 10mM 盐。在一些实施方案中,pH 梯度为 pH 6 至 pH 9,且流动相的

离子强度为约 20mM 盐。在一些实施方案中, pH 梯度为 pH 6 至 pH 9, 且流动相的离子强度为约 30mM 盐。在一些实施方案中, pH 梯度为 pH 6 至 pH 9, 且流动相的离子强度为约 40mM 盐。在一些实施方案中, pH 梯度为 pH 6 至 pH 9, 且流动相的离子强度为约 50mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 11, 且流动相的离子强度为约 10mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 11, 且流动相的离子强度为约 20mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 11, 且流动相的离子强度为约 30mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 11, 且流动相的离子强度为约 40mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 11, 且流动相的离子强度为约 50mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 10, 且流动相的离子强度为约 10mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 10, 且流动相的离子强度为约 20mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 10, 且流动相的离子强度为约 30mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 10, 且流动相的离子强度为约 40mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 10, 且流动相的离子强度为约 50mM 盐。在任何上述实施方案中, 所述盐是 NaCl、KCl 或 Na₂SO₄。在任何上述实施方案中, 所述色谱材料是阳离子交换色谱材料。在任何上述实施方案中, 所述多肽的 pI>9。在任何上述实施方案中, 所述多肽是 pI>9 的抗体。

[0128] 在本发明的一些实施方案中, 通过离子交换色谱来分析多肽和 / 或一种或多种污染物, 其中通过在洗脱期间流动相的离子强度保持基本相同的情况下的 pH 梯度组合来从色谱材料洗脱多肽和 / 或一种或多种污染物。在一些实施方案中, pH 梯度为 pH 8 至 pH 5, 且流动相的离子强度为约 10mM 盐。在一些实施方案中, pH 梯度为 pH 8 至 pH 5, 且流动相的离子强度为约 20mM 盐。在一些实施方案中, pH 梯度为 pH 8 至 pH 5, 且流动相的离子强度为约 30mM 盐。在一些实施方案中, pH 梯度为 pH 8 至 pH 5, 且流动相的离子强度为约 40mM 盐。在一些实施方案中, pH 梯度为 pH 8 至 pH 5, 且流动相的离子强度为约 50mM 盐。在一些实施方案中, pH 梯度为 pH 8 至 pH 5, 且流动相的离子强度为约 100mM 盐。在一些实施方案中, pH 梯度为 pH 8 至 pH 5, 且流动相的离子强度为约 200mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 5, 且流动相的离子强度为约 10mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 5, 且流动相的离子强度为约 20mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 5, 且流动相的离子强度为约 30mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 5, 且流动相的离子强度为约 40mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 5, 且流动相的离子强度为约 50mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 5, 且流动相的离子强度为约 100mM 盐。在一些实施方案中, pH 梯度为 pH 7 至 pH 5, 且流动相的离子强度为约 200mM 盐。在任何上述实施方案中, 所述盐是 NaCl、KCl 或 Na₂SO₄。在任何上述实施方案中, 所述色谱材料是阴离子交换色谱材料。在任何上述实施方案中, 所述多肽的 pI<7。在任何上述实施方案中, 所述多肽是 pI<7 的抗体。

[0129] 在本发明的一些实施方案中, 由流动相的电导率来度量例如洗脱缓冲剂等流动相的离子强度。电导率是指水溶液在两个电极之间的导电能力。在溶液中, 电流通过离子迁移来流动。因此, 通过提高水溶液中存在的离子的量, 溶液将具有更高的电导率。电导率的基本度量单位是 Siemen (或 mho)、mho (mS/cm), 并且可通过使用电导率计来测量, 例如多种型号的 Orion 电导率计。因为电解电导率是溶液中离子运载电流的能力, 所以溶液的电导率可通过改变其中的离子浓度来改变。例如, 可改变缓冲剂浓度和 / 或溶液中的盐 (例如

氯化钠、醋酸钠或氯化钾) 浓度从而达到期望的电导率。优选地, 改变多种缓冲剂的盐浓度来达到期望的电导率。

[0130] 在一些实施方案中, 色谱流动相具有大于约任何以下的初始电导率: 0.0mS/cm、0.5mS/cm、1.0mS/cm、1.5mS/cm、2.0mS/cm、2.5mS/cm、3.0mS/cm、3.5mS/cm、4.0mS/cm、4.5mS/cm、5.0mS/cm、5.5mS/cm、6.0mS/cm、6.5mS/cm、7.0mS/cm、7.5mS/cm、8.0mS/cm、8.5mS/cm、9.0mS/cm、9.5mS/cm、10mS/cm、11mS/cm、12mS/cm、13mS/cm、14mS/cm、15mS/cm、16mS/cm、17.0mS/cm、18.0mS/cm、19.0mS/cm 或 20.0mS/cm。在一些实施方案中, 例如通过离子强度梯度来在色谱过程期间提高流动相的电导率。在一些实施方案中, 在完成洗脱时流动相的电导率大于约任何以下电导率: 1.0mS/cm、1.5mS/cm、2.0mS/cm、2.5mS/cm、3.0mS/cm、3.5mS/cm、4.0mS/cm、4.5mS/cm、5.0mS/cm、5.5mS/cm、6.0mS/cm、6.5mS/cm、7.0mS/cm、7.5mS/cm、8.0mS/cm、8.5mS/cm、9.0mS/cm、9.5mS/cm、10mS/cm、11mS/cm、12mS/cm、13mS/cm、14mS/cm、15mS/cm、16mS/cm、17.0mS/cm、18.0mS/cm、19.0mS/cm 或 20.0mS/cm。在一些实施方案中, 通过线性梯度来提高流动相的电导率。在一些实施方案中, 通过包含一个或多个跨度的阶跃梯度来提高流动相的电导率。

[0131] 在一些实施方案中, 洗脱多肽时色谱流动相的初始电导率和流动相的最终电导率基本相同。在一些实施方案中, 流动相的电导率保持基本为大于约任何以下电导率: 0.0mS/cm、0.5mS/cm、1.0mS/cm、1.5mS/cm、2.0mS/cm、2.5mS/cm、3.0mS/cm、3.5mS/cm、4.0mS/cm、4.5mS/cm、5.0mS/cm、5.5mS/cm、6.0mS/cm、6.5mS/cm、7.0mS/cm、7.5mS/cm、8.0mS/cm、8.5mS/cm、9.0mS/cm、9.5mS/cm、10mS/cm、11mS/cm、12mS/cm、13mS/cm、14mS/cm、15mS/cm、16mS/cm、17.0mS/cm、18.0mS/cm、19.0mS/cm 或 20.0mS/cm。

[0132] 在本文描述的任何方法的一些实施方案中, 以多于任何以下的量将包含多肽和一种或多种污染物的组合物上载至色谱材料上: 约 1 μ g、2 μ g、3 μ g、4 μ g、5 μ g、6 μ g、7 μ g、8 μ g、9 μ g、10 μ g、15 μ g、20 μ g、25 μ g 或 50 μ g。在一些实施方案中, 以多于任何以下的浓度将组合物上载至色谱材料上: 约 0.5mg/mL、1.0mg/mL、1.5mg/mL、2.0mg/mL、2.5mg/mL 和 5.0mg/mL。在一些实施方案中, 在上载至色谱材料上之前稀释组合物; 例如, 稀释 1:1、1:2、1:5、1:10 或大于 1:10。在一些实施方案中, 将组合物稀释至色谱的流动相中。在一些实施方案中, 将组合物稀释至加样缓冲剂中。

[0133] 在本文描述的任何方法的一些实施方案中, 流速为大于约任何以下的流速: 0.5mL/分钟、0.6mL/分钟、0.7mL/分钟、0.8mL/分钟、0.9mL/分钟、1.0mL/分钟、1.1mL/分钟、1.2mL/分钟、1.3mL/分钟、1.4mL/分钟、1.5mL/分钟、1.75mL/分钟和 2.0mL/分钟。

[0134] 在本文描述的方法的一些实施方案中, 色谱材料处于柱中。在一些实施方案中, 所述柱是 HPLC 柱。在一些实施方案中, 所述柱具有任一以下尺寸: 4 $\times$ 50mm、4 $\times$ 100mm、4 $\times$ 150mm、4 $\times$ 200mm、4 $\times$ 250mm 或 2 $\times$ 250mm。

[0135] 在本发明的一些实施方案中, 所述方法是稳健的; 即可干扰一个或多个运行参数而不影响分析结果 (例如主峰与污染物峰的相对百分比)。在一些实施方案中, 加样缓冲剂和 / 或洗脱缓冲剂中的缓冲剂浓度在任一以下范围中变化: 约 10mM 至 50mM、10mM 至 40mM、10mM 至 30mM、10mM 至 20mM、20mM 至 50mM、20mM 至 40mM、20mM 至 30mM、30mM 至 50mM、30mM 至 40mM, 或 40mM 至 50mM。在一些实施方案中, 所述加样缓冲剂和 / 或所述洗脱缓冲剂中的缓冲剂浓度在约 10mM 至约 50mM 之间变化。在一些实施方案中, 第一 pH 在任一以下范围

中变化：约 pH 5.0 至 pH 7.0、pH 5.0 至 pH 6.5、pH 5.0 至 pH 6.0、pH 5.0 至 pH 5.5、pH 5.5 至 pH 7.0、pH 5.5 至 pH 6.5、pH 5.5 至 pH 6.0、pH 6.0 至 pH 7.0、pH 6.0 至 pH 6.5，或 pH 6.5 至 pH 7.0。在一些实施方案中，第一 pH 在约 pH 5.7 至约 pH 6.3 变化。在一些实施方案中，色谱材料的温度在任一以下范围中变化：约 20℃至 50℃、25℃至 50℃、30℃至 50℃、35℃至 50℃、40℃至 50℃、45℃至 50℃、20℃至 45℃、25℃至 45℃、30℃至 45℃、35℃至 45℃、40℃至 45℃、20℃至 40℃、25℃至 40℃、30℃至 40℃、35℃至 40℃、20℃至 35℃、25℃至 35℃、30℃至 35℃、20℃至 30℃、25℃至 30℃，或 20℃至 25℃。在一些实施方案中，色谱材料的温度在约 36℃至约 44℃变化。在一些实施方案中，加样和洗脱在这样的流速下进行，所述流速在任一以下范围中变化：约 0.5ml/分钟至 2.0ml/分钟、0.8ml/分钟至 2.0ml/分钟、1.0ml/分钟至 2.0ml/分钟、1.2ml/分钟至 2.0ml/分钟、1.5ml/分钟至 2.0ml/分钟、1.8ml/分钟至 2.0ml/分钟、0.5ml/分钟至 1.8ml/分钟、0.8ml/分钟至 1.8ml/分钟、1.0ml/分钟至 1.8ml/分钟、1.2ml/分钟至 1.8ml/分钟、1.5ml/分钟至 1.8ml/分钟、0.5ml/分钟至 1.5ml/分钟、0.8ml/分钟至 1.5ml/分钟、1.0ml/分钟至 1.5ml/分钟、1.2ml/分钟至 1.5ml/分钟、0.5ml/分钟至 1.2ml/分钟、0.8ml/分钟至 1.2ml/分钟、1.0ml/分钟至 1.2ml/分钟、0.5ml/分钟至 1.0ml/分钟、0.8ml/分钟至 1.0ml/分钟，或 0.5ml/分钟至 0.8ml/分钟。在一些实施方案中，加样和洗脱在约 0.8ml/分钟至约 1.2ml/分钟的流速进行。在一些实施方案中，加样和洗脱在约 1.5ml/分钟至约 2.0ml/分钟的流速进行。在另一些实施方案中，缓冲剂浓度、起始 pH、色谱材料的温度和 / 或流速的任何组合可根据上述实施方案而不同。

[0136] 电荷变体的检测

[0137] 在一些方面，本发明提供了检测包含多肽和一种或多种多肽组合物中的变体的组合物中的多肽变体的方法。所述方法包括使用具有初始 pH 和初始离子强度的加样缓冲剂使所述多肽以及一种或多种变体结合至离子交换色谱材料，使用洗脱缓冲剂从所述离子交换柱洗脱所述多肽以及一种或多种污染物，其中通过 pH 梯度改变所述洗脱缓冲剂的 pH，且通过离子强度梯度改变所述洗脱缓冲剂的离子强度，从而使所述多肽和所述一种或多种污染物作为不同的分离实体从色谱材料洗脱。然后对色谱洗脱物分析母多肽以及变体的存在。多肽变体可包括多肽的酸性变体和母多肽的碱性变体。酸性变体即 pI 小于母多肽 pI 的变体，其例子包括但不限于其中一个或多个谷氨酰胺和 / 或天冬酰胺残基已被脱酰胺化的多肽。碱性多肽变体即 pI 大于母多肽的 pI 的变体，其例子包括但不限于其中天冬氨酸残基被修饰成琥珀酰亚胺部分的变体。在一些实施方案中，本发明的方法用于检测包含等电点不在中性 pH 范围内的多肽的组合物中的多肽变体。在一些实施方案中，所述方法可用于有效地从污染物中检测包含 pI 大于 9 的多肽组合物中的电荷变体。在一些实施方案中，阳离子交换色谱材料用于有效地检测包含 pI 大于 9 的多肽的组合物中的电荷变体。在其他实施方案中，所述方法可用于有效地从污染物中检测包含 pI 小于 7 的多肽组合物中的电荷变体。在一些实施方案中，阴离子交换色谱材料用于有效地检测包含 pI 小于 7 的多肽的组合物中的电荷变体。多肽的例子包括但不限于抗体和抗体片段。

[0138] 在一些方面，本发明提供了检测包含多肽和一种或多种多肽变体的组合物中的多肽变体的方法。所述方法包括使用具有初始 pH 和初始离子强度的加样缓冲剂使所述多肽以及一种或多种变体结合至离子交换色谱材料，使用洗脱缓冲剂从所述离子交换柱洗脱所

述多肽以及一种或多种污染物,其中通过 pH 梯度改变所述洗脱缓冲剂的 pH,且维持所述洗脱缓冲剂的离子强度与初始离子强度基本相同,从而使所述多肽和所述一种或多种污染物作为不同的分离实体从色谱材料洗脱。然后对色谱洗脱物分析母多肽的存在以及变体的存在。多肽变体可包括多肽的酸性变体和母多肽的碱性变体。酸性变体即 pI 小于母多肽 pI 的变体,其例子包括但不限于其中一个或多个谷氨酰胺和 / 或天冬酰胺残基已经脱酰胺化的多肽。碱性多肽变体即 pI 大于母多肽 pI 的变体,其例子包括但不限于其中天冬氨酸残基被修饰成琥珀酰亚胺部分的变体。在一些实施方案中,本发明的方法用于检测包含等电点不在中性 pH 范围内的多肽的组合物中的多肽变体。在一些实施方案中,所述方法可用于有效地检测包含 pI 大于 9 的多肽组合物中的电荷变体。在一些实施方案中,阳离子交换色谱材料用于有效地检测包含 pI 大于 9 的多肽的组合物中的电荷变体。在其他实施方案中,所述方法可用于有效地检测包含 pI 小于 7 的多肽组合物中的电荷变体。在一些实施方案中,阴离子交换色谱材料用于有效地检测包含 pI 小于 7 的多肽的组合物中的电荷变体。多肽的例子包括但不限于抗体和抗体片段。

[0139] 确定组合物中多肽的纯度

[0140] 在一些方面,本发明提供确定包含多肽的组合物中多肽纯度的方法。所述方法包括使用具有初始 pH 和初始离子强度的加样缓冲剂使所述多肽以及一种或多种污染物结合至离子交换色谱材料,使用洗脱缓冲剂从所述离子交换柱洗脱所述多肽以及一种或多种污染物,其中通过 pH 梯度改变所述洗脱缓冲剂的 pH,且通过离子强度梯度改变所述洗脱缓冲剂的离子强度,从而使所述多肽和所述一种或多种污染物作为不同的分离实体从色谱材料洗脱。多肽的纯度可通过确定从色谱材料洗脱的多肽的量与从色谱材料洗脱的污染物如电荷变体的总量的比率来确定。在一些实施方案中,本发明的方法用于确定包含等电点不在中性 pH 范围内的多肽的组合物中的多肽的纯度。在一些实施方案中,所述方法可用于有效地从污染物中确定包含 pI 大于 9 的多肽组合物中多肽的纯度。在一些实施方案中,阳离子交换色谱材料用于有效地确定包含 pI 大于 9 的多肽的组合物中多肽的纯度。在其他实施方案中,所述方法可用于有效地从污染物中确定包含 pI 小于 7 的多肽组合物中多肽的纯度。在一些实施方案中,阴离子交换色谱材料用于有效地确定包含 pI 小于 7 的多肽的组合物中多肽的纯度。多肽的例子包括但不限于抗体和抗体片段。

[0141] 在一些方面,本发明提供确定包含多肽的组合物中多肽纯度的方法。所述方法包括使用具有初始 pH 和初始离子强度的加样缓冲剂使所述多肽以及一种或多种污染物结合至离子交换色谱材料,使用洗脱缓冲剂从所述离子交换柱洗脱所述多肽以及一种或多种污染物,其中通过 pH 梯度改变所述洗脱缓冲剂的 pH,且维持所述洗脱缓冲剂的离子强度与初始离子强度基本相同,从而使所述多肽和所述一种或多种污染物作为不同的分离实体从色谱材料洗脱。多肽的纯度可通过确定从色谱材料洗脱的多肽的量与从色谱材料洗脱的污染物如电荷变体的总量的比率来确定。在一些实施方案中,本发明的方法用于确定包含等电点不在中性 pH 范围内的多肽的组合物中的多肽的纯度。在一些实施方案中,所述方法可用于有效地从污染物中确定包含 pI 大于 9 的多肽组合物中多肽的纯度。在一些实施方案中,阳离子交换色谱材料用于有效地确定包含 pI 大于 9 的多肽的组合物中多肽的纯度。在其他实施方案中,所述方法可用于有效地从污染物中确定包含 pI 小于 7 的多肽组合物中多肽的纯度。在一些实施方案中,阴离子交换色谱材料用于有效地确定包含 pI 小于 7 的多肽的

组合物中多肽的纯度。多肽的例子包括但不限于抗体和抗体片段。

[0142] 确定组合物中多肽的稳定性

[0143] 在一些方面,本发明提供了用于确定包含多肽的组合物中多肽稳定性的方法。在一些实施方案中,随着时间的进行分析包含多肽的组合物样品。在一些实施方案中,在分析前将组合物温育不同的时间。在一些实施方案中,在分析前,在多于任一以下温度孵育组合物:约 0℃、20℃、37℃或 40℃。在一些实施方案中,在分析前,将组合物温育以下一个或多个时间:1 天、2 天、3 天、5 天、1 周、2 周、3 周、4 周、6 周、2 个月、3 个月、6 个月、1 年。然后如下分析组合物:使用具有初始 pH 和初始离子强度的加样缓冲剂使组合物中的所述多肽以及一种或多种污染物结合至离子交换色谱材料,使用洗脱缓冲剂从所述离子交换柱洗脱所述多肽以及一种或多种污染物,其中通过 pH 梯度改变所述洗脱缓冲剂的 pH,且通过离子强度梯度改变所述洗脱缓冲剂的离子强度,从而使所述多肽和所述一种或多种污染物作为不同的分离实体从色谱材料洗脱。在其他实施方案中,如下分析组合物:使用具有初始 pH 和初始离子强度的加样缓冲剂使组合物中的所述多肽以及一种或多种污染物结合至离子交换色谱材料,使用洗脱缓冲剂从所述离子交换柱洗脱所述多肽以及一种或多种污染物,其中通过 pH 梯度改变所述洗脱缓冲剂的 pH,且维持所述洗脱缓冲剂的离子强度与初始离子强度基本相同,从而使所述多肽和所述一种或多种污染物作为不同的分离实体从色谱材料洗脱。对于任一上述实施方案,多肽与污染物比率的变化指示组合物中多肽的稳定性。例如,如果多肽与污染物的比率不随时间而变化,则多肽可视为稳定的,而组合物中污染物的快速积累以及所伴随的蛋白质质量的降低指示组合物中的多肽不那么稳定。在一些实施方案中,本发明的方法用于确定包含等电点不在中性 pH 范围内的多肽的组合物中的多肽的稳定性。在一些实施方案中,所述方法可用于有效地从污染物中确定包含 pI 大于 9 的多肽组合物中多肽的稳定性。在一些实施方案中,阳离子交换色谱材料用于确定包含 pI 大于 9 的多肽的组合物中多肽的稳定性。在其他实施方案中,所述方法可用于从污染物中确定包含 pI 小于 7 的多肽组合物中多肽的稳定性。在一些实施方案中,阴离子交换色谱材料用于确定包含 pI 小于 7 的多肽的组合物中多肽的稳定性。多肽的例子包括但不限于抗体和抗体片段。

[0144] 多肽的纯化

[0145] 在一些方面,本发明提供了从包含多肽和一种或多种多肽变体的组合物中纯化多肽的方法。所述方法包括使用具有初始 pH 和初始离子强度的加样缓冲剂使所述多肽以及一种或多种变体结合至离子交换色谱材料,使用洗脱缓冲剂从所述离子交换柱洗脱所述多肽以及一种或多种污染物,其中通过 pH 梯度改变所述洗脱缓冲剂的 pH,且通过离子强度梯度改变所述洗脱缓冲剂的离子强度,从而使所述多肽和所述一种或多种污染物作为不同的分离实体从色谱材料洗脱。在色谱洗脱阶段收集级分,将包含多肽并且没有污染物或污染物极少的级分合并,用于进一步处理或用于药物配制。在一些实施方案中,本发明的方法用于纯化包含等电点不在中性 pH 范围内的多肽的组合物中的多肽。在一些实施方案中,所述方法可用于纯化 pI 大于 9 的多肽。在一些实施方案中,使用阳离子交换色谱材料来纯化 pI 大于 9 的多肽。在其他实施方案中,所述方法可用于从污染物纯化 pI 小于 7 的多肽。在一些实施方案中,使用阴离子交换色谱材料来纯化 pI 小于 7 的多肽。多肽的例子包括但不限于抗体和抗体片段。

[0146] 在一些方面,本发明提供了从包含多肽和一种或多种多肽变体的组合物中纯化多肽的方法。所述方法包括使用具有初始 pH 和初始离子强度的加样缓冲剂使所述多肽以及一种或多种变体结合至离子交换色谱材料,使用洗脱缓冲剂从所述离子交换柱洗脱所述多肽以及一种或多种污染物,其中通过 pH 梯度改变所述洗脱缓冲剂的 pH,且所述洗脱缓冲剂的离子强度与初始离子强度基本相同,从而使所述多肽和所述一种或多种污染物作为不同的分离实体从色谱材料洗脱。在色谱洗脱阶段收集级分,将包含多肽但是不含污染物的级分合并,用于进一步处理或用于药物配制。在一些实施方案中,本发明的方法用于纯化包含等电点不在中性 pH 范围内的多肽的组合物中的多肽。在一些实施方案中,所述方法可用于纯化 pI 大于 9 的多肽。在一些实施方案中,使用阳离子交换色谱材料来纯化 pI 大于 9 的多肽。在其他实施方案中,所述方法可用于从污染物纯化 pI 小于 7 的多肽。在一些实施方案中,使用阴离子交换色谱材料来纯化 pI 小于 7 的多肽。多肽的例子包括但不限于抗体和抗体片段。

[0147] III. 多肽

[0148] 提供了用于本文描述的任何离子强度介导的 pH 梯度离子交换色谱方法的多肽。在本发明的一些实施方案中,通过离子强度介导的 pH 梯度离子交换色谱来分析多肽的组合物。这样的方法可用于鉴定组合物中多肽的电荷变体。在一些实施方案中,所述多肽是抗体或其片段。

[0149] 在一些实施方案中,所述多肽是治疗性多肽。所述治疗性多肽可抑制肿瘤细胞生长、诱导凋亡和 / 或诱导细胞死亡。在一些实施方案中,所述多肽是拮抗剂。在一些实施方案中,所述多肽是激动剂。在一些实施方案中,所述多肽是抗体。在一些实施方案中,所述多肽是免疫粘附素。

[0150] 在一些实施方案中,所述多肽的分子量大于约任何以下:5,000 道尔顿、10,000 道尔顿、15,000 道尔顿、25,000 道尔顿、50,000 道尔顿、75,000 道尔顿、100,000 道尔顿、125,000 道尔顿或 150,000 道尔顿。所述多肽可具有任何以下范围的分子量:50,000 道尔顿至 200,000 道尔顿,或者 100,000 道尔顿至 200,000 道尔顿。作为另一选择,用于本文的多肽可具有约 120,000 道尔顿或约 25,000 道尔顿的分子量。

[0151] pI 是等电点,是特定分子或表面不带静电荷时的 pH。在本文描述的任何方法的一些实施方案中,多肽如抗体的 pI 可大于约 9。在一些实施方案中,所述多肽具有约任何以下的 pI:9、9.5、10、10.5、11、11.5 或 12。在一些实施方案中,所述多肽具有约 9 至 12 的 pI。在一些实施方案中,所述多肽具有约 9 至 11 的 pI。在一些实施方案中,所述多肽具有约 9 至 10 的 pI。在一些实施方案中,所述多肽具有约 10 至 12 的 pI。在一些实施方案中,所述多肽具有约 10 至 11 的 pI。在一些实施方案中,所述多肽具有约 11 至 12 的 pI。

[0152] 在本文描述的任何方法的一些实施方案中,多肽如抗体的 pI 可小于约 7。在一些实施方案中,所述多肽具有约任何以下的 pI:7、6.5、6、5.5、5、4.5 或 4。在一些实施方案中,所述多肽具有约 4 至 7 的 pI。在一些实施方案中,所述多肽具有约 4 至 6 的 pI。在一些实施方案中,所述多肽具有约 4 至 5 的 pI。在一些实施方案中,所述多肽具有约 5 至 7 的 pI。在一些实施方案中,所述多肽具有约 5 至 6 的 pI。

[0153] 在本文描述的任何方法的一些实施方案中,包含多肽和一种或多种污染物的组合物中的一种或多种污染物是多肽电荷变体。在一些实施方案中,所述多肽电荷变体是从其

天然状态修饰成使多肽的电荷发生改变的多肽。在一些实施方案中,电荷变体比母多肽更具酸性;即具有比母多肽更低的pI。在其他实施方案中,电荷变体比母多肽更具碱性;即具有比母多肽更高的pI。在一些实施方案中,所述多肽电荷变体是工程化的。在一些实施方案中,所述多肽电荷变体是天然过程的结果;例如氧化、脱酰胺化、赖氨酸残基C端加工、N端焦谷氨酸形成和糖基化。在一些实施方案中,多肽电荷变体是糖蛋白,其中附接至蛋白质的多糖被修饰成该糖蛋白的电荷与母糖蛋白相比发生改变,例如通过添加唾液酸或其衍生物。在一些实施方案中,所述多肽电荷变体是抗体电荷变体。

[0154] 将使用本文描述的方法来分析的多肽一般使用重组技术产生。用于产生重组蛋白质的方法在U. S. Pat No. 5, 534, 615和4, 816, 567中有描述,其通过引用特别并入本文。在一些实施方案中,在CHO细胞中产生感兴趣的蛋白质(参见例如WO 94/11026)。当使用重组技术时,多肽可产生于细胞内、周质空间中或直接分泌至培养基中。

[0155] 可从培养基或宿主细胞裂解物中回收多肽。可通过多种物理或化学方法来破裂用于表达多肽的细胞,例如冻融循环、超声、机械破裂或细胞裂解剂。如果多肽产生于细胞内,则第一步是,例如通过离心或超滤去除颗粒碎片,所述碎片是宿主细胞或者裂解的片段。Carter等Bio/Technology 10:163-167(1992)描述了用于分离分泌至大肠杆菌周质空间的多肽的方法。简略而言,在醋酸钠(pH 3.5)、EDTA和苯甲基磺酰氟(PMSF)存在下,用约30分钟溶解细胞糊状物。通过离心去除细胞碎片。在多肽分泌至培养基中的情况下,一般首先使用市售的多肽浓缩过滤器(例如Amicon或Millipore Pellicon超滤装置)浓缩来自这样的表达系统的上清液。任何前述步骤都可包含例如PMSF等蛋白酶抑制剂以抑制蛋白质水解,且可包含抗生素以防止外来污染物的生长。

[0156] 在一些实施方案中,在通过本发明的方法分析之前已经纯化或部分纯化了包含多肽和一种或多种污染物的组合物中的多肽。例如,所述方法的多肽处于来自亲和色谱、阳离子交换色谱、阴离子交换色谱、混合模式色谱和疏水性相互作用色谱的洗脱剂中。在一些实施方案中,所述多肽处于来自蛋白A色谱的洗脱剂中。

[0157] 可通过本发明方法分析的多肽的例子包括但不限于:免疫球蛋白、免疫粘附素、抗体、酶、激素、融合蛋白、包含Fc的蛋白质、免疫缀合物、细胞因子和白细胞介素。

[0158] 在一些实施方案中,所述多肽处于药物组合物中。在一些实施方案中,所述多肽是处于药物组合物中的抗体或其抗原结合片段。在一些实施方案中,所述药物组合物包含所述多肽和可药用载体,所述可药用载体包括但不限于缓冲剂、赋形剂、稳定剂或防腐剂。在一些实施方案中,所述药物组合物包含抗体或其抗原结合片段,以及可药用载体,所述可药用载体包括但不限于缓冲剂、赋形剂、稳定剂或防腐剂。

[0159] (A) 抗体

[0160] 在本文描述的任何方法的一些实施方案中,用于通过本文描述的方法来分析多肽和包含多肽的制剂的任何方法的多肽是抗体。

[0161] 抗体的分子靶包括CD蛋白质及其配体,例如但不限于:(i)CD3、CD4、CD8、CD19、CD11a、CD20、CD22、CD34、CD40、CD79 α (CD79a)和CD79 β (CD79b);(ii)ErbB受体家族的成员,例如EGF受体、HER2、HER3或HER4受体;(iii)细胞粘附分子,例如LFA-1、Mac1、p150, 95、VLA-4、ICAM-1、VCAM和 α v/ β 3整联蛋白,包括其 α 亚基或 β 亚基(例如抗-CD11a、抗-CD18或抗-CD11b抗体);(iv)生长因子,例如VEGF;IgE;血型抗原;flk2/

flt3 受体 ;肥胖症 (OB) 受体 ;mpl 受体 ;CTLA-4 ;蛋白 C、BR3、c-met、组织因子、 β 7 等 ;以及 (v) 细胞表面的和跨膜的肿瘤相关抗原 (TAA), 例如美国专利 No. 7, 521, 541 中描述的那些。

[0162] 其他示例性的抗体包括但不限于选自以下的那些 :抗 - 雌激素受体抗体、抗 - 孕酮受体抗体、抗 -p53 抗体、抗 -HER-2/neu 抗体、抗 -EGFR 抗体、抗 - 组织蛋白酶 D 抗体、抗 -Bcl-2 抗体、抗 -E- 钙粘着蛋白抗体、抗 -CA125 抗体、抗 -CA15-3 抗体、抗 -CA19-9 抗体、抗 -c-erbB-2 抗体、抗 -P- 糖蛋白抗体、抗 -CEA 抗体、抗 - 成视网膜细胞瘤蛋白抗体、抗 -ras 癌蛋白抗体、抗 -Lewis X 抗体、抗 -Ki-67 抗体、抗 -PCNA 抗体、抗 -CD3 抗体、抗 -CD4 抗体、抗 -CD5 抗体、抗 -CD7 抗体、抗 -CD8 抗体、抗 -CD9/p24 抗体、抗 -CD10 抗体、抗 -CD11a 抗体、抗 -CD11c 抗体、抗 -CD13 抗体、抗 -CD14 抗体、抗 -CD15 抗体、抗 -CD19 抗体、抗 -CD20 抗体、抗 -CD22 抗体、抗 -CD23 抗体、抗 -CD30 抗体、抗 -CD31 抗体、抗 -CD33 抗体、抗 -CD34 抗体、抗 -CD35 抗体、抗 -CD38 抗体、抗 -CD41 抗体、抗 -LCA/CD45 抗体、抗 -CD45RO 抗体、抗 -CD45RA 抗体、抗 -CD39 抗体、抗 -CD100 抗体、抗 -CD95/Fas 抗体、抗 -CD99 抗体、抗 -CD106 抗体、抗 - 泛素抗体、抗 -CD71 抗体、抗 -c-myc 抗体、抗 - 细胞角蛋白抗体、抗 - 波形蛋白抗体、抗 -HPV 蛋白抗体、抗 - κ 轻链抗体、抗 - λ 轻链抗体、抗 - 黑素体抗体、抗 - 前列腺特异性抗原抗体、抗 -S-100 抗体、抗 -tau 抗原抗体、抗 - 纤维蛋白抗体、抗 - 角蛋白抗体和抗 -Tn- 抗原抗体。

[0163] (i) 单克隆抗体

[0164] 在一些实施方案中, 所述抗体是单克隆抗体。单克隆抗体得自基本同质的抗体群的抗体, 即组成群体的单个抗体是相同的和 / 或结合相同表位, 除非在单克隆抗体的生产期间可产生可能的变体, 这样的变体一般以少量存在。因此, 修饰语“单克隆”表示抗体不是独立离散抗体或多克隆抗体的混合物的特征。

[0165] 例如, 单克隆抗体可使用 Kohler 等 Nature 256:495 (1975) 首次描述的杂交瘤法来制成, 或可通过重组 DNA 法 (美国专利 No. 4, 816, 567) 制成。

[0166] 在杂交瘤法中, 如本文所描述来免疫小鼠或例如仓鼠等其他合适的宿主动物, 以诱导产生或能够产生将特异性结合至用于免疫的多肽的淋巴细胞的抗体。作为另一选择, 可体外免疫淋巴细胞。然后使用合适的融合剂如聚乙二醇将淋巴细胞与骨髓瘤细胞融合, 从而形成杂交瘤细胞 (Goding, Monoclonal Antibodies: Principles and Practice, 第 59-103 页 (Academic 出版社, 1986))。

[0167] 将由此制备的杂交瘤细胞接种并使其生长于优选包含一种或多种抑制未融合的亲代骨髓瘤细胞生长或生存的合适培养基中。例如, 若亲代骨髓瘤细胞缺乏酶次黄嘌呤鸟嘌呤磷酸核糖转移酶 (HGPRT 或 HPRT), 则用于杂交瘤的培养基通常将包含次黄嘌呤、氨基蝶呤和胸腺嘧啶 (HAT 培养基), 这些物质阻止 HGPRT 缺陷型细胞的生长。

[0168] 在一些实施方案中, 骨髓瘤细胞是有效融合的细胞, 其支持由所选择的抗体产生性细胞稳定地高水平生产抗体, 并且对培养基如 HAT 培养基敏感。在一些实施方案中, 其中, 骨髓瘤细胞系是鼠科骨髓瘤系, 例如衍生自可得自 Salk Institute Cell Distribution Center, San Diego, California USA 的 MOPC-21 和 MPC-11 小鼠肿瘤, 和可得自 American Type Culture Collection, Rockville, Maryland USA 的 SP-2 或 X63-Ag8-653 细胞的那些。还描述了人骨髓瘤和小鼠 - 人异源骨髓瘤细胞系用于产生人单克隆抗体 (Kozbor, J.

Immunol. 133:3001(1984); Brodeur 等, Monoclonal Antibody Production Techniques and Applications, 第 51-63 页 (Marcel Dekker, Inc., New York, 1987))。

[0169] 对杂交瘤细胞在其中生长的培养基测定针对抗原的单克隆抗体的产生。在一些实施方案中,由杂交瘤细胞产生的单克隆抗体的结合特异性通过免疫沉淀或体外结合测定如放射免疫测定 (RIA) 或酶联免疫吸附测定 (ELISA) 来确定。

[0170] 单克隆抗体的结合亲和力可例如通过 Munson 等 Anal. Biochem. 107:220(1980) 的 Scatchard 分析来确定。

[0171] 在鉴定杂交瘤细胞产生期望特异性、亲和力和 / 或活性的抗体后,可通过有限的稀释方法对该克隆进行亚克隆,并通过标准方法使其生长 (Goding, Monoclonal Antibodies: Principles and Practice, 第 59-103 页 (Academic 出版社, 1986))。用于该目的合适培养基包括例如 D-MEM 或 RPMI-1640 培养基。此外,可使杂交瘤细胞体内生长为动物中的腹水肿瘤。

[0172] 通过例如多肽 A- 琼脂糖、羟磷灰石色谱、凝胶电泳、透析或亲和色谱等常规免疫球蛋白纯化方法适当地将由亚克隆分泌的单克隆抗体从培养基、腹水或血清分离。

[0173] 使用常规方法 (例如通过使用能够特异性结合至编码鼠科抗体重链和轻链的基因的寡核苷酸探针) 容易地分离和测序编码单克隆抗体的 DNA。在一些实施方案中,杂交瘤细胞充当这样的 DNA 来源。在分离后,可将 DNA 放置于表达载体中,然后将其转染至宿主细胞中以实现在重组宿主细胞中合成单克隆抗体,所述宿主细胞例如大肠杆菌细胞、类人猿 COS 细胞、中国仓鼠卵巢 (CHO) 细胞或不另外产生免疫球蛋白多肽的骨髓瘤细胞等。关于编码抗体的 DNA 在细菌中进行重组表达的综述文章包括 Skerra 等, Curr. Opinion in Immunol. 5:256-262(1993), 以及 Plückerthun, Immunol. Revs., 130:151-188(1992)。

[0174] 在另一个实施方案中,可从使用 McCafferty 等 Nature 348:552-554(1990) 中描述的技术生产的抗体噬菌体文库中分离抗体或抗体片段。Clackson 等 Nature 352:624-628(1991) 和 Marks 等 J. Mol. Biol. 222:581-597(1991) 分别描述了使用噬菌体文库来分离鼠科抗体和人抗体。后续的出版物描述了通过链改组 (chain shuffling) (Marks 等, Bio/Technology 10:779-783(1992)) 以及作为用于构建非常大的噬菌体文库的策略的组合感染和体内重组 (Waterhouse 等, Nuc. Acids. Res. 21:2265-2266(1993)) 来产生高亲和力 (nM 范围) 的人抗体。因此,这些技术是用于分离单克隆抗体的传统单克隆抗体杂交瘤技术的可行的备选方案。

[0175] 还可例如通过以下来对 DNA 进行改造:将同源的鼠科序列替换为人重链和轻链恒定结构域的编码序列 (美国专利 No. 4, 816, 567; Morrison 等, Proc. Natl Acad. Sci. USA 81:6851(1984)), 或通过将免疫球蛋白编码序列与全部或部分的非免疫球蛋白多肽编码序列共价结合。

[0176] 通常来说,这样的非免疫球蛋白多肽替换抗体的恒定结构域,或者它们替换抗体的抗原组合位点的可变结构域,以创造嵌合二价抗体,其包含一个具有对抗原的特异性的抗原结合位点和另一个具有对不同抗原的特异性的抗原结合位点。

[0177] 在本文描述的任何方法的一些实施方案中,所述抗体是 IgA、IgD、IgE、IgG 或 IgM。在一些实施方案中,所述抗体是 IgG 单克隆抗体。

[0178] (ii) 人源化抗体

[0179] 在一些实施方案中,所述抗体是人源化抗体。本领域中已经描述了用于人源化非人抗体的方法。在一些实施方案中,人源化抗体具有从非人来源引入其中的一个或多个氨基酸残基。这些非人氨基酸残基常常被称为“输入的”残基,其通常取自“输入的”可变结构域。人源化可基本按照 Winter 及其同事的方法 (Jones 等, *Nature* 321:522-525(1986); Riechmann 等, *Nature* 332:323-327(1988); Verhoeyen 等, *Science* 239:1534-1536(1988))、通过用高变区序列来替换相应的人抗体序列来进行。因此,这样的“人源化”抗体是嵌合抗体 (美国专利 No. 4, 816, 567), 其中基本上小于完整的人可变结构域被来自非人种的相应序列替换。事实上,人源化抗体通常是其中一些高变区残基以及可能的一些 FR 残基被来自啮齿类抗体类似位点的残基所取代的人抗体。

[0180] 对用于制造人源化抗体的人可变结构域——无论是轻链还是重链——的选择对降低抗原性非常重要。根据所谓“最佳配合”法,针对已知的人可变结构域序列的整个文库来筛选啮齿类抗体可变结构域的序列。然后将最接近啮齿类序列的人序列接受为用于人源化抗体的人构架区 (FR) (Sims 等, *J. Immunol.* 151:2296(1993); Chothia 等, *J. Mol. Biol.* 196:901(1987))。另一方法使用衍生自轻链或重链可变区的特定亚组的所有人抗体的共有序列的特定构架区。相同的骨架可用于若干不同的人源化抗体 (Carter 等, *Proc. Natl. Acad. Sci. USA* 89:4285(1992); Presta 等, *J. Immunol.* 151:2623(1993))。

[0181] 在保留对抗原的高亲和力和其他良好生物性质的情况下来人源化抗体也是重要的。为了达到该目标,在所述方法的一些实施方案中,通过使用母序列和人源化序列的三维模型来分析母序列以及多种概念化的人源化产物的方法来制备人源化抗体。三维免疫球蛋白模型通常是可得到的,并且对于本领域技术人员而言是熟悉的。描述和展示所选择的候选免疫球蛋白序列的可能三维构象结构的计算机程序是可得到的。对这些展示的观察使得能够分析残基在候选免疫球蛋白序列发挥功能中的可能作用,即分析影响候选免疫球蛋白结合其抗原的能力的残基。通过此方式,可从接受序列和输入序列选择并组合 FR 残基,以得到期望的抗体特征,例如提高对靶抗原 (多种) 的亲和力。一般而言,高变区残基直接并最大程度地涉及影响抗原结合。

[0182] (iii) 人抗体

[0183] 在一些实施方案中,所述抗体是人抗体。作为人源化的一个备选方案,可生成人抗体。例如,现在能够产生能够基于免疫在不产生内源免疫球蛋白的情况下产生全谱 (full repertoire) 人抗体的转基因动物 (例如小鼠)。例如,据描述,在嵌合的种系突变小鼠中纯合缺失抗体重链结合区 (JH) 基因,导致完全抑制内源抗体产生。将人种系免疫球蛋白基因阵列转入这样的种系突变小鼠中将导致基于抗体激发产生人抗体。参见例如 Jakobovits 等, *Proc. Natl. Acad. Sci. USA* 90:2551(1993); Jakobovits 等, *Nature* 362:255-258(1993); Bruggermann 等, *Year in Immuno.* 7:33(1993); 以及美国专利 No. 5, 591, 669; 5, 589, 369; 和 5, 545, 807。

[0184] 作为另一选择,可使用噬菌体展示技术 (McCafferty 等, *Nature* 348:552-553(1990)) 在体外由来自未免疫供体的免疫球蛋白可变 (V) 结构域基因谱来产生抗体和抗体片段。根据该技术,将抗体 V 结构域基因框内克隆至例如 M13 或 fd 等丝状噬菌体的主要外壳多肽或次要外壳多肽基因中,并在噬菌体颗粒表面上展示为功能性抗体片段。因为所述丝状颗粒包含噬菌体基因组的单链 DNA 拷贝,所以基于抗体功能性质

的选择还导致对编码表现出那些性质的抗体的基因的选择。因此,噬菌体模拟了 B 细胞的一些性质。可以以多种格式来进行噬菌体展示,其综述参见例如 Johnson, Kevin S. 和 Chiswell, David J., *Current Opinion in Structural Biology* 3:564-571(1993)。V- 基因区段的一些来源可用于噬菌体展示。Clackson 等 *Nature* 352:624-628(1991) 从来源于免疫小鼠的脾的小的 V 基因随机组合文库分离了多样的抗 - 噁唑酮抗体阵列。可基本依照 Marks 等 *J. Mol. Biol.* 222:581-597(1991) 或 Griffith 等 *EMBO J.* 12:725-734(1993) 描述的技术来构建来自未免疫的人供体的 V 基因谱以及分离多样抗原(包括自体抗原)阵列的抗体。还参见美国专利 No. 5, 565, 332 和 5, 573, 905。

[0185] 人抗体还可通过体外活化的 B 细胞来生成(参见美国专利 5, 567, 610 和 5, 229, 275)。

[0186] (iv) 抗体片段

[0187] 在一些实施方案中,所述抗体是抗体片段。已经开发了多种技术用于产生抗体片段。在传统上,通过对完整抗体进行蛋白水解消化来衍生这些片段(参见例如 Morimoto 等 *Journal of Biochemical and Biophysical Methods* 24:107-117(1992),以及 Brennan 等, *Science* 229:81(1985))。但是,现在可以通过重组宿主细胞直接产生这些片段。例如,可从上文描述的抗体噬菌体文库分离抗体片段。作为另一选择,可从大肠杆菌直接回收 Fab'-SH 片段,并将其化学耦合以形成 F(ab')₂ 片段(Carter 等, *Bio/Technology* 10:163-167(1992))。根据另一方法,可直接从重组宿主细胞培养物分离 F(ab')₂ 片段。用于产生抗体片段的其他技术对熟练的从业者将是显而易见的。在另一些实施方案中,选择的抗体是单链 Fv 片段(scFv)。参见 WO 93/16185 ;美国专利 No. 5, 571, 894 ;以及美国专利 No. 5, 587, 458。所述抗体片段还可为“线性抗体”,例如,如在美国专利 5, 641, 870 中所述。这样的线性抗体片段可为单特异性或双特异性的。

[0188] 在一些实施方案中,提供了本文描述的抗体片段。在一些实施方案中,所述抗体片段是抗原结合片段。在一些实施方案中,所述抗原结合片段选自下组:Fab 片段、Fab' 片段、F(ab')₂ 片段、scFv、Fv 和双抗体。

[0189] (v) 双特异性抗体

[0190] 在一些实施方案中,所述抗体是双特异性抗体。双特异性抗体是对至少两种不同表位具有结合特异性的抗体。示例性的双特异性抗体可结合两个不同表位。作为另一选择,双特异性抗体结合臂可与结合白细胞上触发分子的臂组合,以将细胞防御机制聚焦至细胞,所述触发分子例如 T- 细胞受体分子(例如 CD2 或 CD3),或 IgG 的 Fc 受体(FcγR),例如 FcγRI(CD64)、FcγRII(CD32) 和 FcγRIII(CD16)。双特异性抗体可制备为全长抗体或抗体片段(例如 F(ab')₂ 双特异性抗体)。

[0191] 用于制备双特异性抗体的方法是本领域已知的。全长双特异性抗体的传统制备基于两条免疫球蛋白重链-轻链对的共表达,其中该两条链具有不同的特异性(Millstein 等, *Nature* 305:537-539(1983))。由于免疫球蛋白重链和轻链的随机分配,这些杂交瘤(四重杂交瘤)产生了潜在的 10 种不同抗体分子的混合物,其中只有一种是正确的双特异性结构。通常由亲和色谱步骤进行的对正确分子的纯化十分繁琐,并且产率低。WO 93/08829 和 Traunecker 等 *EMBO J.*, 10:3655-3659(1991) 中公开了类似方法。

[0192] 根据不同的方法,将具有期望结合特异性的抗体可变结构域(抗体-抗原组合位

点)融合至免疫球蛋白恒定结构域序列。在一些实施方案中,该融合由具有包含至少部分铰链区、CH2 区和 CH3 区的免疫球蛋白重链恒定结构域来进行。在一些实施方案中,包含用于轻链结合所需位点的第一重链恒定区(CH1)存在于至少一种融合物中。将编码免疫球蛋白重链融合物和(如果期望的话)免疫球蛋白轻链的 DNA 插入单独的表达载体中,并将其共转染至合适的宿主生物体中。在构建中使用三种多肽链的不等比率提供最优产率的实施方案中,这在调节三种多肽片段的相互比率方面提供了巨大的灵活性。但是,当以相等比率表达至少两条多肽链导致高产率时,或当该比率没有特别的显著意义时,可将两种或三种多肽链的编码序列插入一个表达载体中。

[0193] 在该方法的一些实施方案中,所述双特异性抗体由在一个臂中的具有第一结合特异性的杂交免疫球蛋白重链和在另一臂中的杂交免疫球蛋白重链-轻链对(提供第二结合特异性)构成。发现,该不对称结构促进期望的双特异性化合物从不需要的免疫球蛋白链组合分离,因为免疫球蛋白轻链存在于仅双特异性分子的一半中提供了容易的分离方法。WO 94/04690 中公开了该方法。产生双特异性抗体的另一些细节参见例如 Suresh 等, *Methods in Enzymology* 121:210(1986)。

[0194] 根据美国专利 No. 5, 731, 168 中描述的另一方法,可工程化抗体分子对之间的界面以使从重组细胞培养物回收的异二聚体的百分比最大化。在一些实施方案中,所述界面包含抗体恒定结构域的至少部分 CH3 结构域。在该方法中,用较大侧链(例如酪氨酸或色氨酸)来替换第一抗体分子的界面的一条或多条小氨基酸侧链。通过用较小的氨基酸侧链(例如丙氨酸或苏氨酸)替换大氨基酸侧链,在第二抗体分子的界面上创造出与一个或多个大侧链相同或相似尺寸的补偿性的“腔”。这提供了提高异二聚物相对于例如同二聚物等其他不需要终产物的产率的机制。

[0195] 双特异性抗体包括交联的抗体或“异源缀合的”抗体。例如,在异源缀合物中,一个抗体耦合至抗生物素蛋白,其他抗体耦合至生物素。已经提出了这样的抗体来使免疫系统细胞靶向不需要的细胞(美国专利 No. 4, 676, 980),以及用于治疗 HIV 感染(WO 91/00360、WO 92/200373 和 EP 03089)。异源缀合抗体可通过使用任何方便的交联法来制备。合适的交联剂是本领域公知,并且在美国专利 No. 4, 676, 980 中公开了交联剂以及一些交联技术。

[0196] 在一些文献中还描述了用于从抗体片段产生双特异性抗体的技术。例如,双特异性抗体可使用化学键合来制备。Brennan 等 *Science* 229:81(1985) 描述了其中以蛋白水解方式切割完整抗体以生成 F(ab')₂ 片段的方法。在二硫络合剂亚砷酸钠存在下还原这些片段,以稳定邻位的二硫醇,并防止形成分子间二硫化物。生成 Fab' 片段,然后将其转化成硫代硝基苯甲酸酯(TNB)衍生物。然后通过用巯基乙胺还原将 Fab'-TNB 衍生物中的一种重转化成 Fab'-巯基,并将其与等摩尔量的另一 Fab'-TNB 衍生物混合,以形成双特异性抗体。所产生的双特异性抗体可用作选择性固定化酶的试剂。

[0197] 也已经描述了多种用于直接从重组细胞培养物制造和分离双特异性抗体的技术。例如,已经使用亮氨酸拉链来产生双特异性抗体。Kostelny 等, *J. Immunol.* 148(5):1547-1553(1992)。通过基因融合将 Fos 和 Jun 蛋白的亮氨酸拉链肽连接至两种不同抗体的 Fab' 部分。在铰链区处还原抗体同二聚物以形成单体,然后再氧化以形成抗体异二聚物。该方法还可用于产生抗体同二聚物。Hollinger 等 *Proc. Natl. Acad. Sci. USA* 90:6444-6448(1993) 描述的“双抗体”技术提供了用于制备双特异性抗体片段的可选机制。

该片段包含由短至不允许相同链上的两个结构域之间配对的接头连接至轻链可变结构域 (V_L) 的重链可变结构域 (V_H)。因此,迫使一个片段的 V_H 和 V_L 结构域与另一片段上的互补 V_L 和 V_H 结构域配对,从而形成两个抗原结合位点。还报道了用于通过使用单链 Fv (sFv) 二聚物来制备双特异性抗体片段的另一策略。参见 Gruber 等, J. Immunol. 152:5368 (1994)。

[0198] 还包括多于二价的抗体。例如,可制备三特异性抗体。Tutt 等, J. Immunol. 147:60 (1991)。

[0199] (v) 多价抗体

[0200] 在一些实施方案中,所述抗体是多价抗体。多价抗体可被表达该抗体所结合的抗原的细胞更快地内化(和/或同化)。本文提供的抗体可为具有三个或更多个抗原结合位点的多价抗体(例如三价抗体)(其为除了 IgM 类以外的抗体),其可通过重组表达编码抗体多肽链的核酸容易地产生。所述多价抗体可包含二聚化结构域和三个或更多个抗原结合位点。优选的二聚化结构域包含 Fc 区或铰链区(或由 Fc 区或铰链区组成)。在该情况下,所述抗体将包含 Fc 区以及 Fc 区氨基末端的三个或更多个抗原结合位点。本文的优选多价抗体包含三个至八个、但优选四个抗原结合位点(或由这些抗原结合位点组成)。多价抗体包含至少一条多肽链(并且优选两条多肽链),其中所述多肽链(多条)包含两个或更多个可变结构域。例如,所述多肽链(多个)可包含 $VD1-(X1)_n-VD2-(X2)_n-Fc$, 其中 VD1 是第一可变结构域, VD2 是第二可变结构域, Fc 是一条 Fc 区多肽链, X1 和 X2 表示氨基酸或多肽,并且 n 为 0 或 1。例如,所述多肽链(多条)可包含: $VH-CH1-$ 柔性接头 $-VH-CH1-Fc$ 区链; 或 $VH-CH1-VH-CH1-Fc$ 区链。本文的多价抗体优选还包含至少两条(并且优选四条)轻链可变结构域多肽。例如,本文的多价抗体可包含约两条至约八条轻链可变结构域多肽。此处预期的所述轻链可变结构域多肽包含轻链可变结构域,并且任选还包含 CL 结构域。

[0201] 在一些实施方案中,所述抗体是多特异抗体。多特异性抗体的例子包括但不限于: 包含重链可变结构域 (V_H) 和轻链可变结构域 (V_L) 的、其中所述 V_HV_L 单元具有多表位特异性的抗体; 具有两个或更多个 VL 和 VH 结构域且各 V_HV_L 单元结合不同表位的抗体; 具有两个或更多个单一可变结构域且各单一可变结构域结合不同表位的抗体; 全长抗体; 抗体片段, 如 Fab、Fv、dsFv、scFv; 双抗体、双特异性双抗体、三抗体、三功能抗体、已经被共价或非共价连接的抗体片段。在一些实施方案中,抗体具有多表位特异性; 例如,特异性结合相同或不同靶(多种)上的两个或更多个不同表位的能力。在一些实施方案中,所述抗体是单特异性的; 例如,结合仅一个表位的抗体。根据一个实施方案,所述多特异抗体是以 $5\ \mu M$ 至 $0.001pM$ 、 $3\ \mu M$ 至 $0.001pM$ 、 $1\ \mu M$ 至 $0.001pM$ 、 $0.5\ \mu M$ 至 $0.001pM$ 或 $0.1\ \mu M$ 至 $0.001pM$ 的亲和力结合各表位的 IgG 抗体。

[0202] (vi) 其他抗体修饰

[0203] 可期望针对效应子功能来修饰本文提供的抗体,例如以此来增强抗体的抗原依赖性细胞介导的细胞毒性 (ADCC) 和/或补体依赖性细胞毒性 (CDC)。这可通过将一个或多个氨基酸替换引入抗体的 Fc 区中来实现。作为另一选择或此外,可将半胱氨酸残基(多个)引入 Fc 区中,从而使得能够在该区中形成链间二硫键。由此生成的同二聚物抗体可改进内化能力和/或提高补体介导的细胞杀伤和抗体依赖性细胞的细胞毒性 (ADCC)。参见 Caron 等, J. Exp. Med. 176:1191-1195 (1992) 和 Shopes, B. J., Immunol. 148:2918-2922 (1992)。如 Wolff 等 Cancer Research 53:2560-2565 (1993) 中所述,还可使用异源双功能交联子

来制备抗肿瘤活性增强的同二聚物抗体。作为另一选择,可工程化具有双 Fc 区的抗体,并因此可增强补体介导的裂解和 ADCC 能力。参见 Stevenson 等, *Anti-Cancer Drug Design* 3:219-230 (1989)。

[0204] 为了提高抗体的血清半衰期,如 US 2006/0067930 所述,可在抗体中进行氨基酸改变,通过引用整体并入本文。

[0205] (B) 多肽变体和修饰

[0206] 本文描述的对包括抗体在内的多肽的氨基酸序列修饰(多种)可用于本文描述的纯化多肽(例如抗体)的方法。

[0207] (i) 变体多肽

[0208] 如本文定义,“多肽变体”意为具有与以下序列至少约 80% 氨基酸序列同一性的多肽,优选活性多肽:全长的天然多肽序列、不含信号肽的多肽序列、多肽的具有或不具有信号肽的胞外结构域。这样的多肽变体包括例如其中在全长天然氨基酸序列的 N 端或 C 端添加或缺失一个或多个氨基酸残基的多肽。一般而言, TAT 多肽变体将具有与以下序列至少约 80% 氨基酸序列同一性,或者至少约任意 85%、90%、95%、96%、97%、98% 或 99% 氨基酸序列同一性:全长天然多肽序列、不含信号肽的多肽、具有或不具有多肽的细胞外结构域。任选地,相比于天然多肽序列,变体多肽将具有不多于一个保守氨基酸替换,或者相比于天然多肽序列具有不多于约任意 2、3、4、5、6、7、8、9 或 10 个保守氨基酸替换。

[0209] 所述变体多肽可在 N 端或 C 端处截短,或可缺失内部残基,例如,当与全长天然多肽相比时。某些变体多肽可缺失不是期望生物学活性必需的氨基酸残基。这些具有截短、缺失和插入的变体可通过任意一些常规技术制备。期望的变体多肽可进行化学合成。另一合适的技术包括通过聚合酶链式反应(PCR),分离并扩增编码期望变体多肽的核酸片段。将限定所述核酸片段的期望末端的寡核苷酸用作 PCR 中的 5' 和 3' 引物。优选地,变体多肽与本文公开的天然多肽共有至少一种生物学活性和/或免疫性活性。

[0210] 氨基酸序列插入物包括长度范围介乎一个残基至包含一百或更多个残基的多肽的氨基端和/或羧基端融合物,以及单个或多个氨基酸残基的序列内插入物。末端插入物的例子包括具有 N 端甲硫氨酰基残基的抗体或融合至细胞毒性多肽的抗体。其他抗体分子插入变体包括抗体的 N 端或 C 端融合至提高抗体的血清半衰期的酶或多肽的融合物。

[0211] 例如,可期望改进多肽的结合亲和力和/或其他生物学性质。多肽的氨基酸序列变体通过将合适的核苷酸变化引入抗体核酸中来制备,或通过肽合成来制备。这样的改造包括例如从多肽的氨基酸序列中缺失残基,和/或向其中插入残基,和/或替换其中的残基。可进行任何缺失、插入和替换的组合来实现最终构建体,只要最终构建体拥有期望的性质即可。氨基酸变化还可改变多肽(例如抗体)的翻译后加工,例如改变糖基化位点的数量或位置。

[0212] 可通过比较多肽序列与同源的已知多肽分子的序列并最小化高同源性区域中的氨基酸序列变化的数量,来指导确定在不有害地影响期望活性的情况下可插入、替换或缺失哪些氨基酸残基。

[0213] 可用于鉴定作为多肽(例如抗体)的优选诱变位置的某些残基或区域的方法称为“丙氨酸扫描诱变”,如 Cunningham 和 Wells *Science* 244:1081-1085 (1989) 所述。此处,鉴定残基或一组靶残基(例如带电残基,如 Arg、Asp、His、Lys 和 Glu),并用中性氨基酸或带

负电的氨基酸（最优选丙氨酸或聚丙氨酸）替换，以影响氨基酸与抗原的相互作用。然后，通过在替换位点处进一步引入变体或对替换位点引入其他变体来改善对替换显示出功能敏感性的这些氨基酸位置。因此，虽然引入氨基酸序列变化的位点是预先确定的，但是突变本身的性质不需要预先确定。例如，为了分析指定位点处的突变性能，在靶密码子或靶区域处进行 ala 扫描或随机诱变，并且针对期望的活性筛选表达的抗体变体。

[0214] 另一类型的变体是氨基酸替换变体。这些变体的抗体分子中的至少一个氨基酸残基由不同残基替换。替换诱变的最感兴趣的位点包括高变区，但是还预期了 FR 改变。标题为“优选替换”的下表 1 示出了保守替换。如果这样的替换导致生物学活性的改变，则可引入更大量的变化，在表 1 中命名为“示例性替换”，或如下文中引用氨基酸种类所进一步描述，并且筛选产物。

[0215] 表 1.

[0216]

初始残基	示例性替换	优选替换
Ala(A)	Val ;Leu ;Ile	Val
Arg(R)	Lys ;Gln ;Asn	Lys
Asn(N)	Gln ;His ;Asp, Lys ;Arg	Gln
Asp(D)	Glu ;Asn	Glu
Cys(C)	Ser ;Ala	Ser
Gln(Q)	Asn ;Glu	Asn
Glu(E)	Asp ;Gln	Asp
Gly(G)	Ala	Ala
His(H)	Asn ;Gln ;Lys ;Arg	Arg
Ile(I)	Leu ;Val ;Met ;Ala ;Phe ;正亮氨酸	Leu
Leu(L)	正亮氨酸 ;Ile ;Val ;Met ;Ala ;Phe	Ile
Lys(K)	Arg ;Gln ;Asn	Arg
Met(M)	Leu ;Phe ;Ile	Leu
Phe(F)	Trp ;Leu ;Val ;Ile ;Ala ;Tyr	Tyr
Pro(P)	Ala	Ala

Ser(S)	Thr	Thr
Thr(T)	Val ;Ser	Ser
Trp(W)	Tyr ;Phe	Tyr
Tyr(Y)	Trp ;Phe ;Thr ;Ser	Phe
Val(V)	Ile ;Leu ;Met ;Phe ;Ala ;正亮氨酸	Leu

[0217] 对多肽生物学性质的大量修饰通过选择其对维持以下具有显著不同效果的替换来实现：(a) 替换区域中多肽骨架的结构，例如，作为叠片或螺旋构象，(b) 分子在靶位点处的电荷或疏水性，或 (c) 侧链的大小。氨基酸可根据其侧链性质的相似性来分类 (A. L. Lehninger, Biochemistry 第二版，第 73-75 页，Worth Publishers, New York (1975))：

[0218] (1) 非极性：Ala(a)、Val(V)、Leu(L)、Ile(I)、Pro(P)、Phe(F)、Trp(W)、Met(M)

[0219] (2) 不带电的极性：Gly(G)、Ser(S)、Thr(T)、Cys(C)、Tyr(Y)、Asn(N)、Gln(Q)

[0220] (3) 酸性：Asp(D)、Glu(E)

[0221] (4) 碱性：Lys(K)、Arg(R)、His(H)

[0222] 作为另一选择，可基于共同的侧链性质来对天然存在的残基进行分类：

[0223] (1) 疏水：正亮氨酸、Met、Ala、Val、Leu、Ile；

[0224] (2) 中性亲水：Cys、Ser、Thr、Asn、Gln；

[0225] (3) 酸性：Asp、Glu；

[0226] (4) 碱性：His、Lys、Arg；

[0227] (5) 影响链朝向的残基：Gly、Pro；

[0228] (6) 芳族：Trp、Tyr、Phe。

[0229] 非保守替换将使这些种类的一种氨基酸换成另一种类。

[0230] 任何不涉及维持抗体正确构象的半胱氨酸残基也可被取代，一般取代为丝氨酸，以改进分子的氧化稳定性并防止异常交联。相反，可向多肽添加半胱氨酸键（多个）来改进其稳定性（特别是在抗体是例如 Fv 片段等抗体片段的情况下）。

[0231] 特别优选的替换变体类型涉及替换母抗体（例如人源化抗体）的一个或多个高变区残基。一般而言，所得的选择用于进一步开发的变体（多个）将在生物学性质方面相对于生成它们的母抗体有所改进。用于生成这样的替换性变体的方便方法涉及使用噬菌体展示的亲和成熟。简略而言，突变一些高变区位点（例如 6 至 7 个位点）以生成各位点处的所有可能的氨基酸替换。由此生成的抗体变体作为与颗粒内包装的 M13 的基因 III 产物的融合物、以单价方式展示于丝状噬菌体颗粒。然后，如本文公开，对噬菌体展示的变体筛选其生物学活性（例如结合亲和力）。为了鉴定用于修饰的候选高变区位点，可进行丙氨酸扫描诱变以鉴定对抗原结合有显著贡献的高变区残基。作为另一选择或此外，分析抗原-抗体复合物的晶体结构对鉴定抗体与靶靶之间的接触点可为有益的。这样的接触残基以及相邻残基是用于根据本文详述的技术的替换候选物。当生成这样的变体后，使该批变体经历如本文描述的筛选，并将在一个或多个相关测定中具有优异性质的抗体用于进一步开发。

[0232] 另一类型的多肽氨基酸变体改变抗体的初始糖基化模式。多肽可包含非氨基酸部分。例如,多肽可被糖基化。这样的糖基化可在宿主细胞或宿主生物体中的多肽表达期间天然发生,或者可通过人为干预来有意修饰。改变意为缺失一个或多个多肽中发现的碳水化合物部分,和/或添加一个或多个多肽中不存在的糖基化位点。

[0233] 多肽的糖基化通常是N-连接或O-连接。N-连接是指碳水化合物部分附接至天冬酰胺残基的侧链上。三肽序列天冬酰胺-X-丝氨酸和天冬酰胺-X-苏氨酸,其中X是除脯氨酸以外的任何氨基酸,是用于将碳水化合物部分酶促附接至天冬酰胺侧链的识别序列。因此,多肽中存在任意这些三肽序列都将创造出潜在的糖基化位点。O-连接的糖基化是指糖N-乙酰半乳糖胺、半乳糖或木糖中的一种附接至羟基氨基酸,最通常为丝氨酸或苏氨酸,但是还可使用5-羟基脯氨酸或5-羟基赖氨酸。

[0234] 通过改变氨基酸序列以使其包含一个或多个上述三肽序列来实现将糖基化位点添加至多肽(针对N-连接的糖基化位点)。还可通过添加一个或多个丝氨酸或苏氨酸残基至初始抗体的序列,或通过用一个或多个丝氨酸或苏氨酸残基替换初始抗体序列来进行该改变(针对O-连接的糖基化位点)。

[0235] 去除存在于多肽上的碳水化合物部分可通过化学方式或酶方式或通过突变替换编码充当糖基化标靶的氨基酸残基的密码子来实现。对多肽上碳水化合物部分的酶切割可通过使用多种内-糖苷酶和外-糖苷酶来实现。

[0236] 其他修饰包括:将谷氨酰胺和天冬酰胺残基脱酰胺成相应的谷氨酰胺和天冬酰胺残基,羟化脯氨酸和赖氨酸,对丝氨酸或苏氨酸残基的羟基的磷酸化,对赖氨酸、精氨酸和组氨酸侧链的 α -氨基的甲基化,对N端胺的乙酰化,以及对任何C端羧基的酰胺化。

[0237] (ii) 嵌合多肽

[0238] 可以以形成包含融合至另一异源多肽或氨基酸序列的多肽的嵌合分子的方式来修饰本文描述的多肽。在一些实施方案中,嵌合分子包含这样的多肽融合物,其具有提供可被抗标签抗体选择性结合的表位的标签多肽。所述表位标签一般位于多肽的氨基端或羧基端。可通过使用标签多肽的抗体来检测这样的表位标记形式的多肽的存在。此外,提供表位标签使能够容易地通过使用抗标签抗体或结合表位标签的另一类型亲和基质的亲和纯化来纯化多肽。

[0239] 在一个可选实施方案中,所述嵌合分子可包含多肽与免疫球蛋白或免疫球蛋白特定区的融合物。二价形式的嵌合分子称为“免疫粘附素”。

[0240] 本文所用术语“免疫粘附素”表示组合了异源多肽的结合特异性与免疫球蛋白恒定结构域的效应子功能的类抗体分子。从结构上而言,免疫粘附素包含氨基酸序列与免疫球蛋白恒定结构域序列的融合物,所述氨基酸序列具有期望的结合特异性,其不同于抗体的抗原识别,和结合位点(即是“异源的”)。免疫粘附素分子的粘附素部分通常是至少包含受体或配体的结合位点的连续的氨基酸序列。免疫粘附素中的免疫球蛋白恒定结构域序列可得自任何免疫球蛋白,例如IgG-1、IgG-2、IgG-3或IgG-4亚型、IgA(包括IgA-1和IgA-2)、IgE、IgD或IgM。

[0241] Ig融合优选包括可溶性形式(缺失或失活跨膜结构域)的多肽替换Ig分子内的至少一个可变区。在一个特别优选的实施方案中,免疫球蛋白融合物包含IgG1分子的铰链区、CH₂区和CH₃区,或者铰链区、CH₁区、CH₂区和CH₃区。

[0242] (iii) 多肽缀合物

[0243] 用于多肽制剂的多肽可缀合至细胞毒性试剂,例如化疗剂、生长抑制剂、毒素(细菌、真菌、植物或动物来源的酶活化毒素或其片段),或放射性同位素(即放射性缀合物)。

[0244] 可使用可用于生产这样的缀合物的化疗剂。此外,可使用的酶活化毒素及其片段包括:白喉 A 链、白喉毒素的非结合活化片段、外毒素 A 链(来自绿脓假单胞菌(*Pseudomonas aeruginosa*))、蓖麻毒素 A 链、红豆因(abrin)A 链、蒴莲根毒素(modeccin)A 链、 α -帚曲霉素(sarcin)、油桐(*Aleutites fordii*)蛋白、香石竹毒(dianthin)蛋白、美洲商陆(*Phytolacca americana*)蛋白(PAP1、PAPII 和 PAP-S)、苦瓜(*Momordica charantia*)抑制剂、麻疯树毒蛋白(curcin)、巴豆毒蛋白(crotonin)、肥皂草(*Saponaire officinalis*)抑制剂、白树毒蛋白(gelonin)、丝林霉素(mitogellin)、局限曲菌素(restrictocin)、酚霉素(phenomycin)、新霉素(enomycin)和单端孢菌素(tricothecenes)。多种放射性核素可用于生成放射性缀合的抗体。例子包括 ^{212}Bi 、 ^{131}I 、 ^{131}In 、 ^{90}Y 和 ^{186}Re 。使用多种双官能蛋白质偶联剂来制备抗体和细胞毒性剂的缀合物,所述双官能偶联剂例如 N-琥珀酰亚胺基-3-(2-吡啶基二硫基)丙酸酯(SPDP)、亚氨基硫烷(iminothiolane)(IT)、亚氨酸酯(例如盐酸二甲基己二亚胺酸酯)、活性酯类(例如辛二酸二琥珀酰亚胺酯)、醛(例如戊二醛)、双叠氮化合物(例如双(对叠氮苯甲酰基)己二胺)、双-重氮鎓衍生物(例如双(对重氮苯甲酰基)-乙二胺)、二异硫氰酸酯(例如甲苯 2,6-二异氰酸酯)和双活性氟化合物(例如 1,5-二氟-2,4-二硝基苯)的双官能衍生物。例如,可如 Vitetta 等 Science 238:1098(1987)中所述来制备蓖麻毒素免疫毒素。碳-14 标记的 1-异硫氰酸苯甲基-3-甲基二亚乙基三胺五乙酸(MX-DTPA)是用于将放射性核苷酸缀合至抗体的示例性螯合剂。

[0245] 本文还预期了多肽与一种或多种小分子毒素的缀合物,所述小分子毒素例如卡里奇霉素(calicheamicin)、类美登素(maytansinoids)、单端孢霉烯(trichothene)和 CC1065,以及这些毒素的具有毒素活性的衍生物的缀合物。

[0246] 类美登素是通过抑制微管蛋白聚合来起作用的有丝分裂抑制剂。美登素首次分离于东非灌木齿叶美登木(Maytenus serrata)。随后,发现某些微生物也产生类美登素,例如美登醇(maytansinol)和 C-3 美登醇酯。还预期了合成美登醇及其衍生物和类似物。存在许多本领域已知的用于制备多肽-类美登素缀合物的连接基团,包括例如美国专利 No. 5,208,020 中公开的那些。如上文指定的专利所公开,所述连接基团包括:二硫基、硫醚基、酸不稳定基团、光不稳定基团、肽酶不稳定基团或酯酶不稳定基团,优选二硫基和硫醚基。

[0247] 接头可根据连接的类型在多种位置附接于类美登素分子。例如,可使用常规的耦合技术、通过与羟基的反应来形成酯链接。反应可发生于具有羟基的 C-3 位、被羟甲基修饰的 C-14 位、被羟基修饰的 C-15 位和具有羟基的 C-20 位。在一个优选实施方案中,在美登醇或美登醇类似物的 C-3 位形成连接。

[0248] 另一感兴趣的缀合物包含缀合至一个或多个卡里奇霉素分子的多肽。抗生素的卡里奇霉素家族能够在皮摩尔浓度下产生双链 DNA 断裂。卡里奇霉素家族的缀合物的制备参见例如美国专利 No. 5,712,374。可使用的卡里奇霉素的结构类似物包括但不限于 γ_1^I 、 α_2^I 、 α_3^I 、N-乙酰基- γ_1^I 、PSAG 和 θ_1^I 。抗体可缀合的另一抗肿瘤药物是抗叶酸剂 QFA。卡

里奇霉素和 QFA 二者都具有细胞内作用部位,并且不容易穿过质膜。因此,通过多肽(例如抗体)介导的内化作用来细胞内摄入这些试剂大大地增强了它们的细胞毒性。

[0249] 可缀合至本文描述的多肽的其他抗肿瘤剂包括 BCNU、链佐星、长春新碱和 5- 氟尿嘧啶、已知的统称为 LL-E33288 复合物的药剂家族,以及埃斯波霉素 (esperamicins)。

[0250] 在一些实施方案中,所述多肽可为多肽与具有溶核活性的化合物(例如核糖核酸酶或 DNA 核酸内切酶如脱氧核糖核酸酶;DNA 酶)的缀合物。

[0251] 在另一个实施方案中,可将多肽(例如抗体)缀合至“受体”(例如链霉亲和素)用于肿瘤预靶向,其中将多肽受体缀合物施用至患者,接着使用清除剂从循环系统去除未结合的缀合物,然后施用缀合至细胞毒性剂(例如放射性核苷酸)的“配体”(例如抗生物素蛋白)。

[0252] 在一些实施方案中,所述多肽可缀合至前药活化性酶,后者可将前药(例如肽基化疗剂)转化成活性抗癌药物。免疫缀合物的酶组分包括能够以将前药转化成其更高活性、细胞毒性形式的方式来作用于前药的任何酶。

[0253] 可用的酶包括但不限于:可用于将包含磷酸盐的前药转化成游离药物的碱性磷酸酶;可用于将包含硫酸盐的前药转化成游离药物的芳基硫酸酶;可用于将无毒 5- 氟胞嘧啶转化成抗癌药物 5- 氟尿嘧啶的胞嘧啶脱氨酶;可用于将包含肽的前药转化成游离药物的蛋白酶,例如沙雷氏菌蛋白酶、嗜热菌蛋白酶 (thermolysin)、枯草杆菌蛋白酶 (subtilisin)、羧肽酶和组织蛋白酶(如组织蛋白酶 B 和 L);可用于转化包含 D- 氨基酸取代基的前药的 D- 丙氨酰基羧肽酶;可用于将糖基化的前药转化成游离药物的碳水化合物切割性酶,例如 β - 半乳糖苷酶和神经酰胺酶;可用于将用 β - 内酰胺衍生的前药转化成游离药物的 β - 内酰胺酶;以及可用于将分别用苯氧基乙酰基或苯基乙酰基在其胺氮处衍生的药物转化成游离药物的青霉素酰胺酶,例如青霉素 V 酰胺酶或青霉素 G 酰胺酶。作为另一选择,可使用具有酶活性的抗体——在本领域也称为“抗体酶”——来将前药转化成活性药物。

[0254] (iv) 其他

[0255] 对多肽的另一类型的共价修饰包括将多肽连接至多种非蛋白聚合物中的一种,例如聚乙二醇、聚丙二醇、聚氧化烯或聚乙二醇与聚丙二醇的共聚物。多肽还可以包载于例如通过凝聚技术或通过界面聚合制备的微胶囊(例如分别地羟甲基纤维素或明胶-微胶囊和聚-(甲基丙烯酸甲酯)微胶囊)中,胶状药物递送系统(例如脂质体、白蛋白微球、微乳液、纳米-颗粒和纳米胶囊)中,或粗乳液中。Remington's Pharmaceutical Sciences, 第 18 版, Gennaro, A. R., 编 (1990) 中公开了这样的技术。

[0256] IV. 得到用于制剂和方法的多肽

[0257] 可通过使用包括重组法在内的本领域公知方法来得到用于本文描述的分析方法中的多肽。以下部分提供了关于这些方法的指导。

[0258] (A) 多核苷酸

[0259] “多核苷酸”或“核酸”在本文中可互换使用,是指任何长度的核苷酸聚合物,并且包括 DNA 和 RNA。

[0260] 编码多肽的多核苷酸可来自任何来源,包括但不限于被视为拥有多肽 mRNA 并且以可检测水平进行表达的组织制备的 cDNA 文库。因此,编码多核苷酸的多肽可方便地得自

从人组织制备的 cDNA 文库。编码多肽的基因还可得自基因组文库或通过已知的合成方法得到（例如自动核酸合成）。

[0261] 例如，多核苷酸可编码完整的免疫球蛋白分子链，例如轻链或重链。完整的重链不仅包含重链可变区 (V_H)，还包含通常包含三个恒定结构域 C_H1 、 C_H2 和 C_H3 的重链恒定区 (C_H)；以及“铰链”区。在一些情况下，期望存在恒定区。

[0262] 可通过多核苷酸来编码的其他多肽，包括抗原结合性的抗体片段，例如单一结构域抗体（“dAb”）、Fv、scFv、Fab' 和 F(ab')₂，以及“微型抗体”。微型抗体（通常）是已将 C_H1 和 C_H 或 C_L 结构域结构域从其切离的二价抗体片段。因为微型抗体小于常规抗体，所以它们将在临床 / 诊断用途中实现更佳的组织渗透性，但是由于是二价的，所以它们将保留比一价抗体片段更高的结合亲和力，例如 dAb。因此，除非上下文另有指明，否则本文所用术语“抗体”不仅涵盖完整的抗体分子，还涵盖上述类型的抗原结合性的抗体片段。优选地，编码多肽中存在的各构架区都将包含相对于相应的人受体构架的至少一个氨基酸替换。因此，例如，构架区可包含相对于受体构架区的总共三、四、五、六、七、八、九、十、十一、十二、十三、十四或十五个氨基酸替换。

[0263] VI. 示例性实施方案

[0264] 1. 在一个实施方案中，本发明提供了用于分析包含多肽以及一种或多种污染物的组合物的方法，所述方法包括 a) 使用加样缓冲剂使所述多肽以及一种或多种污染物结合至离子交换色谱材料，其中所述加样缓冲剂处于第一 pH，且包含第一离子强度；b) 使用洗脱缓冲剂从所述离子交换色谱材料洗脱所述多肽以及一种或多种污染物，其中在 pH 梯度中改变所述洗脱缓冲剂的 pH，并且在离子强度梯度中改变所述洗脱缓冲剂的离子强度，其中通过所述 pH 梯度和离子强度梯度的组合分离所述多肽以及所述一种或多种污染物；c) 检测所述多肽和所述一种或多种污染物。在该实施方案的一些方面，本发明提供用于分析包含多肽以及一种或多种污染物的组合物中的所述多肽的方法，其中所述方法将一种或多种污染物从所述多肽分离，所述方法包括 a) 使用加样缓冲剂使所述多肽以及一种或多种污染物结合至离子交换色谱材料，其中所述加样缓冲剂处于第一 pH，且包含第一离子强度；b) 使用洗脱缓冲剂从所述离子交换色谱材料洗脱所述多肽以及一种或多种污染物，其中在 pH 梯度中改变所述洗脱缓冲剂的 pH，且在离子强度梯度中改变所述洗脱缓冲剂的离子强度，其中通过所述 pH 梯度和离子强度梯度的组合分离所述多肽以及所述一种或多种污染物；c) 检测所述多肽和所述一种或多种污染物，其中所述方法用于分析 pI 介于约 7.0 至约 9.5 范围的多肽。

[0265] 2. 在实施方案 1 的另一实施方案中，所述多肽是抗体或免疫粘附素，或其片段。

[0266] 3. 在实施方案 1 或 2 的另一实施方案中，所述多肽是单克隆抗体或其片段。

[0267] 4. 在实施方案 2 或 3 的另一实施方案中，所述抗体是人抗体。

[0268] 5. 在实施方案 2 或 3 的另一实施方案中，所述抗体是人源化抗体。

[0269] 6. 在实施方案 2 或 3 的另一实施方案中，所述抗体是嵌合抗体。

[0270] 7. 在实施方案 2 至 6 中任一项的另一实施方案中，所述抗体是抗体片段。

[0271] 8. 在实施方案 1 至 7 中任一项的另一实施方案中，所述污染物是多肽的变体。

[0272] 9. 在实施方案 1 至 7 中任一项的另一实施方案中，所述污染物是多肽的降解产物。例如，电荷变体。

- [0273] 10. 在实施方案 1 至 9 中任一项的另一实施方案中,所述多肽具有大于约 9.0 的 pI。
- [0274] 11. 在实施方案 1 至 10 中任一项的另一实施方案中,所述色谱材料是阳离子交换色谱材料。
- [0275] 12. 在实施方案 11 的另一实施方案中,所述阳离子交换色谱材料是磺化色谱材料或羧基化色谱材料。
- [0276] 13. 在实施方案 1 至 12 中任一项的另一实施方案中,所述 pH 梯度是线性梯度。
- [0277] 14. 在实施方案 1 至 12 中任一项的另一实施方案中,所述 pH 梯度是阶跃梯度。
- [0278] 15. 在实施方案 13 或 14 的另一实施方案中,所述 pH 梯度包含从约 pH 5 至约 pH 11 的升高。
- [0279] 16. 在实施方案 1 至 15 中任一项的另一实施方案中,所述 pH 梯度通过使用一种或多种缓冲剂来产生。
- [0280] 17. 在实施方案 15 的另一实施方案中,所述一种或多种缓冲剂选自哌嗪、咪唑、tris、磷酸盐或 CAPS。
- [0281] 18. 在实施方案 1 至 17 中任一项的另一实施方案中,所述离子强度梯度是线性梯度。
- [0282] 19. 在实施方案 1 至 17 中任一项的另一实施方案中,所述离子强度梯度是阶跃梯度。
- [0283] 20. 在实施方案 18 或 19 的另一实施方案中,所述离子强度梯度包含从约 0mM 至约 200mM 的盐浓度升高。
- [0284] 21. 在实施方案 18 至 20 中任一项的另一实施方案中,所述离子强度梯度是 NaCl 梯度、KCl 梯度或 Na₂SO₄梯度。
- [0285] 22. 在实施方案 1 至 9 中任一项的另一实施方案中,所述多肽具有小于约 7.0 的 pI。
- [0286] 23. 在实施方案 22 的另一实施方案中,所述色谱材料是阴离子交换色谱材料。
- [0287] 24. 在实施方案 23 的另一实施方案中,所述阴离子交换色谱材料是季胺色谱材料或叔胺色谱材料。
- [0288] 25. 在实施方案 22 至 24 中任一项的另一实施方案中,所述 pH 梯度是线性梯度。
- [0289] 26. 在实施方案 22 至 24 中任一项的另一实施方案中,所述 pH 梯度是阶跃梯度。
- [0290] 27. 在实施方案 25 或 26 的另一实施方案中,所述 pH 梯度包含从约 pH 8 至约 pH 5 的降低。
- [0291] 28. 在实施方案 22 至 27 中任一项的另一实施方案中,所述 pH 梯度通过使用一种或多种缓冲剂来产生。
- [0292] 29. 在实施方案 28 的另一实施方案中,所述一种或多种缓冲剂选自哌嗪、咪唑或 Tris。
- [0293] 30. 在实施方案 22 至 29 中任一项的另一实施方案中,所述离子强度梯度是线性梯度。
- [0294] 31. 在实施方案 22 至 29 中任一项的另一实施方案中,所述离子强度梯度是阶跃梯度。

[0295] 32. 在实施方案 30 或 31 的另一实施方案中,所述离子强度梯度包含从约 0mM 至约 200mM 的盐浓度升高。

[0296] 33. 在实施方案 30 至 32 中任一项的另一实施方案中,所述离子强度梯度是 NaCl 梯度、KCl 梯度或 Na_2SO_4 梯度。

[0297] 34. 在一个实施方案中,本发明提供了用于分析包含多肽以及一种或多种污染物的组合物的方法,所述方法包括 a) 使用加样缓冲剂使所述多肽以及一种或多种污染物结合至离子交换色谱材料,其中所述加样缓冲剂处于初始 pH,且包含初始离子强度;b) 使用洗脱缓冲剂从所述离子交换色谱材料洗脱所述多肽以及一种或多种污染物,其中在 pH 梯度中改变所述洗脱缓冲剂的 pH,且其中所述洗脱缓冲剂的离子强度基本与所述加样缓冲剂的初始离子强度相同,其中通过在约所述初始离子强度下的 pH 梯度来分离所述多肽以及所述一种或多种污染物;c) 检测所述多肽和所述一种或多种污染物。在该实施方案的一些方面中,本发明提供了用于分析包含多肽以及一种或多种污染物的组合物中的多肽的方法,所述方法包括 a) 使用加样缓冲剂使所述多肽以及一种或多种污染物结合至离子交换色谱材料,其中所述加样缓冲剂处于初始 pH,且包含初始离子强度;b) 使用洗脱缓冲剂从所述离子交换色谱材料洗脱所述多肽以及一种或多种污染物,其中在 pH 梯度中改变所述洗脱缓冲剂的 pH,且其中所述洗脱缓冲剂的离子强度基本与所述加样缓冲剂的初始离子强度相同,其中通过在约所述初始离子强度下的 pH 梯度来分离所述多肽以及所述一种或多种污染物;c) 检测所述多肽和所述一种或多种污染物,其中所述方法用于分析 pI 介于约 7.0 至约 9.5 范围的多肽。

[0298] 35. 在实施方案 34 的另一实施方案中,所述多肽是抗体或免疫粘附素,或其片段。

[0299] 36. 在实施方案 34 或 35 的另一实施方案中,所述多肽是单克隆抗体或其片段。

[0300] 37. 在实施方案 35 或 36 的另一实施方案中,所述抗体是人抗体。

[0301] 38. 在实施方案 35 或 36 的另一实施方案中,所述抗体是人源化抗体。

[0302] 39. 在实施方案 35 或 36 的另一实施方案中,所述抗体是嵌合抗体。

[0303] 40. 在实施方案 35 至 39 中任一项的另一实施方案中,所述抗体是抗体片段。

[0304] 41. 在实施方案 34 至 40 中任一项的另一实施方案中,所述污染物是多肽的变体。

[0305] 42. 在实施方案 34 至 40 中任一项的另一实施方案中,所述污染物是多肽的降解产物。

[0306] 43. 在实施方案 34 至 42 中任一项的另一实施方案中,所述多肽具有大于约 9.0 的 pI。

[0307] 44. 在实施方案 34 至 43 中任一项的另一实施方案中,所述色谱材料是阳离子交换色谱材料。

[0308] 45. 在实施方案 44 的另一实施方案中,所述阳离子交换色谱材料是磺化的色谱材料或羧基化的色谱材料。

[0309] 46. 在实施方案 34 至 45 中任一项的另一实施方案中,所述 pH 梯度是线性梯度。

[0310] 47. 在实施方案 34 至 45 中任一项的另一实施方案中,所述 pH 梯度是阶跃梯度。

[0311] 48. 在实施方案 46 或 47 的另一实施方案中,所述 pH 梯度包含从约 pH 5 至约 pH 11 的升高。

[0312] 49. 在实施方案 34 至 48 中任一项的另一实施方案中,所述 pH 梯度通过使用一种

或多种缓冲剂来产生。

[0313] 50. 在实施方案 49 的另一实施方案中,所述一种或多种缓冲剂选自哌嗪、咪唑、tris、磷酸盐或 CAPS。

[0314] 51. 在实施方案 34 至 50 中任一项的另一实施方案中,所述洗脱缓冲剂的离子强度为约 0mM 至约 100mM。

[0315] 52. 在实施方案 51 的另一实施方案中,所述洗脱缓冲剂包含约 0mM NaCl 至约 100mM NaCl、约 0mM KCl 至约 100mM KCl,或约 0mM Na₂SO₄至约 100mM Na₂SO₄。

[0316] 53. 在实施方案 34 至 42 中任一项的另一实施方案中,所述多肽具有小于约 7.0 的 pI。

[0317] 54. 在实施方案 53 的另一实施方案中,所述色谱材料是阴离子交换色谱材料。

[0318] 55. 在实施方案 54 的另一实施方案中,所述阴离子交换色谱材料是季胺色谱材料或叔胺色谱材料。

[0319] 56. 在实施方案 53 至 55 中任一项的另一实施方案中,所述 pH 梯度是线性梯度。

[0320] 57. 在实施方案 53 至 55 中任一项的另一实施方案中,所述 pH 梯度是阶跃梯度。

[0321] 58. 在实施方案 56 或 57 的另一实施方案中,所述 pH 梯度包含从约 pH 8 至约 pH 5 的降低。

[0322] 59. 在实施方案 53 至 58 中任一项的另一实施方案中,所述 pH 梯度通过使用一种或多种缓冲剂来产生。

[0323] 60. 在实施方案 59 的另一实施方案中,所述一种或多种缓冲剂选自哌嗪、咪唑或 Tris。

[0324] 61. 在实施方案 53 至 60 中任一项的另一实施方案中,所述洗脱缓冲剂的离子强度为约 0mM 至约 100mM。

[0325] 62. 在实施方案 61 的另一实施方案中,所述洗脱缓冲剂包含约 10mM NaCl 至约 100mM NaCl。

[0326] 63. 在实施方案 1 至 62 中任一项的另一实施方案中,所述分析通过高效液相色谱来进行。

[0327] 64. 在实施方案 1 至 63 中任一项的另一实施方案中,所述加样缓冲剂和 / 或所述洗脱缓冲剂中的缓冲剂浓度为 10mM 至约 50mM。

[0328] 65. 在实施方案 1 至 64 中任一项的另一实施方案中,所述第一 pH 为约 pH 5.0 至约 pH 7.0。

[0329] 66. 在实施方案 1 至 65 中任一项的另一实施方案中,所述色谱材料的温度为约 20℃至约 50℃。

[0330] 67. 在实施方案 1 至 66 中任一项的另一实施方案中,所述加样和洗脱在约 0.5ml/分钟至约 2.0ml/ 分钟的流速进行。

[0331] 68. 在另一实施方案中,本发明提供了一种确定组合物中多肽的纯度的方法,其包括根据实施方案 1 至 67 的方法中的任一种来分析所述组合物,并确定所述组合物中多肽与污染物的比率。

[0332] 69. 在另一实施方案中,本发明提供了一种确定包含多肽的组合物中所述多肽的稳定性的方法,所述方法包括:a) 将所述包含多肽的组合物于 0℃至 40℃温育一周至六周,

b) 通过实施方案 1 至 67 所述的方法中的任一方法来分析步骤 a) 的所述组合物, 以及 c) 确定所述组合物中变体与多肽的比率, 其中, 与未进行温育的组合物相比, 所述组合物中变体与多肽的比率的升高指示所述组合物所述多肽的降解。

[0333] 在本说明书中公开的所有特征可以任意组合。在本说明书中公开的各特征可由服务于相同、等效或相似目的的替选特征来代替。因此, 除非另有说明, 公开的各特征仅为一系列等效或类似特征中的一个例子而已。

[0334] 本发明的进一步细节由下面的非限制性实施例来描述。说明书中的所有文献的公开内容都清楚地通过引用并入本文。

实施例

[0335] 以下实施例旨在纯粹示范本发明, 并且因此不应当以任何方式视为对本发明的限制。以下实施例和详述是以举例说明的方式而不是以限制的方式给出。

[0336] 实施例的材料和方法

[0337] 除非另有说明, 否则以下材料和方法用于实施例。

[0338] 材料

[0339] 所有 mAb 都使用稳定的中国仓鼠卵巢 (CHO) 细胞系于 Genentech (South San Francisco, CA) 内部制造。通过使用来自仪器制造商的 icIEF 方案 (Wu, J 和 Huang, T (2006) Electrophoresis 27:3584)、使用七个 pI 标志物来以实验确定所使用 mAb 的 pI 值。通过在 40°C 分别温育 mAb 3 周和 6 周来得到热胁迫的样品。在色谱分析前将胁迫的 mAb 储存于 -80°C。

[0340] Propac WCX-10 柱购自 Dionex。咪唑得自 EMD Biosciences 或 Fluka。哌嗪 (无水) 得自 Tokyo Chemical Industry Co. LTD。Trisma (Tris) 得自 Mallinckrodt Baker Inc. 或 Sigma (St. Louis, MO)。Trizma 碱和 CAPS 得自 Sigma。氯化钠、氢氧化钠 (10N) 和盐酸 (12N) 得自 Mallinckrodt Baker Inc.。磷酸 (85%) 得自 EMD Millipore。

[0341] HPLC 设置

[0342] 阳离子交换色谱实验主要在 Waters 2796BioAlliance 液相色谱仪或 UltiMate 3000Quaternary Rapid Separation LC (Thermo Scientific Dionex) 上进行。该仪器包括低压四元梯度泵、具有温度控制能力的自动进样器、用于精确控制温度的热柱室和双波长二极管阵列 UV 检测器。在柱的出口处, 以串联方式连接在线 pH 传感器 (Sensorex 的 Model S450CD) 和电导率传感器 (Amber Science, Eugene, OR 的 Model 529)。pH 传感器由 Mettler Toledo 的模型 Seven Multi pH 计控制; 电导率传感器由 Amber Science 的模型 1056 数字电导计来控制。将来自这两个仪表的 pH 和电导率读数通过 Dionex UCI 50 类似物 / 数字转换器收集进 Chromeleon 中。用 6.8 版的 Dionex Chromeleon 软件来进行仪器控制、数据获取和数据分析。

[0343] 流动相制备

[0344] tris 和咪唑的单独的储存缓冲剂溶液制备为 1.0M, 且 CAPS 溶液制备为 0.1M 的浓度, 不调节 pH 值, 并储存于室温。首先在不调节 pH 值的情况下制备包含 40mM 哌嗪、40mM 咪唑和 40mM Tris (都为游离碱) 缓冲剂储液, 并储存于室温。在色谱实验之前, 通过用去离子水稀释缓冲剂储液来分别制备包含等摩尔浓度的 1、2mM、4mM 或 8mM 哌嗪、咪唑和 Tris

的系列流动相缓冲剂。然后,分别使用盐酸将缓冲剂的 pH 值分别调节至 5.0(缓冲剂 A)和 10.8(缓冲剂 B)。用去离子水制备 0.5M 的氯化钠溶液(盐溶液)。然后,在使用前通过 0.2 μ M 尼龙过滤器单独过滤流动相。

[0345] 如文献报道制备具有 11.6mM 哌嗪、1.5mM 咪唑和 2.4mM Tris 的流动相缓冲剂(Farnan, D 和 Moreno, GT(2009) Anal. Chem. 81:8846 ;Rea, JC 等, (2010) J. Pharm. Biomed. Anal. 54:317)。首先制备包含 116mM 哌嗪、15mM 咪唑和 24mM Tris 的十倍浓缩储备液,并储存于室温。在每个实验之前,用去离子水将两等份储液稀释 10 倍,然后使用盐酸将其 pH 调节为 5.0(缓冲剂 C)和 9.5(缓冲剂 D)。然后,在使用前通过 0.2 μ M 尼龙过滤器单独过滤流动相。

[0346] 阴离子交换色谱

[0347] 除非另有说明,否则色谱条件如下。用去离子水将 mAb 样品(对照样品和胁迫样品)稀释至 2mg/mL,并于 $5 \pm 3^\circ\text{C}$ 保存于自动进样器中。作为另一选择,用缓冲剂 A 与 D 的 1:1 混合物将 mAb 样品稀释至 1mg/mL,并在 $5 \pm 3^\circ\text{C}$ 保存于自动进样器中。将 Propac WCX-10HT, $4 \times 50\text{mm}$ 柱放置于温度设置为 $40 \pm 1^\circ\text{C}$ 的柱室中。使用 $4 \times 250\text{mm}$ Dionex Propac WCX 柱来进行色谱分离,并将其放置于温度设置为 $40 \pm 1^\circ\text{C}$ 的柱室中。每次色谱运行注射 10 μ L 蛋白(20 μ g)。

[0348] 通过使用在四元泵上以缓冲剂 A、B 和盐溶液(0.5M NaCl)形成的三元梯度来建立盐介导的 pH 梯度。用 58 分钟来递送 100% 的 A 至 96.8% 的 B 的线性梯度和 3.2% 的盐溶液,以建立 5.0 至 10.8 的 pH 梯度(0.1pH 单位/分钟)以及 0mM NaCl 至 16mM NaCl 的介导盐梯度(0.28mM/分钟)。最终梯度(分钟, % B 和 C)如下:0, 100% A;2, 100% A;60, 96.8% B 和 3.2% C;64, 96.8% B 和 3.2% C;65, 100% A;75, 100% A。流动相流速为 1.0mL/分钟。

[0349] 用缓冲剂 C 和 D 建立 5.0 至 9.5 的报道 pH 梯度(Farnan, D 和 Moreno, GT(2009) Anal. Chem. 81:8846)。用 45 分钟递送 0 至 100% 的缓冲剂 D 线性升高,以建立 5.0 至 9.5 的 pH 梯度(0.1pH 单位/分钟)。流动相流速为 1.0mL/分钟。通过 280nm 的紫外(UV)吸光度来检测蛋白质。

[0350] 通过使用以缓冲剂 A、B、C 和 D 的四元泵上形成的四元梯度来建立混合 pH 梯度。该布置提供了调节以下的灵活性:1) 起始 pH 和终止 pH,使用缓冲剂 A 和 B 调节,以及 2) 用于各梯度的盐的量,使用缓冲剂 C 和 D 调节。作为这些实验中使用的一个例子,通过将缓冲剂 B 从 0 增加至 40%,同时保持缓冲剂 C 和 D 分别为 10% 和 40% 来建立 6 至 10 的 pH 梯度以及恒定的 10mM 盐浓度。使用的梯度列于表 2 中。

[0351] pH-IEC 造模

[0352] 基于理想溶液模型、使用 Henderson - Hasselbalch, H-H 方程对各组分估计两种 pH 缓冲剂的线性混合梯度的 pH。首先对各起始缓冲剂确定可获得/可解离质子的数量,然后对以 0.1pH 单位跨度的两种 pH 缓冲剂之间的各 pH 值确定可获得/可解离质子的数量。基于需要的质子数量,导出两种缓冲剂的摩尔比。得到获得梯度中 pH 点的各缓冲剂的百分比。在各 pH 点,使用估计的离子组分来计算离子强度。

[0353] 实施例 1. pH-IEC 法的评估

[0354] 评估 pH-IEC 法的性能。虽然所报道的 pH-IEC 法示出了描述多种 mAb 的电荷不均

匀性的能力,但是主要旨在用于 pI 值为约 7 至 9 的 mAb。对于超出该范围 (pI<7 或 pI>9, 也称为极限 pI 值) 的 mAb, 该 pH-IEC 法常常得到不可接受的电荷不均匀性分布。为了评估该方法, 依照以前报道的方法来产生 5.0 至 9.5 的 pH 梯度 (Farnan, D 和 Moreno, GT (2009) Anal. Chem. 81:8846 ;Rea, JC 等, (2010) J. Pharm. Biomed. Anal. 54:317)。所述缓冲剂由 11.4mM 哌嗪、1.5mM 咪唑和 2.4mM Tris 构成, 其 pH 分别调节至 5.0 和 9.5。分析横跨宽范围 pI (6.2、8.2 和 9.4) 的三种 mAb, 图 1 示出了所得色谱图。这些 mAb 中, 仅 mAb2 (pI 8.2) 示出了以良好电荷变体分离为特征的可接受的电荷不均匀性分布。低 pI mAb1 (pI = 6.2) 的电荷变体分离不良; 高 pI mAb3 (pI 9.4) 在 pH 梯度期间不洗脱。即使当将 pH 梯度扩展至 pH 10.8 时洗脱了 mAb3, 柱背压也接近柱的压力上限, 且色谱图在不同的运行之间不一致。该实验清楚地证实, 虽然所报道的 pH-IEC 法对 pI 值介于 7 至 9 之间的 mAb 很有效, 但是其不能描述具有极限 pI 值的 mAb 的电荷不均匀性。

[0355] 表 2. 样品梯度

[0356] pI 值 7.2–8.3 的样品

[0357]

pH 6-9, 10 mM NaCl; 22 min run					pH 6-9, 10 mM NaCl; 15 min run				
时间 min	%B	%C	%D	曲线	时间 min	%B	%C	%D	曲线
0.0	0.0	10	40	5	0.0	0.0	10	40	5
0.2	0.0	10	40	5	0.2	0.0	10	40	5
16.0	30.0	10	40	5	10.0	30.0	10	40	5
18.0	30.0	10	40	5	12.0	30.0	10	40	5
18.1	0.0	10	40	5	12.1	0.0	10	40	5
22.0	0.0	10	40	5	15.0	0.0	10	40	5

[0358] pI 值 8.3–9.0 的样品

[0359]

pH 6-10, 20 mM NaCl; 22 min run					pH 6-10, 20 mM NaCl; 15 min run				
时间 min	%B	%C	%D	曲线	时间 min	%B	%C	%D	曲线
0.0	0.0	20	30	5	0.0	0.0	20	30	5
0.2	0.0	20	30	5	0.2	0.0	20	30	5
16.0	40.0	20	30	5	10.0	40.0	20	30	5
18.0	40.0	20	30	5	12.0	40.0	20	30	5
18.1	0.0	20	30	5	12.1	0.0	20	30	5
22.0	0.0	20	30	5	15.0	0.0	20	30	5

[0360] pI 值 9.0–9.2 的样品

[0361]

pH 7-10, 50 mM NaCl; 22 min run					pH 7-10, 50 mM NaCl; 15 min run				
时间 min	%B	%C	%D	曲线	时间 min	%B	%C	%D	曲线
0.0	10.0	50	0	5	0.0	10.0	50	0	5
0.2	10.0	50	0	5	0.2	10.0	50	0	5
16.0	40.0	50	0	5	10.0	40.0	50	0	5
18.0	40.0	50	0	5	12.0	40.0	50	0	5
18.1	10.0	50	0	5	12.1	10.0	50	0	5
22.0	10.0	50	0	5	15.0	10.0	50	0	5

[0362] pI 值 9.3–9.4 的样品

[0363]

pH 9-11, 10 mM NaCl, 22 min run					pH 9-11, 10 mM NaCl, 15 min run				
时间 min	%B	%C	%D	曲线	时间 min	%B	%C	%D	曲线
0.0	30.0	10	40	5	0.0	30.0	10	40	5
0.2	30.0	10	40	5	0.2	30.0	10	40	5
16.0	50.0	10	40	5	10.0	50.0	10	40	5
18.0	50.0	10	40	5	12.0	50.0	10	40	5
18.1	30.0	10	40	5	12.1	30.0	10	40	5
22.0	30.0	10	40	5	15.0	30.0	10	40	5

[0364] 柱出口处的 pH 从 5.0 升高至 9.5, 溶剂的电导率以近线性的方式从 $2700 \mu\text{S/m}$ 降低至 $800 \mu\text{S/m}$ (注意, 5mM KCl 的电导率为 $720 \mu\text{S/m}$, 而去离子水的电导率为 $5.5 \mu\text{S/m}$)。三种 pH 缓冲剂组分都是 pK_a 跨越宽范围的胺类: 哌嗪 $\text{pK}_{a1} = 5.68$ 且 $\text{pK}_{a2} = 9.82$, 咪唑 $\text{pK}_a = 6.95$ 且 Tris $\text{pK}_a = 8.10$ (室温)。当溶液 pH 低于其 pK_a 时, 这些化合物被质子化 (或带正电), 而当 pH 高于其 pK_a 时变为中性。当溶剂 pH 升高时, 缓冲剂组分逐渐从质子化变为中性, 因此缓冲剂的电导率降低。值得注意的是, pH 6 附近的 pH 分布是凹的, 因为哌嗪是缓冲剂中最丰富的组分, 使 pH 曲线在其两个 pK_a 5.68 和 9.82 附近相对较平。

[0365] 还基于如图 2A 中虚线所示的理想溶液模型计算了 pH 梯度的 pH 和离子强度分布。模型 pH 曲线与实验 pH 分布非常相似, 不同之处在于实验分布因系统保压体积 (system dwell volume) 和柱体积而延迟 5 分钟。同样地, 模型离子强度曲线示出了与实验观察到的电导率分布相似的形状。模型数据和实验数据之间的一致性提示, 基于胺的缓冲剂组分的混合遵循理想溶液模型。因此, 建立的模型可用于在其他色谱条件下估计实验 pH 和离子强度分布。

[0366] 此外, pH-IEC 期间的柱背压随着流动相的 pH 显著升高 (图 2B)。鉴于在 pH 梯度期间流动相的组分是恒定的, 所以这归因于离子强度的降低。根据双层模型, 当流动相离子强度低时, 固定相表面的静电势变高 (Staahlberg, J(1994) Anal. Chem. 66:440; Stahlberg, J(1999) J. Chromatogr. A 855:3)。该高的静电势可改变树脂的构象 (例如使树脂膨胀从而降低表面电荷密度), 这很可能提高柱背压 (Product Manual for Propac WCX-10 and Propac SCX-10, 第 7 版, Dionex Incorporation, Sunnyvale, CA, 2007)。

[0367] 实验电导率和模型离子强度分布可用于解释具有极限 pI 值的 mAb 的不良电荷不均匀性分布。低 pI mAb 于低 pH 区中洗脱, 其中缓冲剂组分被质子化, 且流动相具有相对较高的离子强度。因为 pH 梯度 IEC 分离似乎涉及离子强度基洗脱机制与 pH 基洗脱机制的组合 (Anderson, DJ 和 Shan, L(2001) Clin. Chem. 47:128; Shan, L 和 Anderson, DJ(2001) J. Chromatogr. A 909:191; Shan, L 和 Anderson, DJ(2002) Anal. Chem. 74:5641), 所以高离子强度基洗脱可干扰 pH 基洗脱, 导致这些低 pI 电荷变体拆分不良。另一方面, 高 pI mAb 通常在高 pH 区中洗脱, 其中缓冲剂组分变成中性。由于流动相中的低离子强度, 这些高 pI mAb 难以从阳离子交换柱洗脱。为了证实离子强度显著影响 pH-IEC 分离, 并且为了改进用于具有极限 pI 值的 pH-IEC 法, 如下文描述来调节 pH 梯度 IEC 法中的 pH 缓冲剂的离子强度。

[0368] 实施例 3. 通过控制离子强度来改进 pH-梯度 IEC 法

[0369] pH 梯度过程期间, 以两种方式调节离子强度。第一种, 通过使用不同浓度的缓冲剂来控制低 pH 区的离子强度。如下文描述, 测试一系列缓冲剂浓度以评估其对 pH 梯度 IEC 的影响。第二种, 通过将盐梯度添加至 pH 梯度来调节高 pH 区的离子浓度。还研究了盐浓

度的影响。因此,该新方法称为“离子强度介导的 pH-IEC”法。

[0370] 缓冲剂浓度:在该方法中,使用等摩尔的 pH- 梯度而不是所报道方法中使用的混合比率 pH 梯度 (Farnan, D 和 Moreno, GT (2009) Anal. Chem. 81:8846) 这基于两点考虑:首先,近线性的 pH 梯度可通过使用等摩尔浓度的哌嗪、咪唑和 Tris 来得到 (图 2C)。建立的 pH 的跨越宽范围的线性梯度将不牺牲指定 pH 区域的分离 (Tsonev, LI and Hirsh, AG (2008) J. Chromatogr. A 1200:166)。其次,其提供对梯度斜率的优化。

[0371] 研究了由 1mM、2mM、4mM 和 8mM 的等摩尔浓度的哌嗪、咪唑和 tris 的四种缓冲剂。这些缓冲剂被称为 1mM、2mM、4mM 和 8mM 缓冲剂,每种缓冲剂都用 pH 5.0 至 10.8 的 pH 梯度和 0mM NaCl 至 16mM NaCl 的线性盐梯度来介导。图 3A 中展示了使用这四种缓冲剂的 mAb1 (pI = 6.2) 的色谱图。电荷变体之间的分辨率明显依赖于缓冲剂浓度。使用 1mM 缓冲剂,电荷变体分离不良。使用 2mM 缓冲剂分辨率改进,且使用 4mM 缓冲剂分辨率达到峰值。然而,使用 8mM 缓冲剂,分辨率显著降低。相反,mAb2 (pI = 8.2) 的分辨率对缓冲剂浓度没有 mAb1 那么敏感 (图 3B)。使用所有四种缓冲剂都达到对 mAb2 (pI = 8.2) 的良好分辨率,尽管 4mM 缓冲剂比其他三种缓冲剂提供了稍微更好的分辨率。基于目视检查,4mM 缓冲剂似乎提供了对 mAb1 和 mAb2 的最佳分辨率。

[0372] 为了更好地显示缓冲剂浓度对 pH-IEC 的影响,图 3C 示出了 mAb 的主峰的半峰全宽 (FWHM) 作为缓冲剂浓度的函数。主峰的 FWHM 一般与 pH-IEC 的分辨率相关,因为 FWHM 越低表示分辨率越高。对于 mAb1 和 mAb2 二者而言,在四种缓冲剂中,使用 4mM 缓冲剂的主峰 FWHM 最低,这提示,4mM 缓冲剂提供了最窄的峰宽,因此提供了良好的分辨率。对 mAb3 (pI = 9.4) 而言相反,当缓冲剂浓度从 1mM 升高至 8mM 时,主峰的 FWHM 稍微降低。因此,8mM 缓冲剂很可能提供了对 mAb3 的最佳分辨率。

[0373] 缓冲剂浓度对 mAb pH-IEC 的影响取决于 mAb 的 pI 值。具有低 pI (6.2) 和中 pI (8.2) 值的 mAb 示出使用 4mM 缓冲剂的最优分离,而具有高 pI 值 (9.4) 的 mAb 似乎倾向更高浓度的缓冲剂。这是有道理的,因为高 pI mAb 强结合至柱,并因此可能需要比低 pI 和中 pI mAb 更高的离子强度基洗脱以达到最优分辨率。因为在 pH 梯度 IEC 中缓冲剂浓度和电导率明显影响 mAb 的分辨率,所以每当期望高分辨率的时候都应当对每种单独的 mAb 进行优化。开发了可对跨越宽 pI 范围的 mAb 从主峰解析酸性变体和碱性变体的 IEC 法。4mM 缓冲剂似乎满足了该要求,并因此选择用于多产物盐介导的 pH-IEC 法。

[0374] 盐浓度:为了研究离子强度如何影响 pH-IEC 分离,通过 pH 5.0 至 10.8 的线性梯度,将五种不同的氯化钠水平 (0mM、8mM、16mM、32mM 和 64mM) 添加至 pH 梯度 (由 4mM 缓冲剂建立)。平行分析 mAb1、mAb2 和 mAb3。如图 3D 所示,将 mAb 的主峰 FWHM 作为盐浓度的函数进行作图。对于 mAb1,主峰的 FWHM 对盐浓度高度敏感,并且使用 8mM NaCl 其达到最小值。这提示,8mM NaCl 提供了对 mAb1 最佳的分辨率。对于 mAb2,主峰的 FWHM 在整个盐浓度范围内是基本平的,这提示,盐浓度不明显影响 mAb2 的分辨率。对 mAb3 而言相反,主峰的 FWHM 随着盐浓度从 8mM 升高至 32mM 而降低,且在 32mM 至 64mM 盐之间保持不变。这提示,mAb3 需要 32mM 的盐来达到最优分辨率。

[0375] 离子强度对 mAb 的 pH 梯度 IEC 的影响还与 mAb 的 pI 相关。使用 8mM 的盐,低 pI mAb 显示分离;高 pI mAb 示出在更高盐浓度下的分离,而中 pI mAb 的分辨率不依赖于盐浓度。由于对分辨率的明显影响,每当期望高分辨率时,可针对每种单独的 mAb 来优化盐浓

度。在五种盐浓度中,使用 16mM NaCl 盐梯度的 pH-IEC 法提供了对 pI 值跨越从 6.2 至 9.2 的宽范围的 mAb 的可接受分辨率,并因此在本文中将其选为多产物盐介导的 pH-IEC 法。

[0376] 优化的离子强度介导的 pH-IEC 法:优化的盐介导的 pH-IEC 法使用 4mM 哌嗪、4mM 咪唑和 4mM Tris 来建立 pH 梯度,并用 0mM 至 16mM NaCl 的线性盐梯度来介导。该方法的 pH 和电导率分布在图 2C 中示出为实线。为了比较,将模型 pH 和离子强度示出为虚线。除 8.5 至 9.0 的 pH 处的小凹外,柱出口处的实验 pH 以大致线性的方式随保留时间而升高,这与模型 pH 一致,不同之处在于因系统空隙体积导致的 5 分钟延时。通过用盐介导,流动相的实验电导率在 pH 梯度期间示出稍微升高 (1570 μ S 至 1800 μ S)。相似地,模型离子强度在 pH 梯度期间基本一致。通过降低缓冲剂浓度和添加线性盐梯度,成功地控制了基于胺的 pH 梯度的离子强度。通过使用受控的离子强度,在高 pH 区将柱背压维持低于 95 巴 (图 2D)。所得的 mAb1-3 色谱图 (未示出) 是可重现的,说明阳离子交换柱在该压力范围内是稳定的。

[0377] 实施例 4. 描述 16 种 mAb 的电荷不均匀性

[0378] 为了进一步该新的盐介导的 pH-IEC 法的多产物能力,分析了 pI 值从 6.2 至 9.4 的 16 种 mAb,并在图 4 中示出了它们的色谱图。用 pH 5 至 pH 10.8 的 pH 梯度和 0mM NaCl 至 16mM NaCl 的盐梯度从柱洗脱抗体。对于低 pI mAb1 (6.2) 和高 pI mAb3 (9.4) 二者,电荷变体分离良好,得到可接受的电荷不均匀性分布。相比于所报道的 pH-IEC 法 (图 1),这是大幅的改进。所有 16 种 mAb 的电荷变体分离良好,这说明所开发的盐介导的 pH-IEC 法能够在无需任何附加方法开发努力的情况下描述多种 mAb 产物的电荷不均匀性。

[0379] 除了更宽的适用性外,盐介导的 pH 梯度还提供了比所报道的 pH-IEC 法更好的分辨率。对于 mAb2 (pI = 8.2),盐介导的 pH-IEC 法提供了电荷变体之间的基线分辨率 (图 4)。但是,由以前的 pH-IEC 法得到的分辨率低得多 (图 1)。虽然所述盐介导的 pH 梯度 (58 分钟) 比以前的 pH 梯度 (45 分钟) 更长,但是两种方法的梯度斜率相同 (0.1pH 单位 / 分钟)。因此,由盐介导的 pH-IEC 法得到的改进的分辨率不是梯度长度变化的结果,而是控制离子强度的影响的结果。

[0380] 实施例 5. 监测 mAb 的热稳定性

[0381] 通常使用阳离子交换色谱来评估生物药物蛋白质在制备期间的降解和批与批之间的变化 (Vlasak, J 和 Ionescu, R (2008) Curr. Pharm. Biotechnol. 9:468)。为了显示监视蛋白降解的能力,将所开发的离子强度介导的 pH-IEC 法用于描述 mAb1 在热胁迫后的电荷不均匀性。在该研究中选择 mAb1,因为在这些 mAb 中其具有最低的保留,并且其 pH-IEC 分布最易受色谱参数变化的影响。用 pH 5 至 pH 10.8 的 pH 梯度和 0mM NaCl 至 16mM NaCl 的盐梯度从柱洗脱抗体。

[0382] 用主峰归一化 mAb1 的对照材料和胁迫材料的色谱图 (图 5)。在热胁迫后,酸性变体和碱性变体二者都升高。胁迫样品主峰的右边还出现了肩。这些分布变化明显说明, mAb1 于 40°C 温育 3 周和 6 周后降解。相似地, mAb2 和 mAb3 在热胁迫下的降解也通过所述盐介导的 pH-IEC 法来检测 (数据未示出)。

[0383] 实施例 6. 离子强度介导的 pH-IEC 的稳健性测试

[0384] 由于所述盐介导的 pH-IEC 法的复杂洗脱过程,需要确保其用于日常样品测试的稳健性。如上所述,我们知道, pH 缓冲剂组成和盐浓度影响对 mAb 的保留和分辨率。此处

进一步研究的目的是研究当使用最优 pH 缓冲剂组成和盐浓度时来源于柱、缓冲剂批次和仪器的变异性。在四个不同的天中测试了三根柱、三个缓冲剂批次、两个仪器。表 3 示出了实验设计。在该研究中再次选择 mAb1, 因为其 pH-IEC 分布最易受色谱参数变化的影响。

[0385] 使用不同的柱和缓冲剂批次得到的 mAb1 色谱图是相当的, 但是使用不同的仪器得到的色谱图示出了稍微不同的保留时间。仪器之间预期的延迟体积方面的差异导致了保留时间的变化, 但其不影响该方法的性能。表 4 中总结了对 mAb1 电荷变体的定量。对于使用两个不同的仪器、三根柱和三种缓冲剂制备物得到的 16 个色谱图, 电荷变体的定量是一致的, 这说明, 盐介导的 pH-IEC 在这些色谱条件下是强健的。

[0386] 表 3. 使用 mAb1 的盐介导的 pH-IEC 的强健性测试的实验设计

[0387]

	第 1 天	第 2 天	第 3 天	第 4 天
Waters 2796	X	X	X	
Dionex U3000				X
柱 1	X			
柱 2		X	X	
柱 3				X
缓冲剂 1	X	X		
缓冲剂 2			X	
缓冲剂 3				X

[0388] 表 4. 针对 mAb1 得到的盐介导的 pH 梯度的强健性数据 (n = 16) 的总结

[0389]

	酸性变体	主峰	碱性变体
平均	12.32	78.99	8.69
最高	13.20	79.92	9.54
最低	10.54	77.91	8.14
STD 标准差	0.78	0.56	0.55
% RSD	6.3	0.7	6.4

[0390] 盐介导的 pH 梯度 IEC 在上载至柱上的宽范围的样品质量范围内也是强健的。如图 6 所示, 当将 5 μ g 至 200 μ g 的 mAb1 上载至柱上时, 观察到一致的洗脱分布。虽然当柱加样超过 50 μ g 时主峰稍微变宽, 但是在所有测试的柱加样中, 电荷变体的定量是一致的 (表 5)。

[0391] 在强健性研究过程中, 得到足够的数量以覆盖典型 HPLC 实验中经历的大多数变

量。盐介导的 pH-IEC 法针对典型 mAb 提供了相当的色谱图和一致的电荷变体定量结果,这说明该方法在此处研究的所有色谱条件下是强健的。

[0392] 表 5. 在不同柱加样下 mAb1 的电荷不均匀性的强健性

[0393]

柱加样(μg)	酸性变体	主峰	碱性变体
5	12.66	79.04	8.30
10	13.04	78.84	8.12
50	13.27	78.14	8.59
100	13.58	77.80	8.62
200	13.55	77.86	8.59
平均	13.22	78.34	8.44
STD 标准差	0.38	0.57	0.22
% RSD	2.9	0.7	2.6

[0394] 实施例 7. 减少离子强度介导的 pH-IEC 的运行时间

[0395] 以前, pH 介导的 IEC 法使用吡啶、咪唑、tris(PIT) 作为缓冲剂 (Farnan, D 和 Moreno), GT 2009 Anal. Chem. 81:884-8857)。为了生成 pH 梯度, 生成 pH 6 至 9.5 的半线性 pH 曲线。如图 7 所示, 当流动相 pH 小于试剂的 pKa 时, 各缓冲剂的氨基官能团维持正电, 当流动相 pH 更大时, 各缓冲剂的氨基官能团为中性。使用缓冲剂 A (pH 5.0) 和缓冲剂 B (pH 9.5) 生成 35 分钟内的梯度为 pH 5 至 pH 9.5 的 pH 梯度。柱为 Propac WCX-10, 4 $\times$ 250mm。该缓冲剂体系 pH 梯度对具有 7 至 8.5 的 pI 值的大范围分子有效。但是, 超出该工作范围的分子的电荷变体没有得到同样的解析。前期的工作揭示, 缓冲剂的浓度和离子强度影响 pH 曲线的线性和样品的保留时间。如实施例 2 中所示, 离子强度随着 pH 随时间的升高而大大降低。调节缓冲剂浓度并引入同时的盐梯度提供了十分稳定的贯穿 pH 梯度的离子强度。通过使用同时的盐梯度, pH 梯度分离现在可解析 pI 值为 6.2 至 9.4 的分子变体。如图 8 中所示, 通过使用 58 分钟的长梯度, 其中在 PIT 缓冲剂 (4mM 吡啶、4mM 咪唑和 4mM Tris) 中 pH 以 0.1pH 单位 / 分钟的斜率从 pH 5 升高至 pH 10.8 并且盐浓度从 0mM NaCl 升高至 16mM NaCl, 解析了 pI 值为 6.2 (Mab1)、8.2 (Mab2) 和 9.4 (Mab3) 的单克隆抗体。该改进的方法称为“离子强度介导的 pH 梯度”或“盐介导的 pH 梯度”。

[0396] 为了减少运行时间和提高通量, 评价了更短柱上的盐介导的 pH 梯度。Farnan, D 和 Moreno, GT (2009) Anal. Chem. 81:8846-8857 指出, 无论柱长度是多少, 只要其是相同的 pH-IEC 分离模式 (WCX 或 SCX), 则使用 pH-IEC 得到相似的洗脱谱。为了确定更短的柱是否可减少洗脱时间, 对比了使用 WCX-10, 4 $\times$ 50mm 柱、使用初始 pH-IEC 和盐介导的 pH-IEC 流动相二者对具有不同 pI 值的三种 Mab 的分离。所述 mAb 是 Mab 1, pI 7.6; Mab 2, pI 8.6 至 9.3; 以及 Mab 3, pI 9.1。pH 梯度为 0 至 16 分钟内 pH 6 至 11, 在 pH 11 保持 2 分钟, 然后于 pH 6 重平衡 4 分钟, 总运行时间 22 分钟。用于初始 pH-IEC 的缓冲剂为 2.4mM Tris、

1. 5mM 咪唑和 11. 6mM 哌嗪。用于离子强度介导的 pH-IEC 的缓冲剂为 4mM Tris、4mM 咪唑和 4mM 哌嗪, 以及 16mM NaCl。如在图 9 中所见, 虽然对这三种 Mab 得到相似的峰分布, 但是电荷变体的分辨率随着分子 pI 的升高而降低。为了实现在更短运行时间内对具有宽 pI 范围的分子的充分分离, 启动了对变体分离机制和不同缓冲体系的研究。

[0397] 为了协助缓冲剂筛选, 使用 pH 和电导率预测工具。可计算常用的不同组合、pH 范围和梯度时间的缓冲剂和 NaCl 盐的 pH 和电导率曲线, 并基于缓冲剂的 pKa 作图。该形象化预测工具使得能够快速优化具有例如线性 pH 曲线、随着时间稳定或升高的离子强度和更低的缓冲剂毒性等期望性质的缓冲体系。使用该模型筛选了多种缓冲剂组合。据发现, Tris、哌嗪和磷酸盐 (TPP) 缓冲剂的组合形成跨越 16 分钟梯度的 pH 梯度 (图 10)。缓冲剂包括具有 5. 33pKa (范围 5. 0 至 6. 0) 的哌嗪 pK1、具有 7. 20pKa (范围 5. 8 至 8. 0) 的磷酸盐 pK2、具有 8. 06pKa (范围 7. 5 至 9. 0) 的 Tris 碱、具有 9. 73pKa (范围 9. 5 至 9. 8) 的哌嗪 pK2 和具有 12. 33pKa 的磷酸盐 pK3。图 10 中的表显示, 磷酸盐分子贯穿 pH 范围都保持带电, 并且在一定程度上弥补了因 Tris 和哌嗪中的氨基官能团随着 pH 升高去质子化而导致的离子强度损失。色谱条件如下:

[0398] 柱: Propac WCX-10HT, 4×50mm

[0399] pH 梯度: 5. 0mM Tris、哌嗪、磷酸盐 (TPP)

[0400] 缓冲剂 A = pH 6. 0

[0401] 缓冲剂 B = pH 11. 0 梯度: 16 分钟内 6 至 11

[0402] 缓冲剂 C = 60mM NaCl

[0403] 缓冲剂 D = MilliQ 水

[0404] 预测的 pH 和电导率曲线 (图 11 的右图) 与通过实验得到的曲线一致 (左图)。缓冲体系包含 5mM Tris、哌嗪和磷酸盐, 并且 NaCl 浓度为 10mM、20mM 或 30mM (模型中使用)。实验流速为 1ml/ 分钟或 2ml/ 分钟。

[0405] 实施例 8. 确定优化的离子强度范围

[0406] 使用 16 分钟梯度盐介导的 TPP 法分析多种样品。图 12 中示出了分别来自 4×50 和 4×250 Propac WCX-10 柱的 22 分钟和 58 分钟之间的一致分布的例子。用 4×50 柱的洗脱使用跨越 16 分钟的盐介导的 Tris/ 哌嗪/ 磷酸盐 (TPP)、pH 6 至 11 和 0mM NaCl 至 30mM NaCl。用 4×250 柱的洗脱使用跨越 58 分钟的盐介导的哌嗪/ 咪唑/ Tris (PIT)、pH 5 至 10. 8 和 0-16mM NaCl。据确定, 盐介导的 TPP 法对主要处于 7 至 8. 5 的 pI 范围内的分子是足够的, 所述 pI 范围是用初始 PIT 法得到的相似范围。得出结论, 缓冲剂和离子强度在分离中起作用, 但是可涉及其他因素。

[0407] 对跨越 pH 范围的 MAb 的静电荷状态进行造模。图 13 示出了对于具有不同 pI 的 mAb 随 pH 的静电荷叠加。在阳离子交换模式中, mAb 带有正电荷直至 pH 降低至其 pI (x- 截距), 然后从柱洗脱。随着 pH 升高, mAb 变得带负电。对于指定蛋白质分子的电荷变体分布由主峰、酸性区和碱性区组成 (如底图所示)。通常来说, 电荷变体和主峰差一个或一些电荷, 从最酸性变体到最碱性变体的整个电荷包 (charge envelop) 跨越 5 至 7 个电荷。最优的 pH 梯度分离窗可能处于曲线的 pH 6. 5 至 pH 8. 5 的平区域 (图 13); 例如, 对于 pI 值小于约 8. 5 的分子, 紧低于其理论 pI 的静电荷变化非常小, 曲线在 pH 6. 5 至 8. 5 之间相对较平。在 pH 梯度运行中, 有足够的时间让电荷变体随着 pH 随时间的升高来以不同保留时间

分别从柱洗脱。

[0408] 相反地,对于 pI 值大于 8.5 和小于 6.5 的分子,在其理论 pI 处,静电荷变化大(图 14)。在平台线性 pH 梯度运行期间,在这些 pH 下,电荷每分钟的斜率太陡使得不能充分分离,并且观察到一个峰(插图)。

[0409] 因为在电荷对 pH 曲线的相对较平部分中实现了更好的峰分离和强健性,所以问题是,如何在该区域中洗脱蛋白质电荷变体而不管其 pI。对于较高 pI 的分子,这需要使用除 pH 以外的因素来预先修饰电荷状态。该因素是缓冲剂离子强度。通过流动相中的更高的离子强度,从固定性屏蔽了分子表面上的更多电荷。因此,表观静电荷降低,使得变体能够在低于其 pI 的 pH 下进行洗脱。

[0410] 在实施例 1 至 6 示出的工作中,通过校正 PIT 流动相中离子强度缺陷并组合盐梯度(盐介导的 pH 梯度),良好分离了 pI 值大于 9(阳离子交换模式)和小于 7(阴离子交换模式)的分子的电荷变体。与单独的 pH 梯度相比,组合的 pH 与盐梯度的驱动力使分子的表观电荷状态随时间降低得更快。这可描述为电荷对于 pH 曲线得到垂向的拉伸,更窄的 pH 7 至 8 之间的平区域,以及在大于 9 的 pH 下更浅的斜率。因此,对于具有更高 pI 的分子,该分离发生于稍低于其 pI 的 pH 下。通过使用较长的柱(250mm)和长运行时间(60 分钟),有足够的时间用于电荷变体洗脱。但是,盐介导的 pH 梯度法未能在较短的柱(50mm)上在短时间(20min)内解析高 pI 分子的电荷变体。

[0411] 因为短的运行时间是关键的,所以不是使用跨越 60 分钟的盐梯度来逐渐降低表观电荷,而是可通过早期引入盐并保持全程恒定从而在运行的开始时达到期望的电荷状态。

[0412] 图 15 是在不同浓度的 NaCl 盐存在下对高 pI mAb(pI 9.2)的 pH 梯度分离的叠加。通过使用四元体系,使用 A 线和 B 线制造 pH 6 至 11 的 pH 梯度,并且使用 C 线和 D 线来维持 0mM、10mM、20mM、30mM、40mM 或 50mM 的 NaCl 盐浓度。梯度条件和运行时间如下:仪器:U3000 2DLC;流动相:10mM Tris、哌嗪、磷酸盐,A)pH 6.0,B)pH 11.0;C)100mM NaCl,D)milliQ 水;柱:Propac WCX-10,4×50mm,10 μm;柱温度:40℃;流速:1ml/分钟;pH 梯度:10%至 50% B;盐常数:0mM = 0% C,50% D;10mM = 10% C,40% D;20mM = 20% C,30% D,30mM = 30% C,20% D,40mM = 40% C,10% D,50mM = 50% C,0% D;样品浓度:1 μg/μL;加样体积:20 μL。

[0413] 在较低的盐浓度下,观察到非常小的分离。在随后较高的盐浓度下,分离得到改进,同时洗脱时间减少。洗脱时间随着盐浓度升高而减少,支持了以下:分子表面上的额外电荷被从固定相屏蔽,导致表观电荷降低,从而使得能够在低于其 pI 的 pH 进行变体分离。在这种情况下,于 40-50mM NaCl 实现最优分离,在该盐浓度下,推测该 mAb 的表观电荷处于电荷对 pH 曲线的相对较平部分。

[0414] 在不同的恒定盐浓度下但是施加相同的 pH 梯度来进行对具有不同 pI 的多种 mAb 的分析。一般而言,在使表观电荷状态落在电荷对 pH 曲线的平部分中的恒定盐浓度下实现了最优分离。例如,处于 7 至 8.3 的 pI 范围的 mAb 不需要盐,处于 8.3 至 8.8 的 pI 范围中的 mAb 需要 20mM 盐,并且处于 8.8 至 9.0 的 pI 范围中的 mAb 需要 50mM 盐(图 16 和 17)。在电荷对 pH 曲线上,盐的添加基本使 x 轴向上移动至分子的期望电荷状态,这使得能够在曲线的平区域中进行最优分离。该类型的分离成为“混合”pH 梯度(图 18)。

[0415] 改进的分离通过改变梯度条件来实现。无论是与初始 pH-IEC 法还是与盐介导的 pH-IEC 法相比, 浅的混合 pH-IEC 梯度 (使用 TPP 流动相) 都导致改进的峰分离 (图 19 和 20)。通过使用 TIC 缓冲剂 (Tris、咪唑、CAPS), 用 10mM NaCl 替代哌嗪 (出于有害处理的担心) 以及通用生物缓冲剂磷酸盐 (其可因缓冲剂催化的翻译后修饰而有害地引起测定伪象), 实现了相似的分离 (图 21)。

[0416] 可进一步挑战短的混合 pH-IEC 法以确定更快的分析时间是否可行。将 pH 梯度设置为 10 分钟内 pH 7 至 10, 在 pH 10 保持 2 分钟, 且平衡时间 3 分钟, 总运行时间 15 分钟。

[0417] 色谱条件如下: 仪器: U3000 2DLC; 流动相: 10mM Tris、哌嗪、磷酸盐, A) pH 6.0, B) pH 11.0; C) 100mM NaCl, D) milliQ 水; 柱: Propac WCX-10, 4×50mm, 10 μm; 柱温度: 40°C; 流速: 1ml/分钟; pH 梯度: 0% 至 30% B; 盐常数: 0mM = 0% C, 50% D; 10mM = 10% C, 40% D; 20mM = 20% C, 30% D, 30mM = 30% C, 20% D, 40mM = 40% C, 10% D, 50mM = 50% C, 0% D; 样品浓度: 1 μg/μL; 加样体积: 20 μL。

[0418] 如图 22 和 23 所示, 对于具有 8.8 和 9.0 的 pI 值的两种不同 MAb 而言, 15 分钟和 22 分钟运行之间的分布相似。但是, 由于运行时间变得更短, 需要更具产物特异性的起始/终止 pH 值和盐浓度。

[0419] 当开发产物特异性方法时, 可通过小心地基于分子 pI 选择合适的 pH 梯度斜率和盐浓度来实现最优分辨率。如果在序列中只分析仅一种产物, 则产物特异性方法将是有益的。对于分析多种 mAb、每种都具有不同 pI 的实验室而言, 期望使用可递送 pH 梯度同时维持某些盐浓度的四元体系。否则, 可能需要更长的 22 分钟的运行时间, 如平台方法使用在固定盐浓度下递送 pH 梯度的二元体系。

[0420] 实施例 9. 离子强度介导的 pH 梯度离子交换色谱的强健性

[0421] 为了显示离子强度介导的 pH 梯度离子交换色谱的强健性窗口和标靶运行条件适于使用实验设计 (DOE) 方法来分析具有跨越宽范围的 pI 的多种单克隆抗体产物。如果在包括主峰 (主峰%)、酸性变体峰 (AV%) 和碱性变体 (BV%) 峰的相对峰面积在内的可报道值方面没有显著变化, 则该方法适当强健的。此外, 一般峰分布通过峰值分辨率来度量 (Rs1 和 Rs2)。为了测试强健性, 故意扰动运行参数, 例如盐浓度、缓冲剂浓度、pH、柱温度和流速。

[0422] 材料与方法

[0423] 测试了以下三种抗体:

[0424] mAb1, pI = 8.2

[0425] mAb2, pI = 8.5

[0426] mAb3, pI = 9.0

[0427] 使用配备有通向柱的 8 接口 3 通转换阀、用于线 C 和 D 的 6 接口溶剂选择阀以及 Waters 2487Dual λUV 检测器的 Waters 2796Bioseparations Module 来进行色谱。

[0428] 色谱柱为 Dionex ProPac WCX-10HT, 4×50mm 柱。

[0429] 缓冲体系如下: 对于缓冲剂 A 和 B, 等摩尔的咪唑、Tris 和 CAPS。缓冲剂 C 为 100mM NaCl, 并且缓冲剂 D 是水。

[0430] 缓冲剂 A 的 pH 如实验设计表 (表 6) 之规定, 缓冲剂 B 的 pH 为 10.0。实验设计表中示出了所使用的总缓冲剂强度和盐浓度。

[0431] 梯度如下:0-2 分钟,处于起始 pH;2-16 分钟,从起始 pH 至 pH 10;16-18 分钟,处于 pH 10;18-22 分钟,处于起始 pH。选择的盐浓度在梯度中维持恒定。

[0432] 所使用的柱温度和流速如表 6 中所指定。

[0433] 3 种 mAb:

[0434] mAb1, pI = 8.2

[0435] mAb2, pI = 8.5

[0436] mAb3, pI = 9.0

[0437] Waters 2796Bioseparations Module 配备有通向柱的 8 接口 3 通转换阀、用于线 C 和 D 的 6 接口溶剂选择阀以及 Waters 2487Dual λ UV 检测器。

[0438] Dionex ProPac WCX-10HT, 4×50mm 柱

[0439] 缓冲体系:对于缓冲剂 A 和 B, 等摩尔的咪唑、Tris 和 CAPS。缓冲剂 C 为 100mM NaCl, 并且缓冲剂 D 是水。

[0440] 实验设计表规定了缓冲剂 A 的 pH, 缓冲剂 B 的 pH 为 10.0。实验设计表中示出了总缓冲剂强度和盐浓度。

[0441] 梯度:0-2 分钟,处于起始 pH;2-16 分钟,从起始 pH 至 10;16-18 分钟,处于 pH 10;18-22 分钟,处于起始 pH。选择的盐浓度在梯度中维持恒定。

[0442] 柱温度和流速:参见实验设计表。

[0443] 表 6. 实验设计(运行参数)

[0444]

模式	盐 (mM)	缓冲剂 (mM)	起始 pH	柱温度 (°C)	流量 (ml/ 分钟)
+++ - -	25	20	6.3	36	0.8
- - + - -	15	10	6.3	36	0.8
- + - - -	15	20	5.7	36	0.8
+ - + - +	25	10	6.3	36	1.2
- + + - +	15	20	6.3	36	1.2
+ - - + +	25	10	5.7	44	1.2
- - - - +	15	10	5.7	36	1.2
0	20	15	6.0	40	1
+++++	25	20	6.3	44	1.2
++ - + -	25	20	5.7	44	0.8
- - +++	15	10	6.3	44	1.2

- - - + -	15	10	5.7	44	0.8
- + - ++	15	20	5.7	44	1.2
+ - ++ -	25	10	6.3	44	0.8
- +++ -	15	20	6.3	44	0.8
++ - - +	25	20	5.7	36	1.2
+ - - - -	25	10	5.7	36	0.8
0	20	15	6.0	40	1

[0445] 通过系统地从标靶运行条件扰动参数来测试用于分析多种单克隆抗体产物的色谱法的强健性,所述标靶运行条件为 20mM 盐、15mM 缓冲剂、起始 pH 6.0、柱温度 40℃以及 1.0ml/ 分钟的流速(在表 6 中命名为 0)。测试了 pI 为 8.2、8.5 和 9.0 的三种不同 MAb。图 24 中示出了在标靶运行条件下进行的两次重复色谱所得的色谱图。图 25 中示出了根据表 6 扰动运行条件来测试标靶运行条件针对 MAb3 的强健性的例子。虽然在分辨率方面有一些小差异,但是保持了一般峰分布,尤其是三个区域(酸性峰、主峰和碱性峰)的峰分布,并且使得能够定量。

[0446] 运行参数对方法性能的影响可通过使用分布表制图来显示(图 26)。在该表制图中,用于在不同条件下分析的可报道值(主峰%、AV%和 BV%)接近地分布于标靶条件的值(处于每个图的中心,即 20mM 盐)周围。该结果示出,盐浓度对可报道值没有显著影响。但是,在分辨率上,随着盐浓度的升高存在这样的趋势:MAb1(pI = 8.2) 和 MAb2(pI = 8.5) 的稍微下降的趋势,以及 MAb3(pI = 9) 的上升趋势。这提示,对于产物特异性的方法而言,随着分子的 pI 升高,可通过提高盐浓度来进一步改进分辨率。作为多产物方法,将盐浓度优化用于具有跨越宽范围 pI 的 mAb。这些研究的结果示出,20mM 是可用于分析具有范围介于 7.0 至 9.5 的 pI 的抗体的盐浓度。

[0447] 还对其他运行参数的影响进行了作图,并在图 27 中示出。在这些表制图中,用于在不同条件下分析的可报道值(主峰%、AV%和 BV%)也围绕标靶条件下的值周围。这些结果显示,运行条件的故意扰动对可报道值没有显著影响。因此,所述多产物方法在标靶运行条件下是适当稳健的。

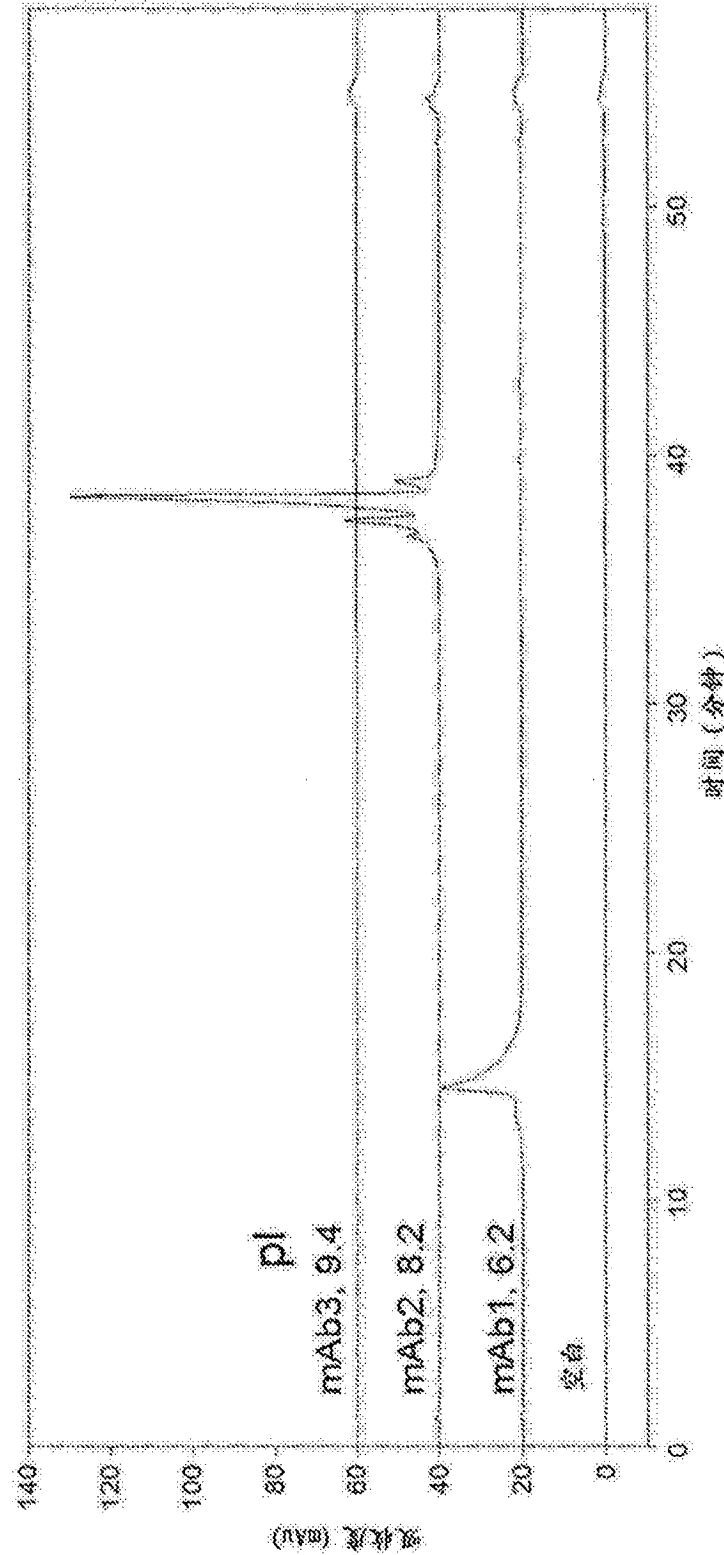
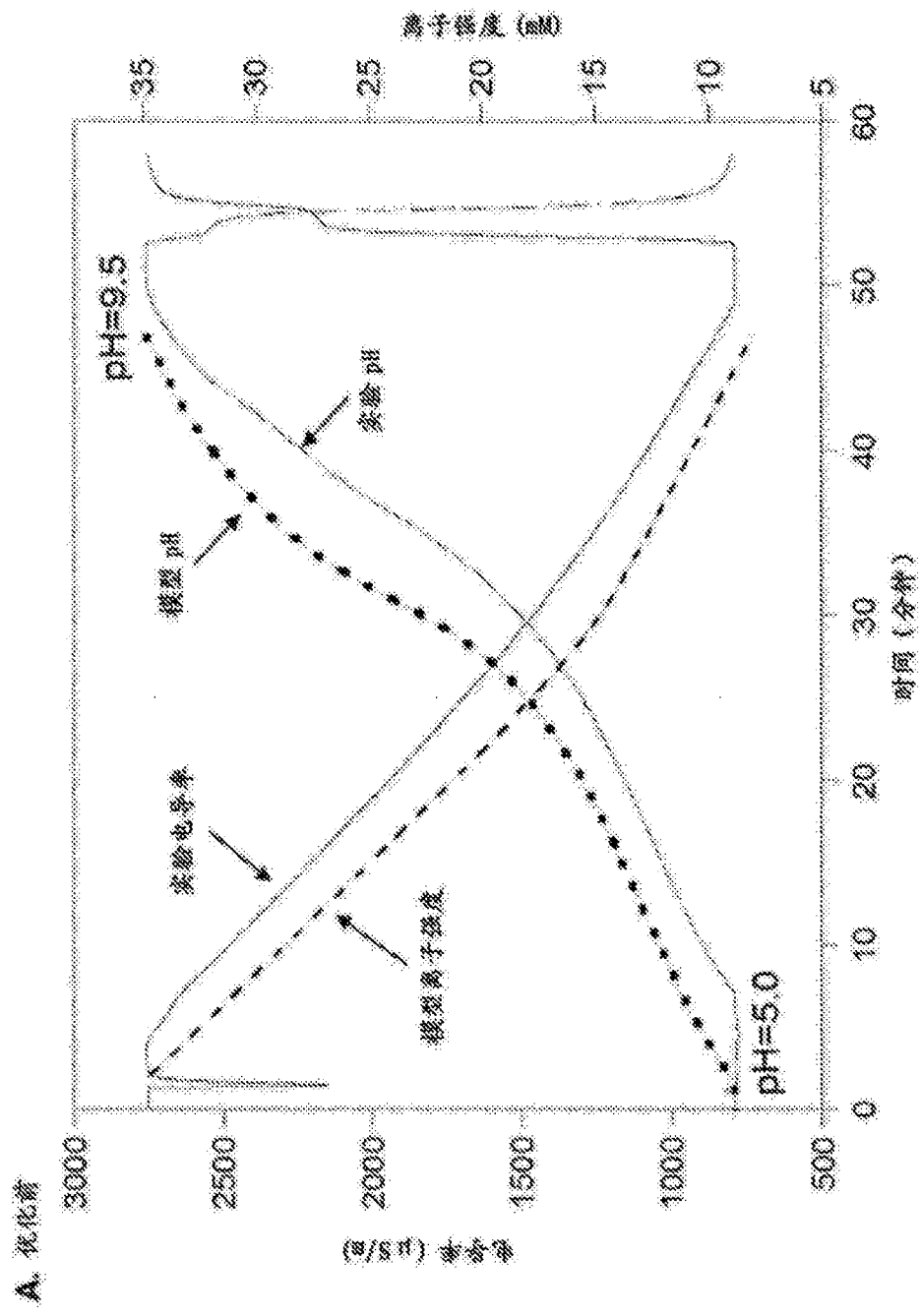
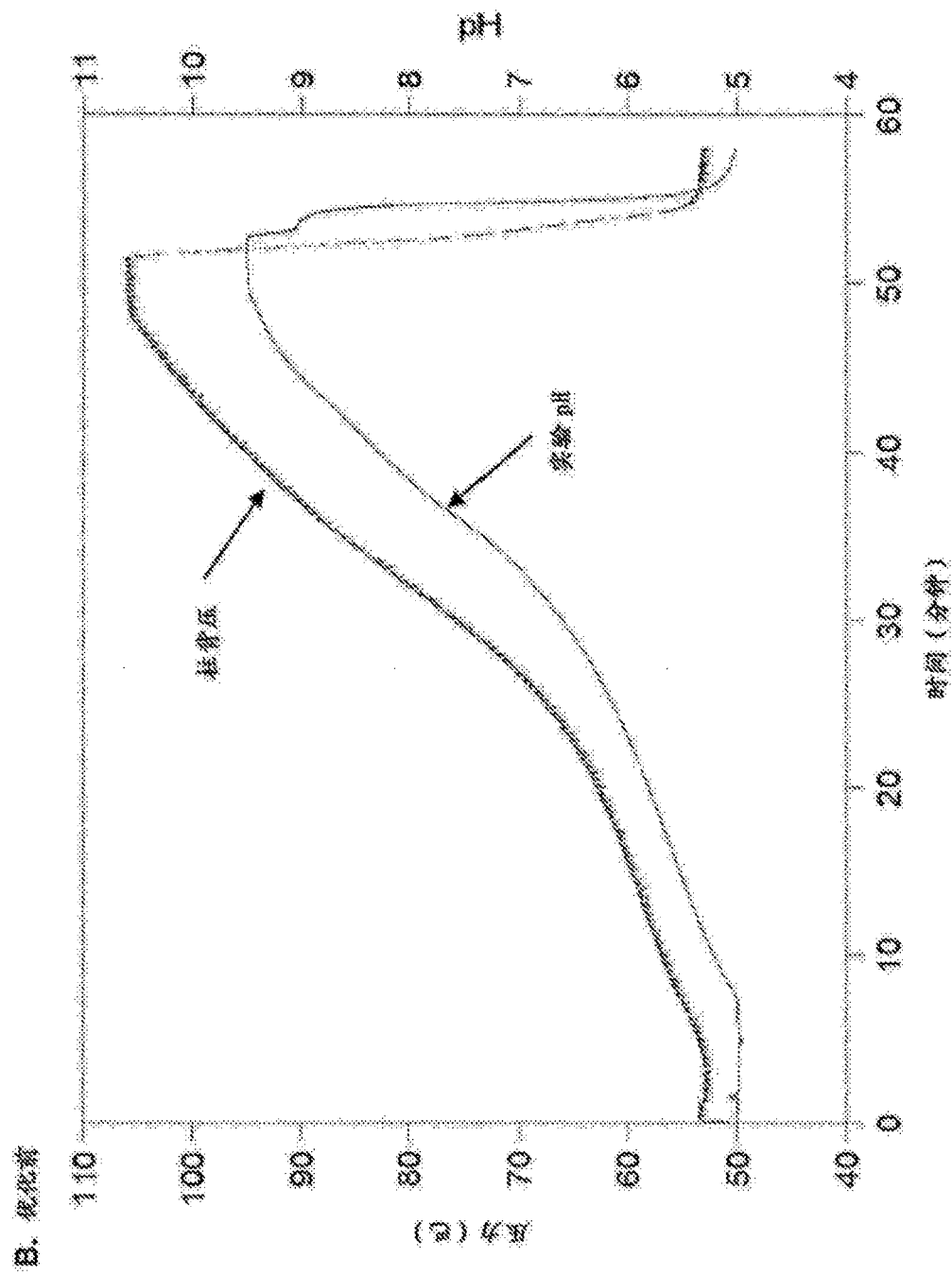
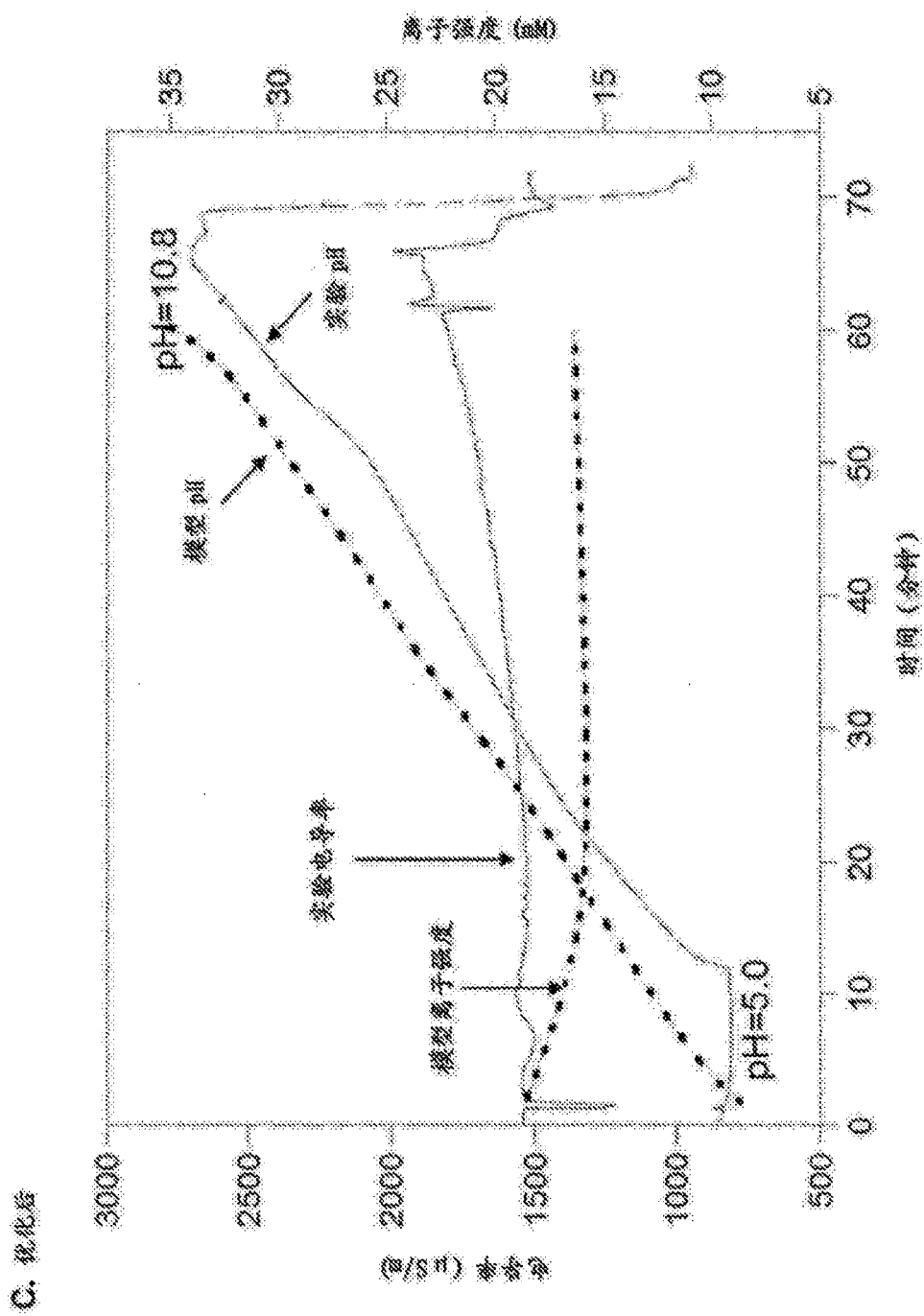


图 1







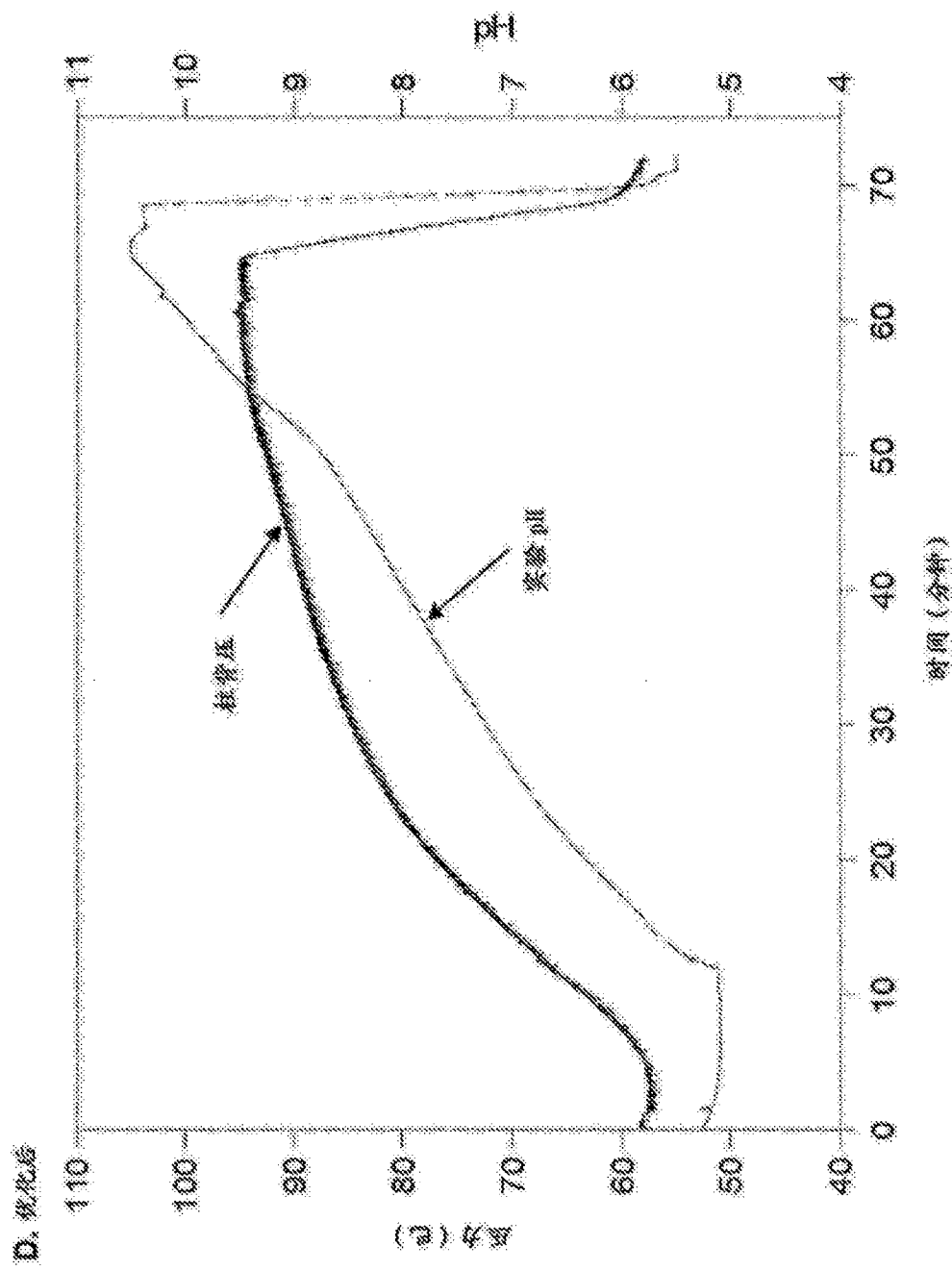
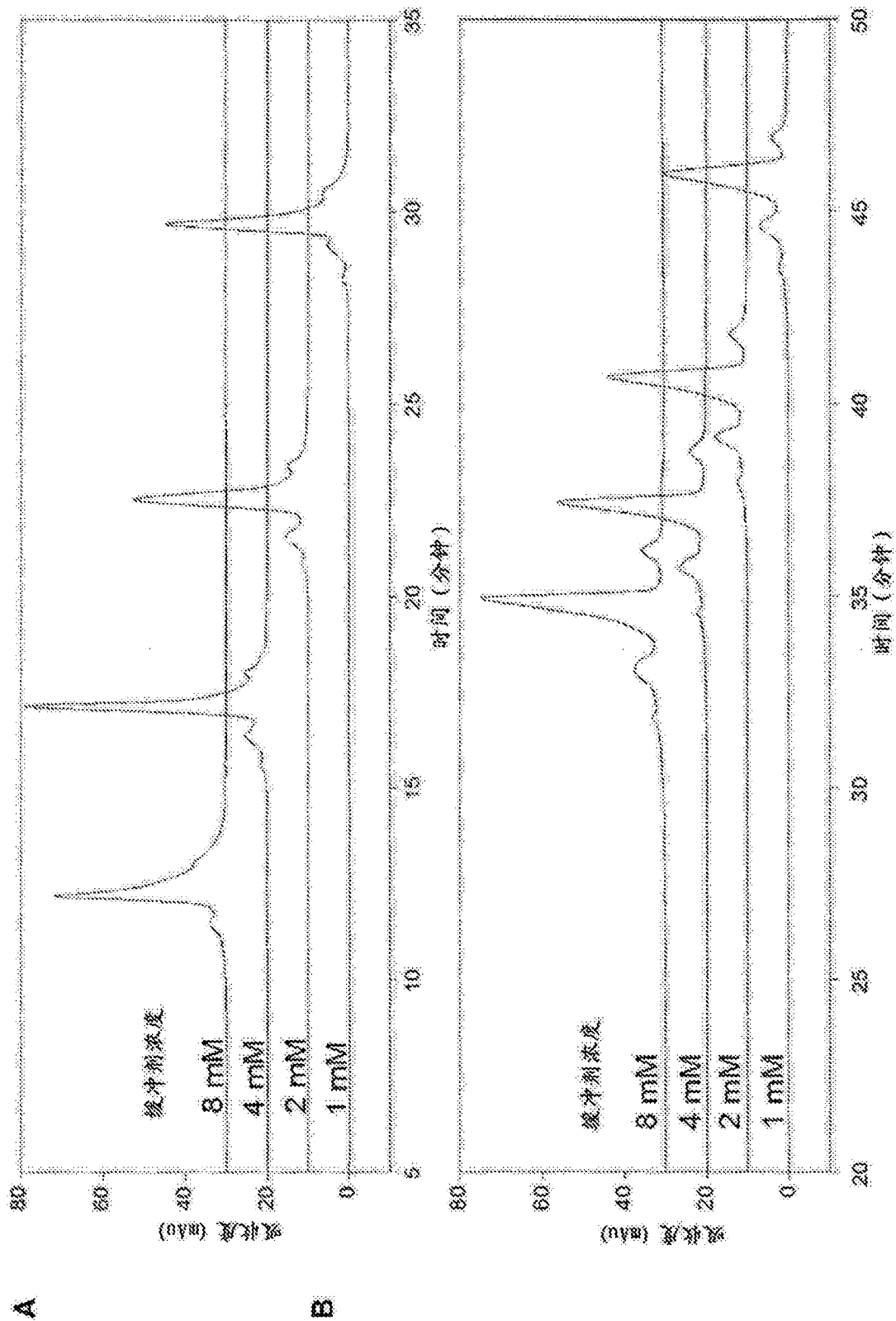


图 2



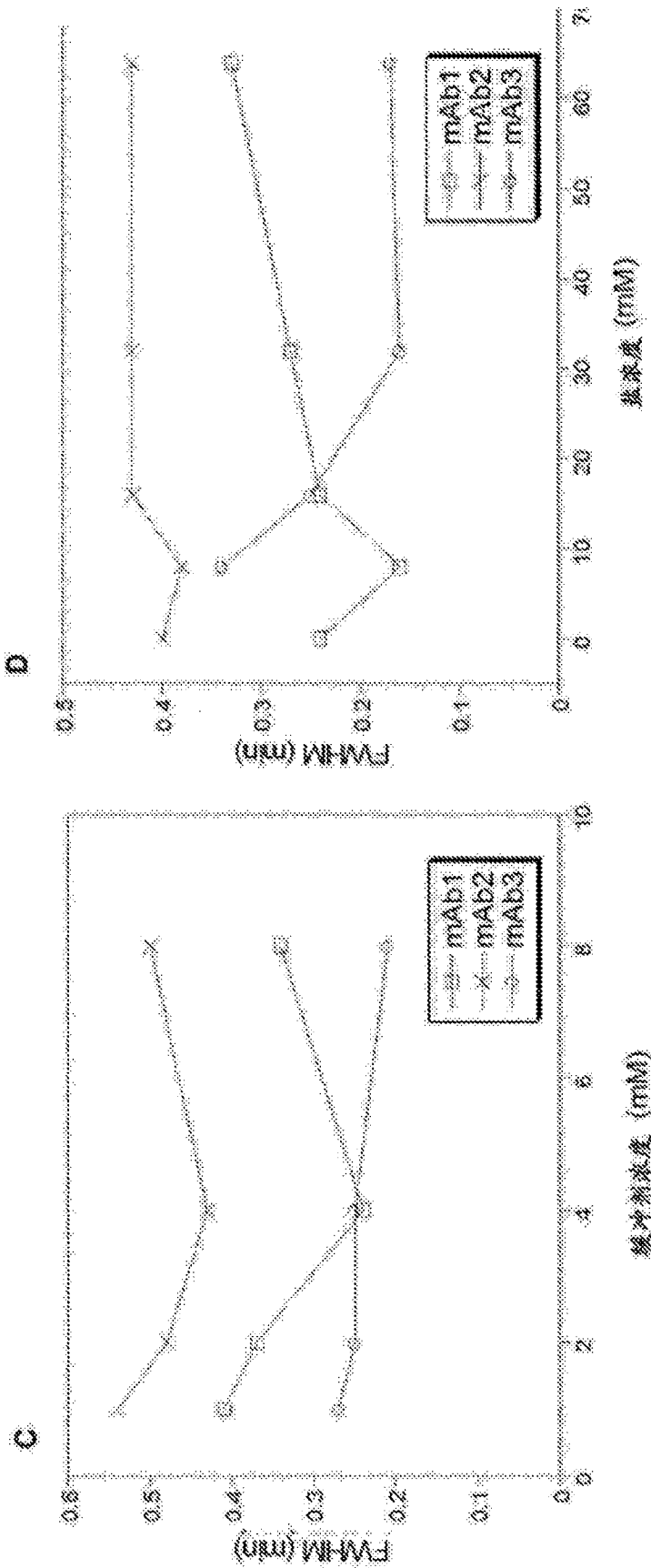


图 3

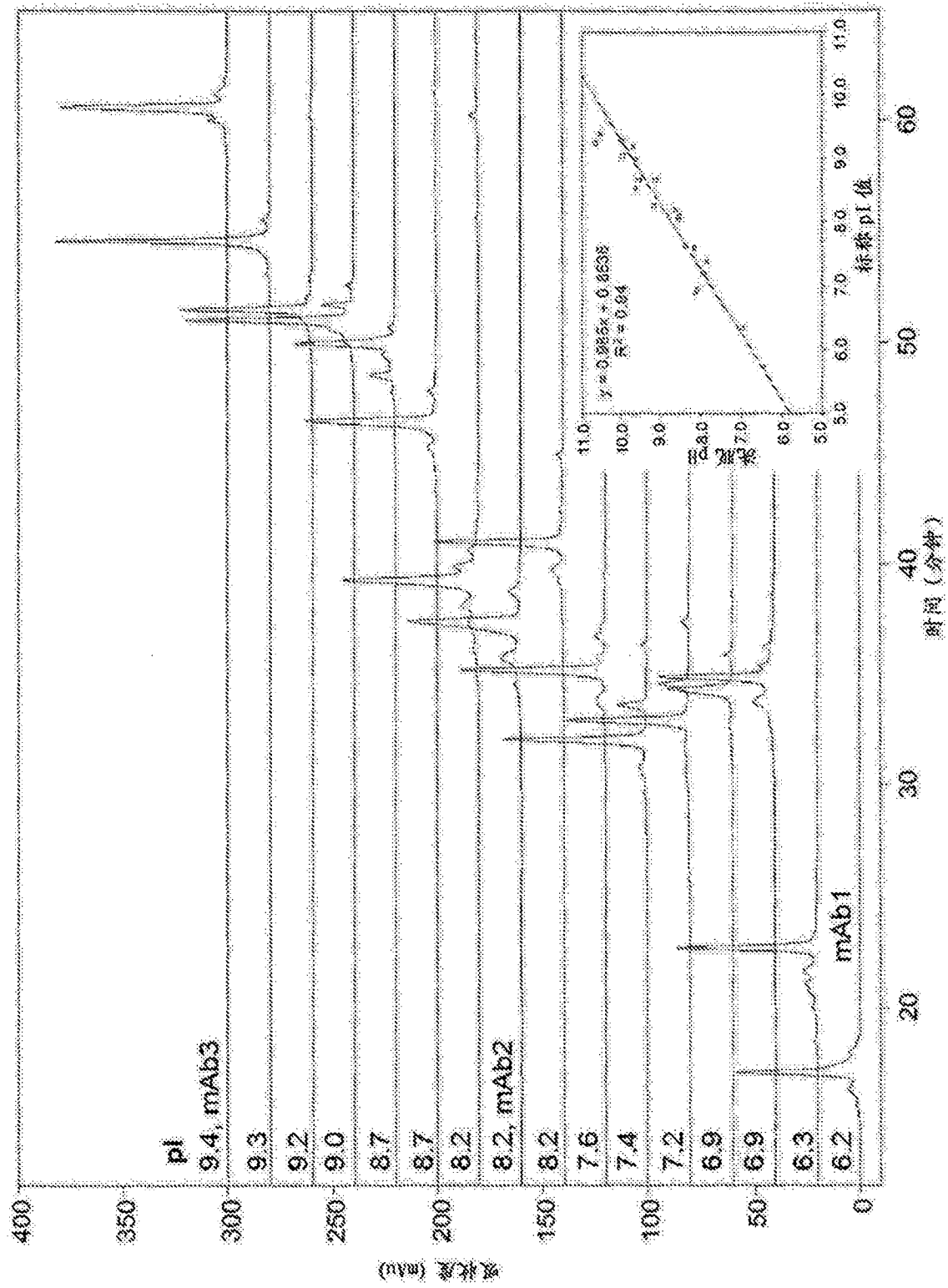


图 4

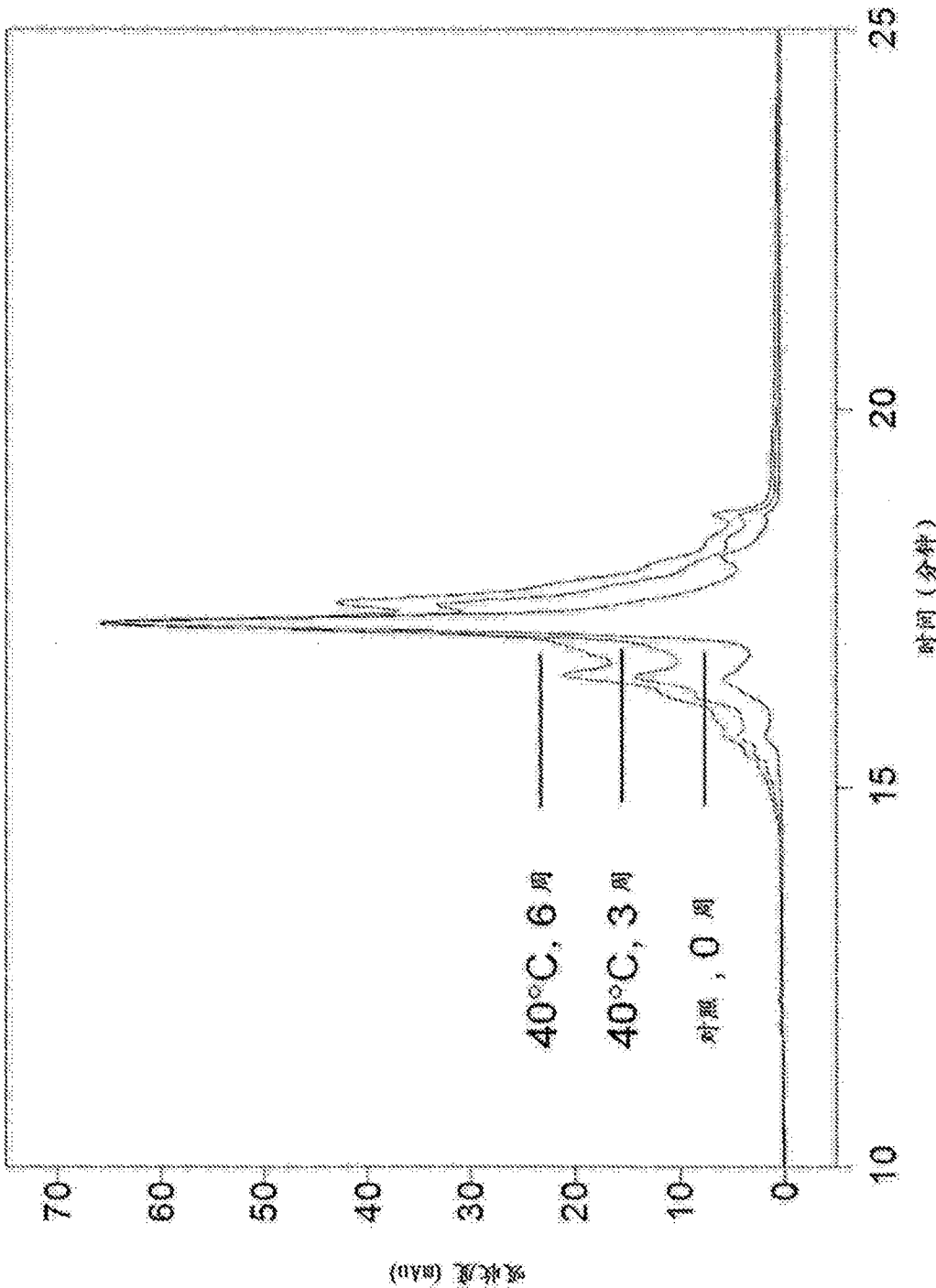


图 5

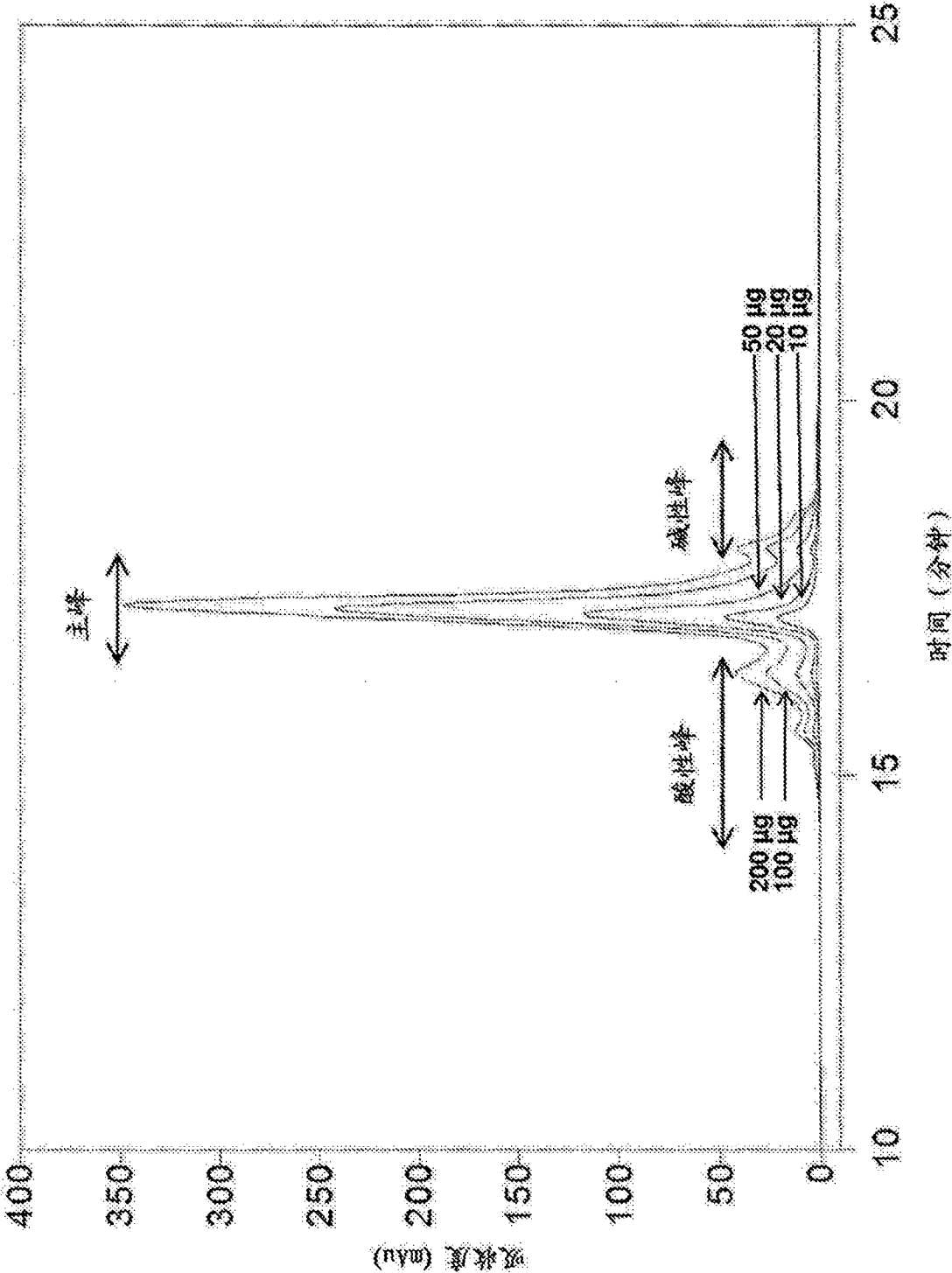


图 6

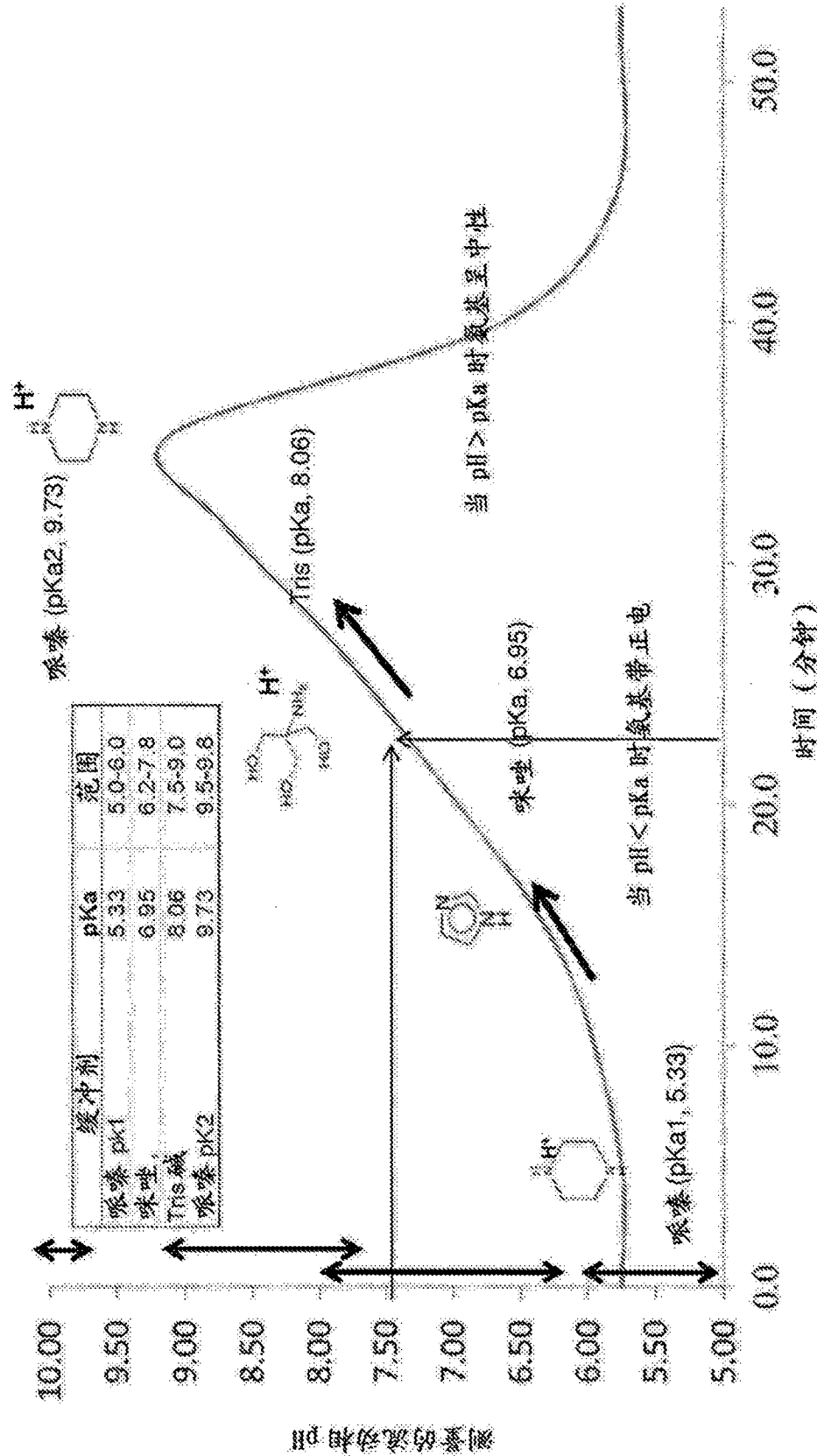


图 7

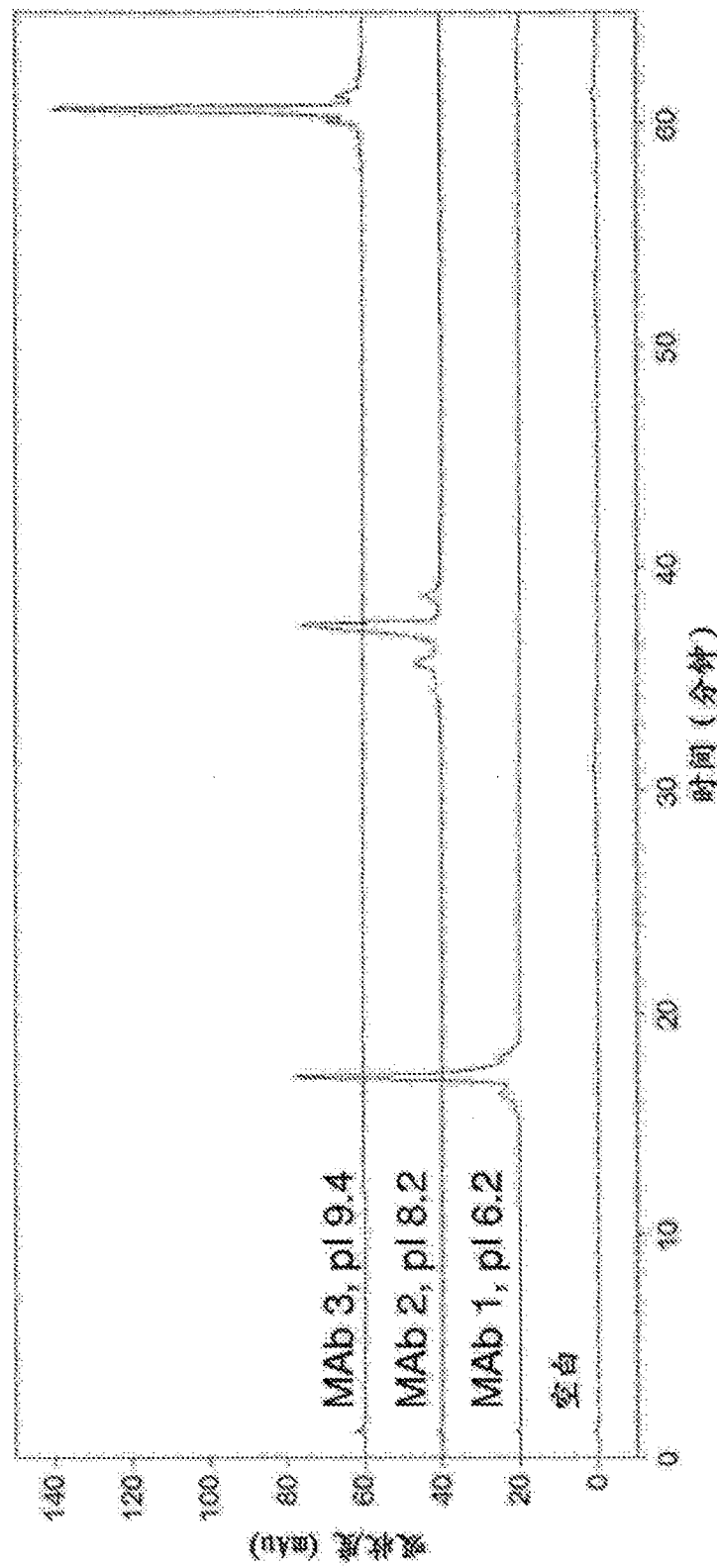


图 8

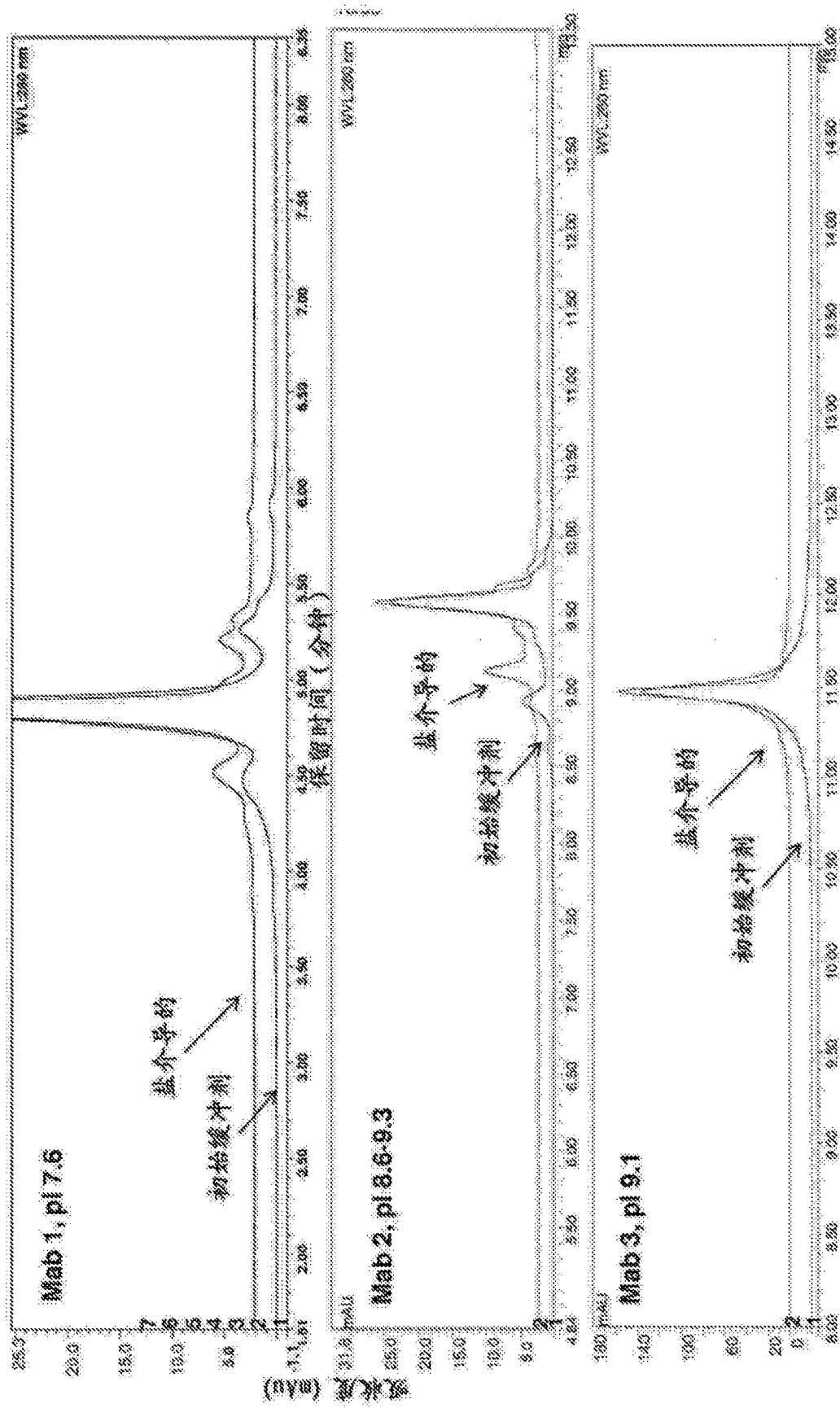


图 9

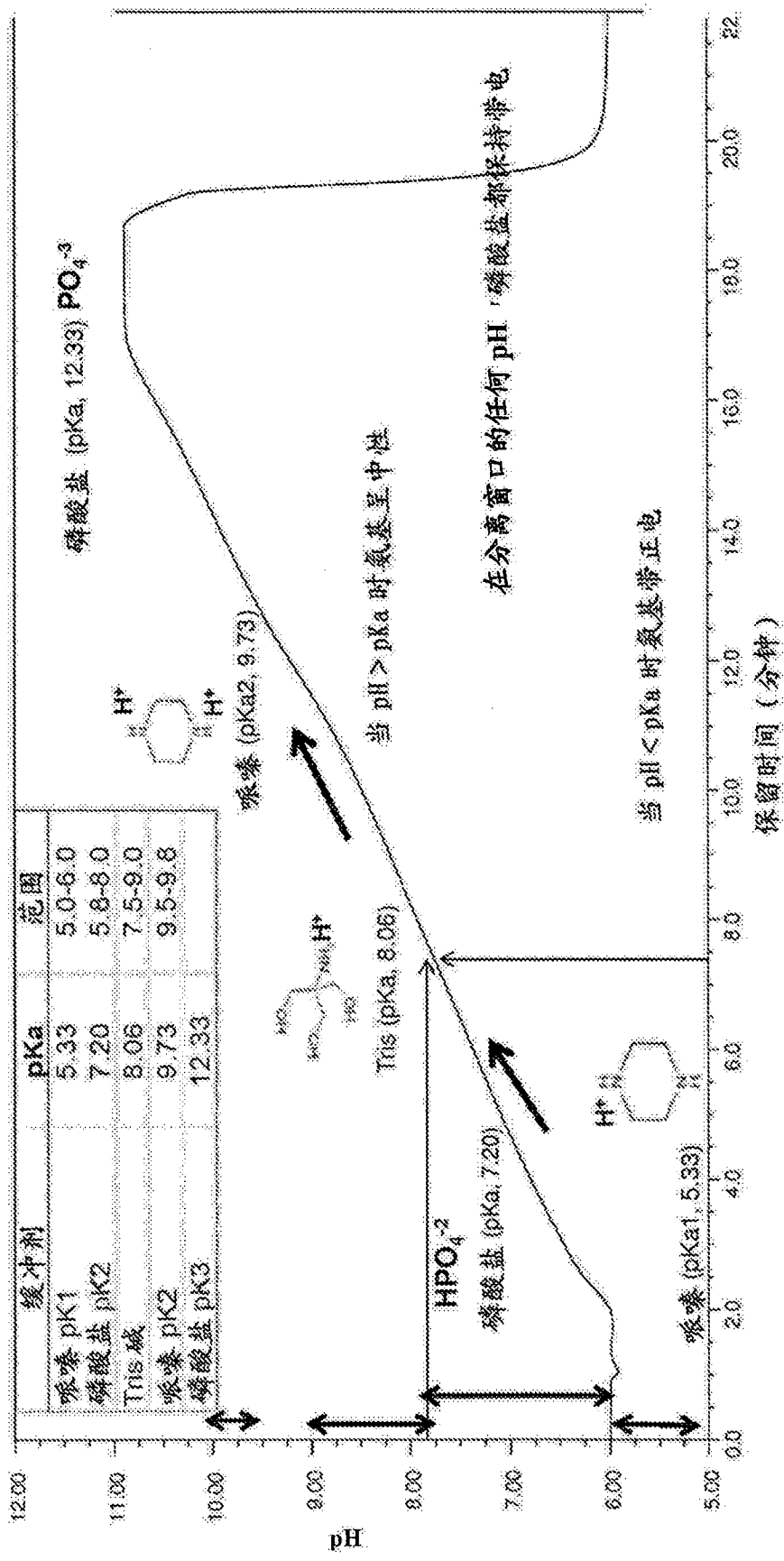
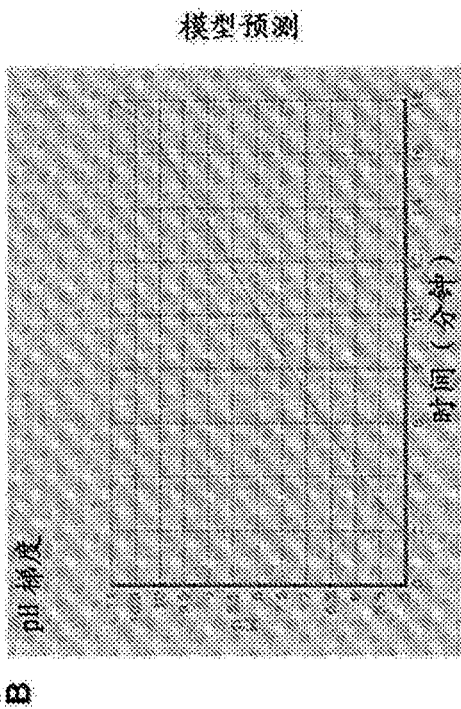
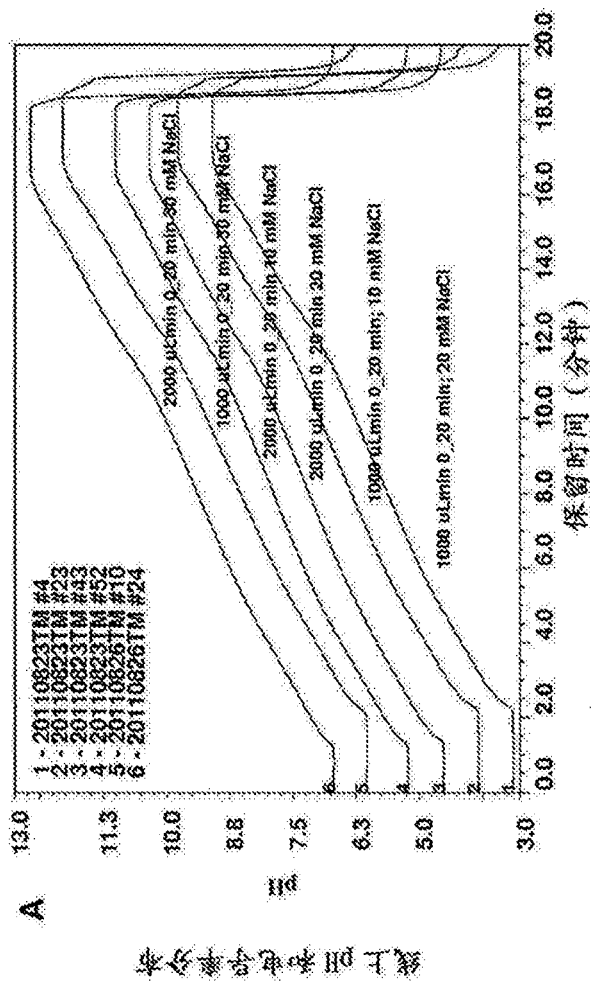
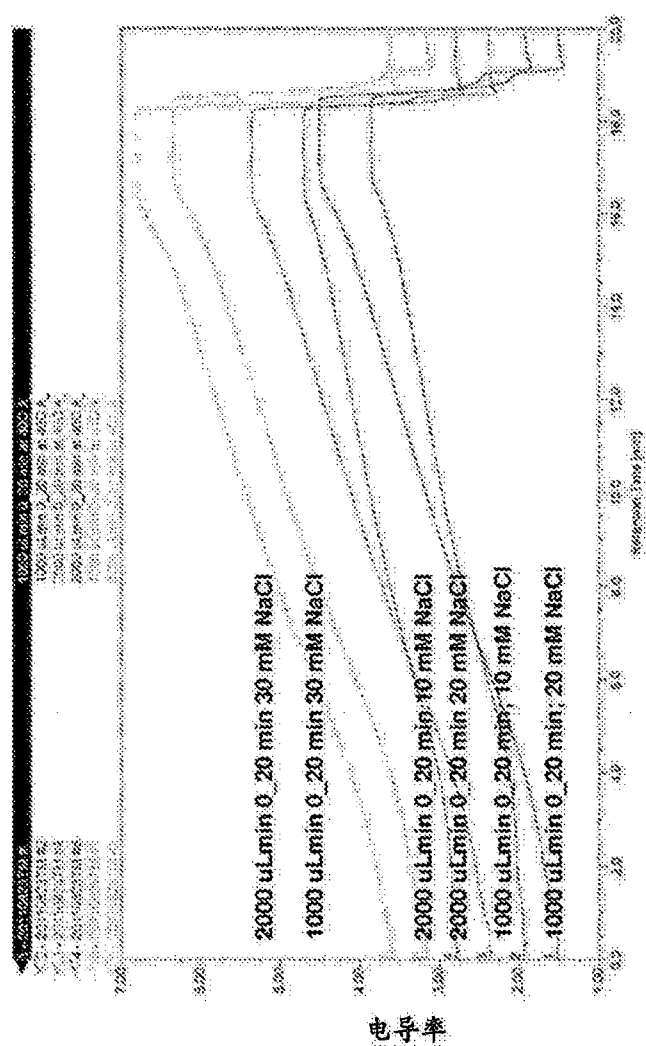


图 10





线上产品和零售分布

模型预测

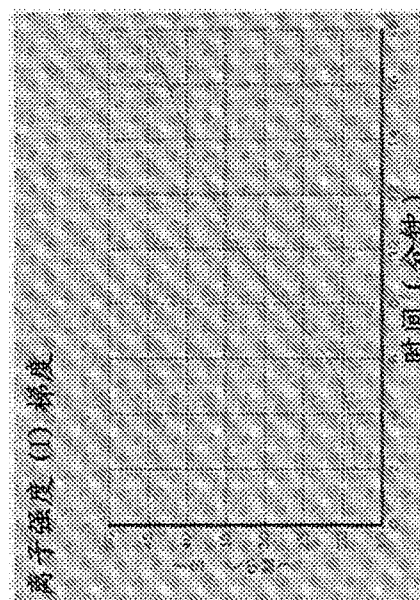


图 11

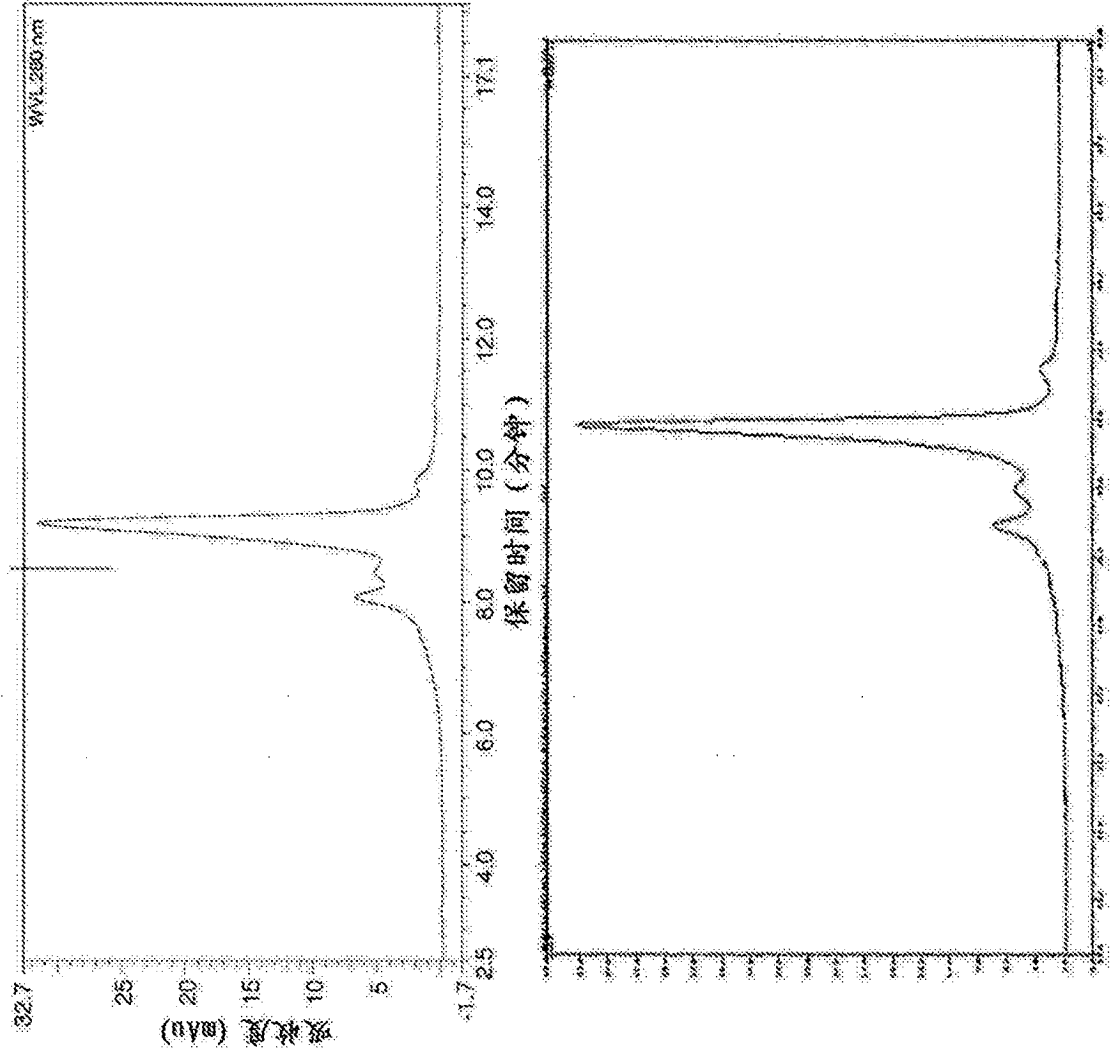


图 12

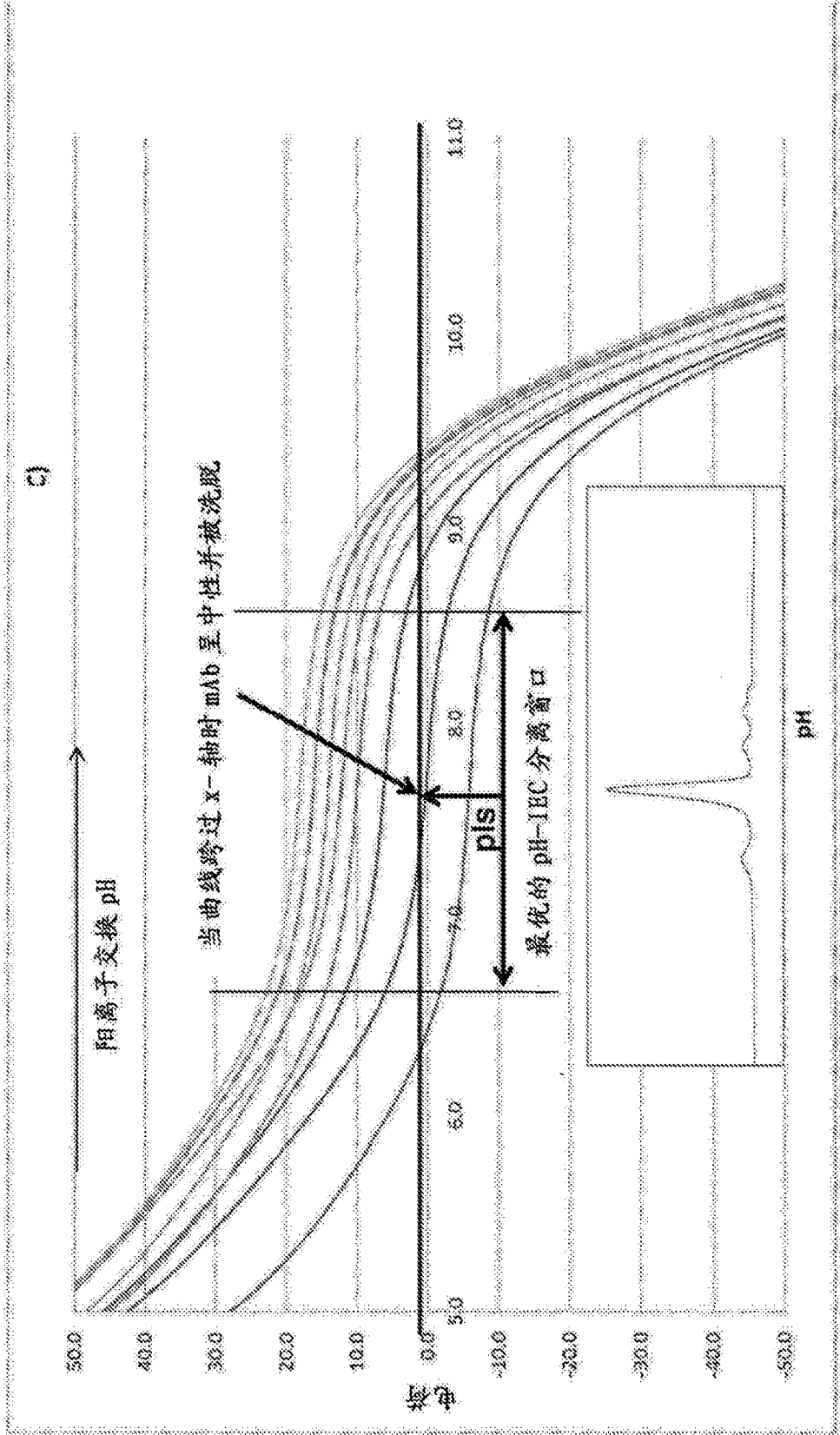


图 13

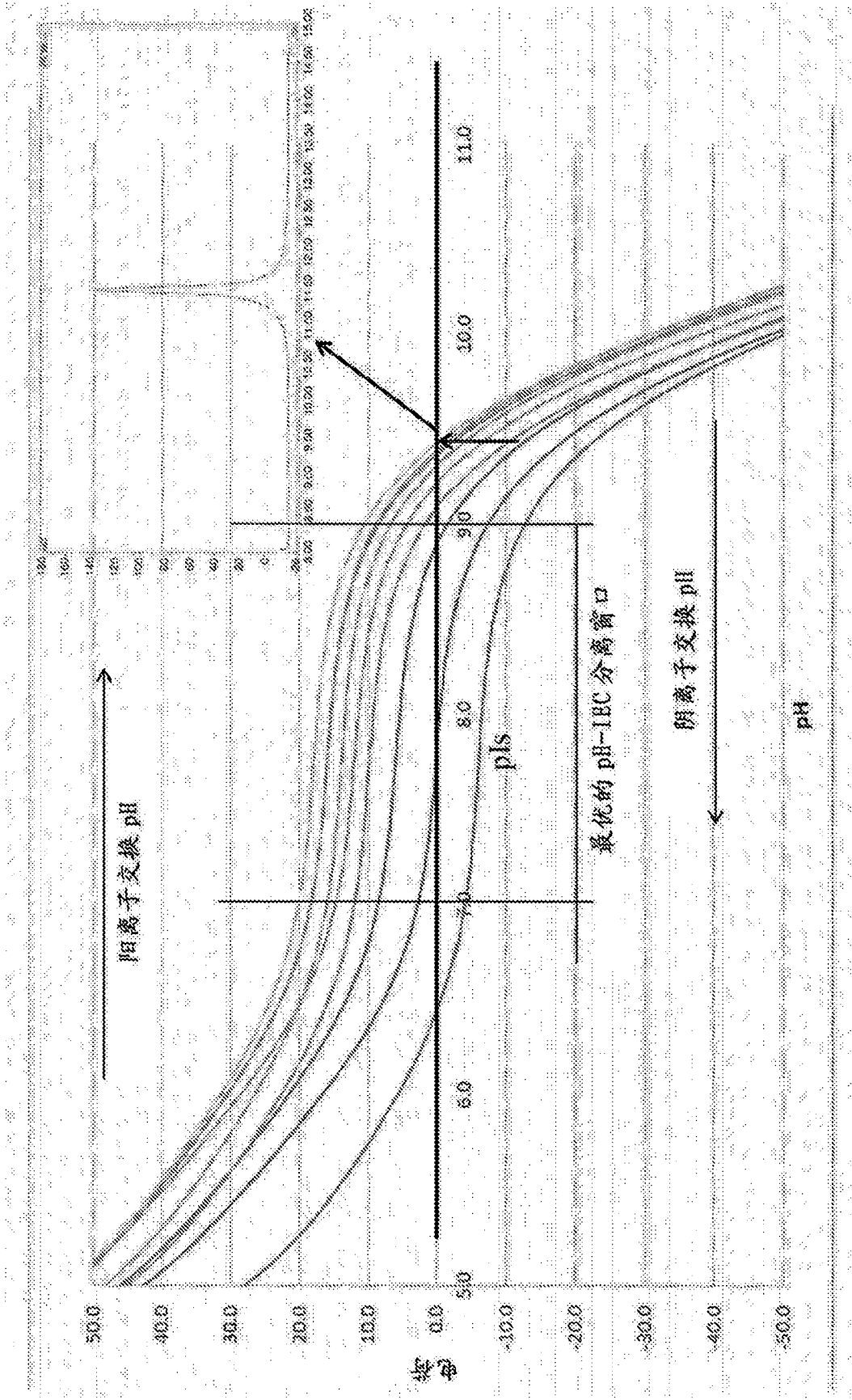


图 14

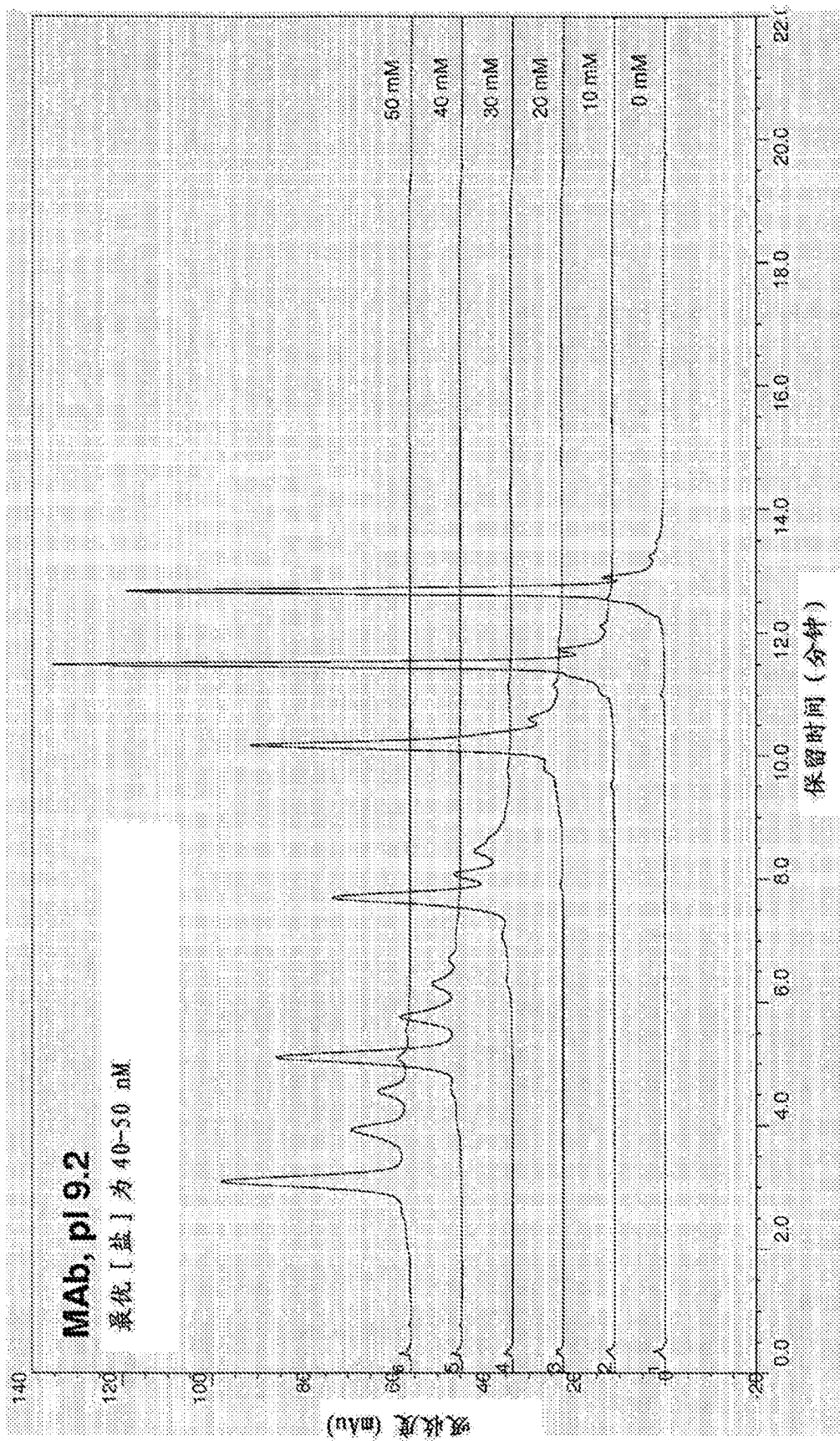


图 15

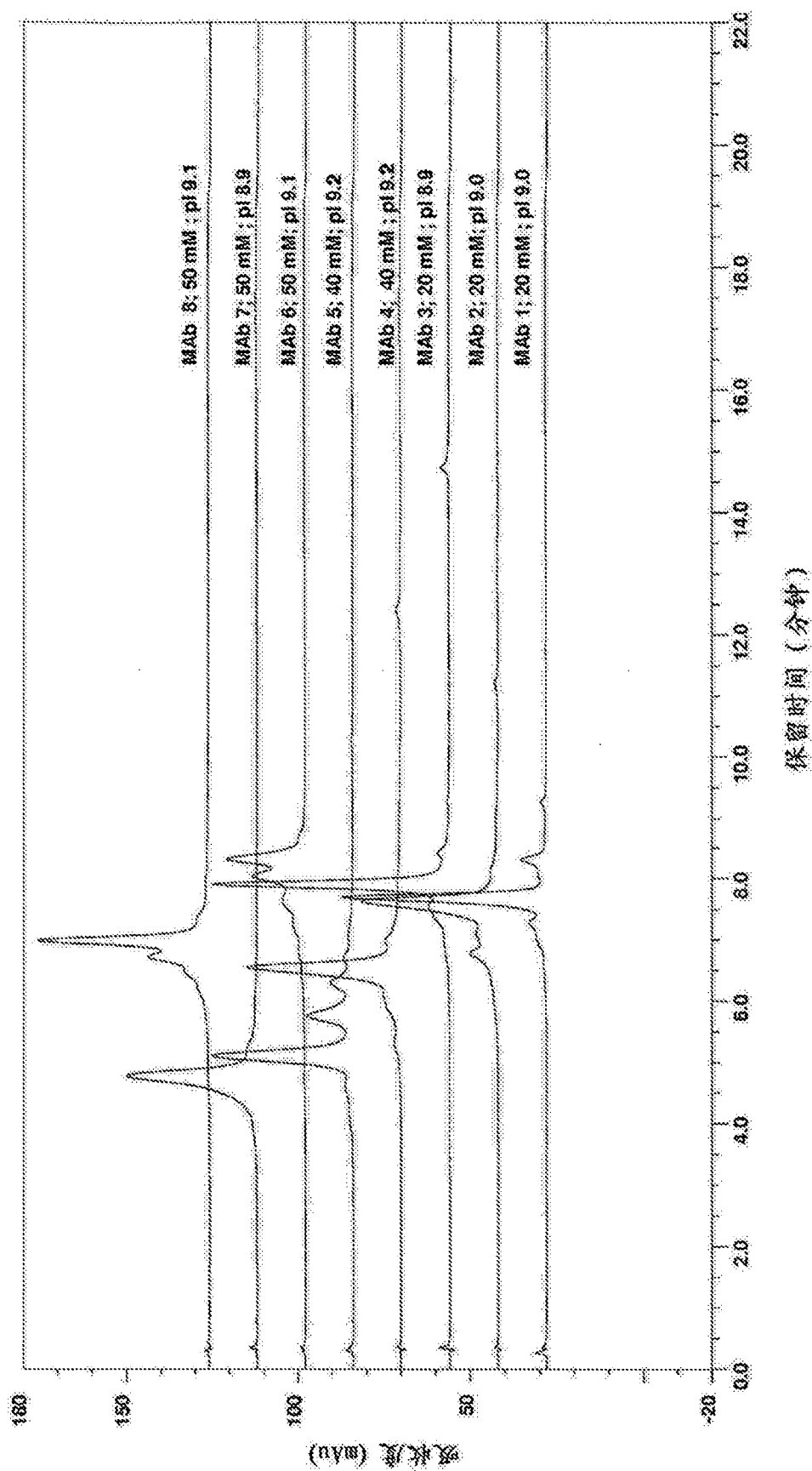


图 16

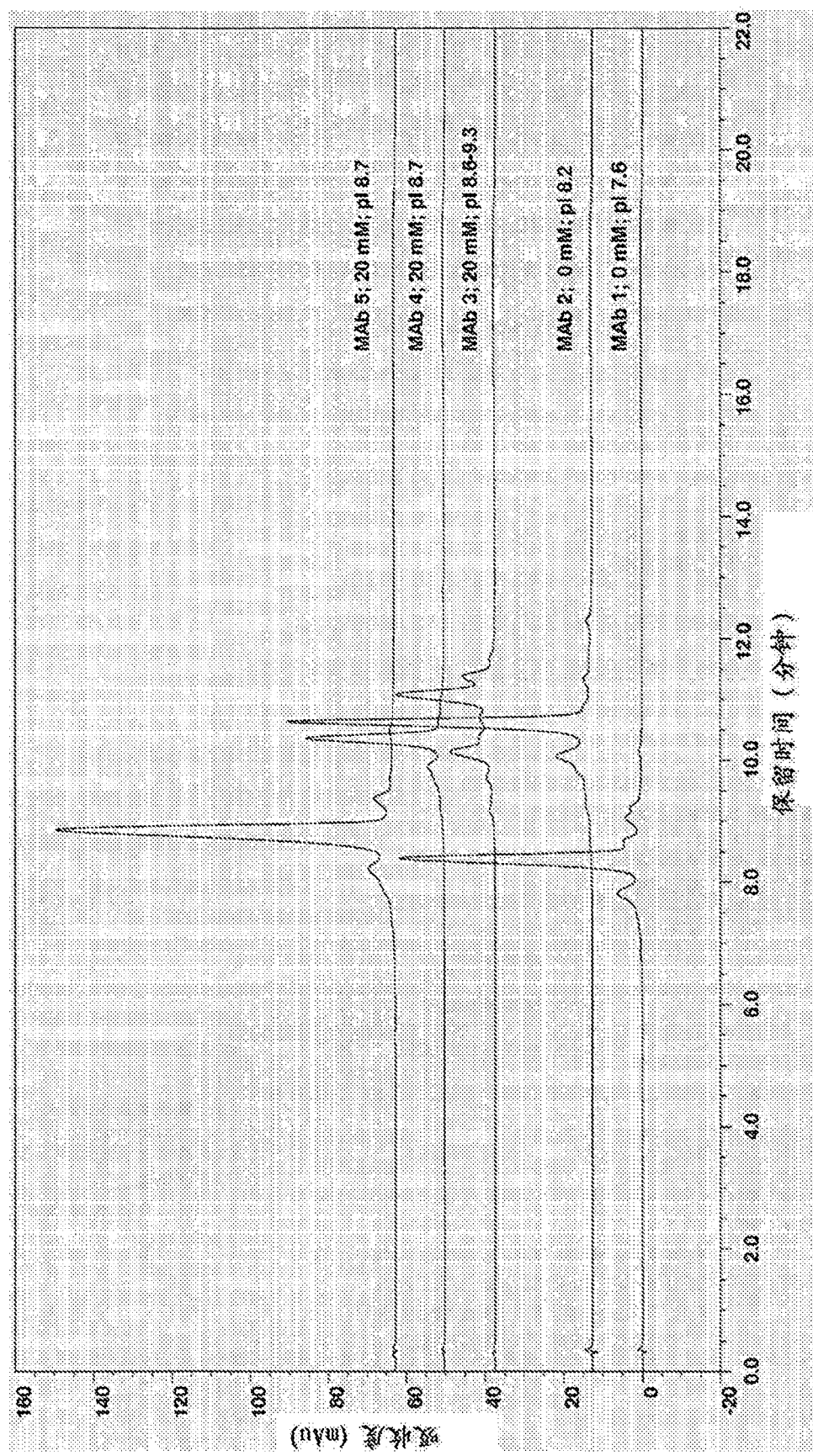


图 17

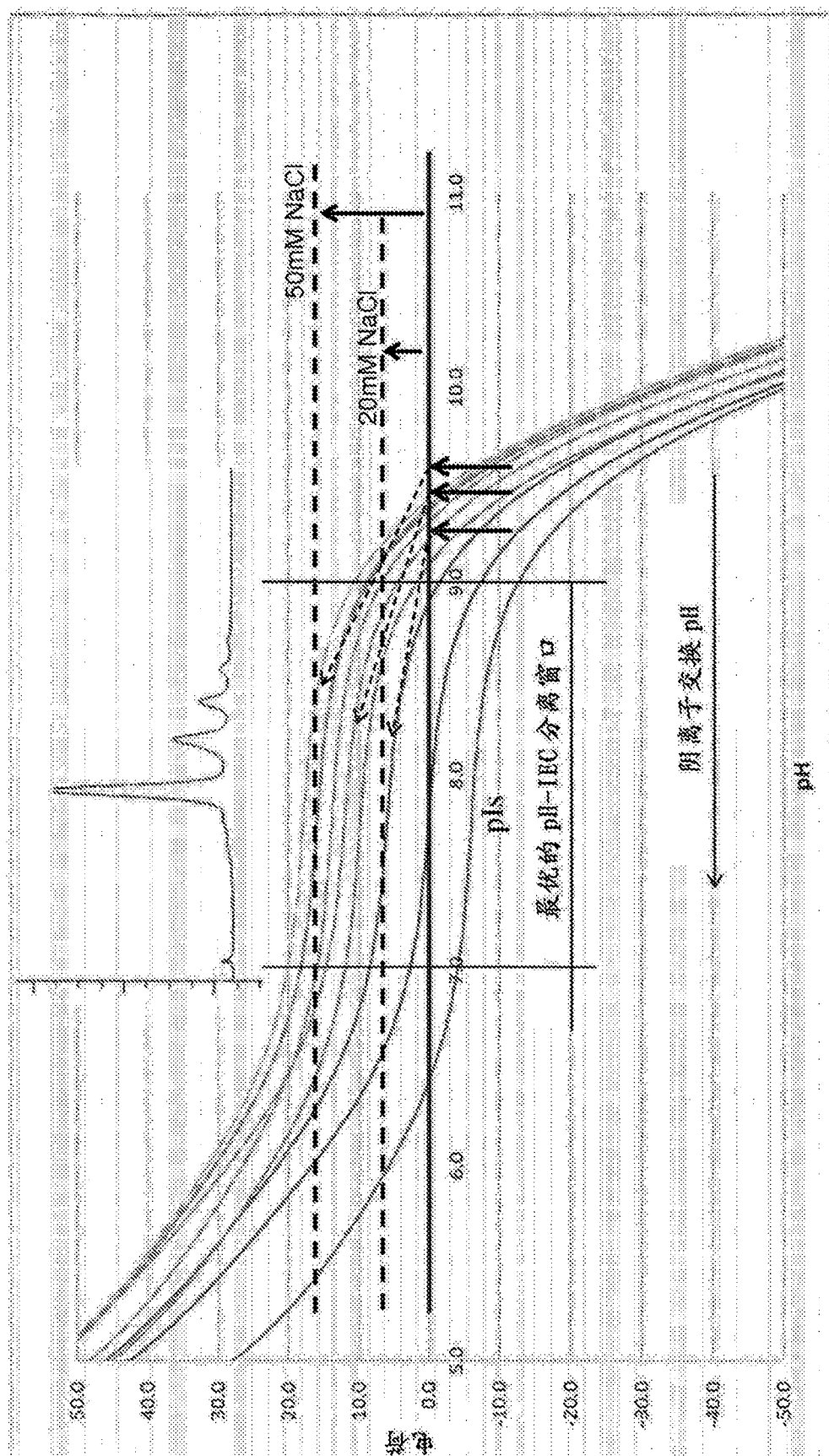


图 18

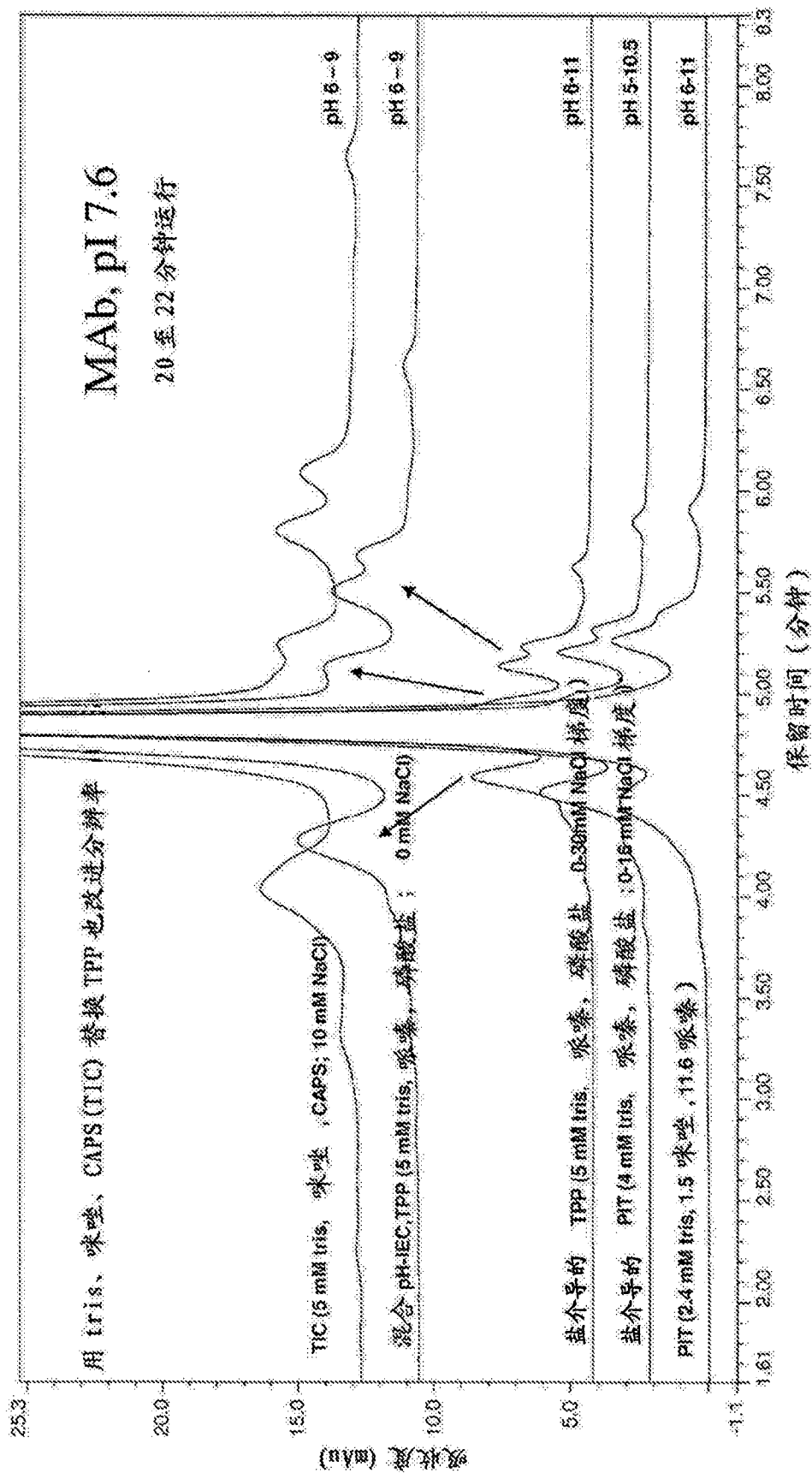


图 19

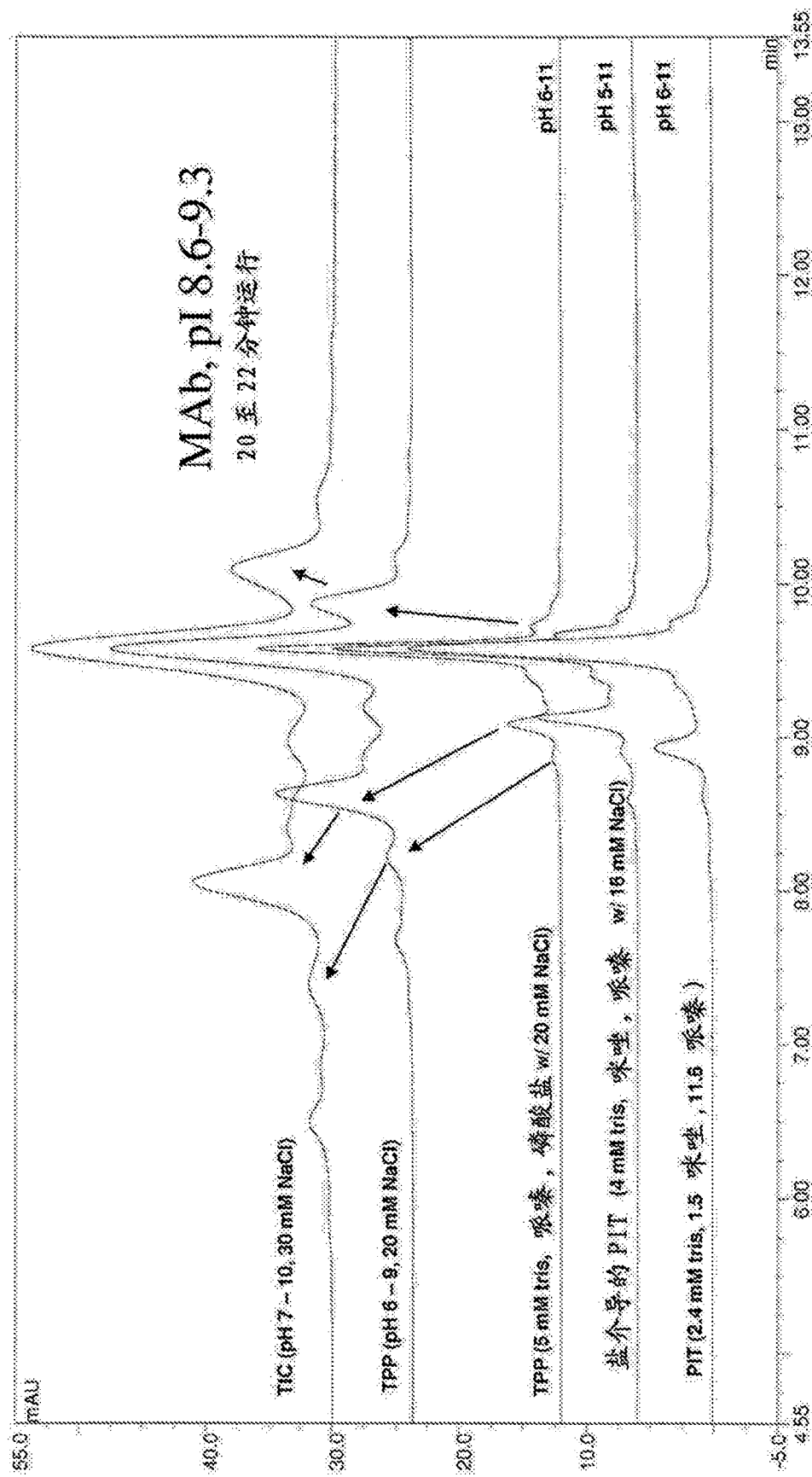


图 20

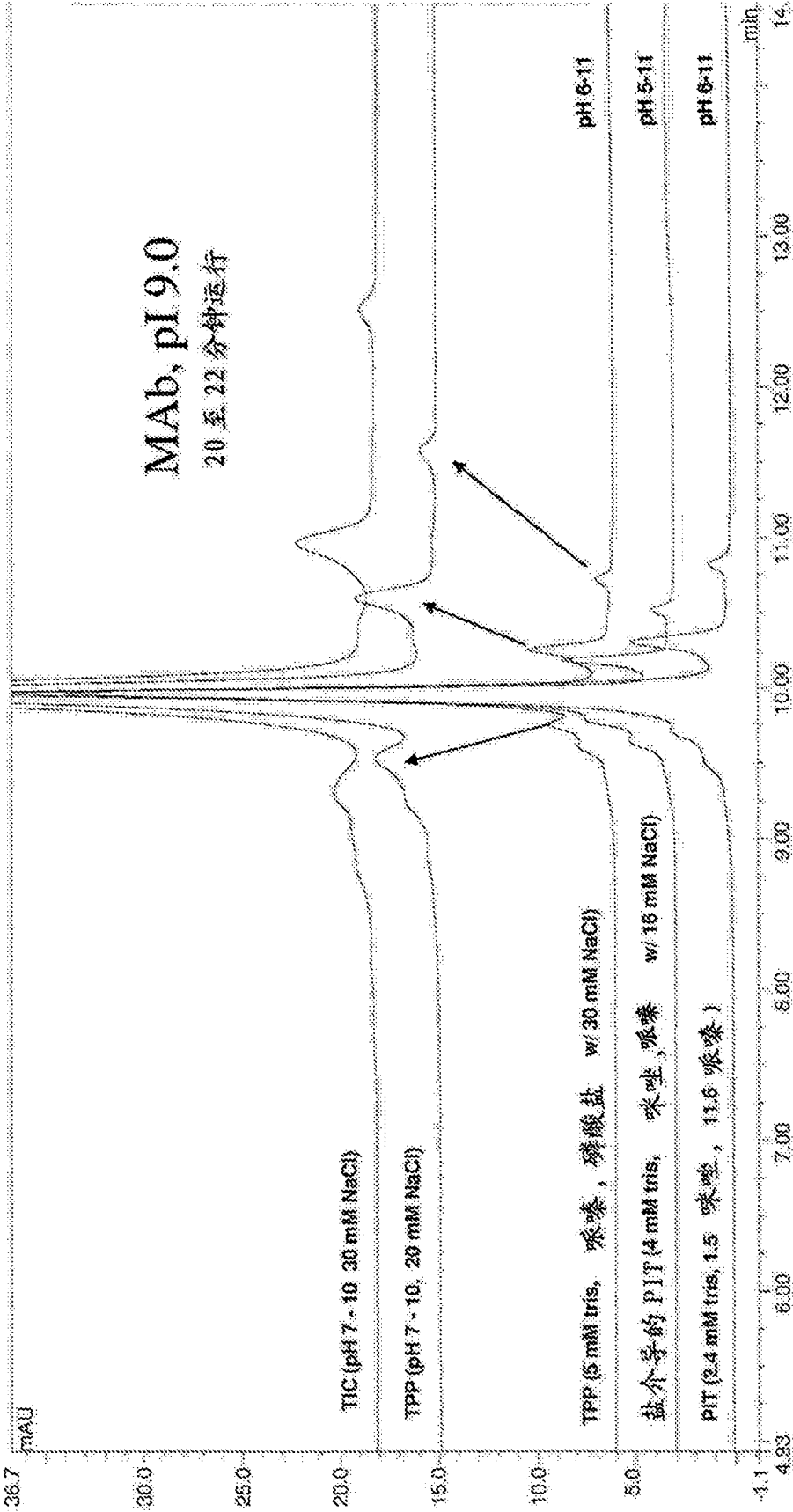


图 21

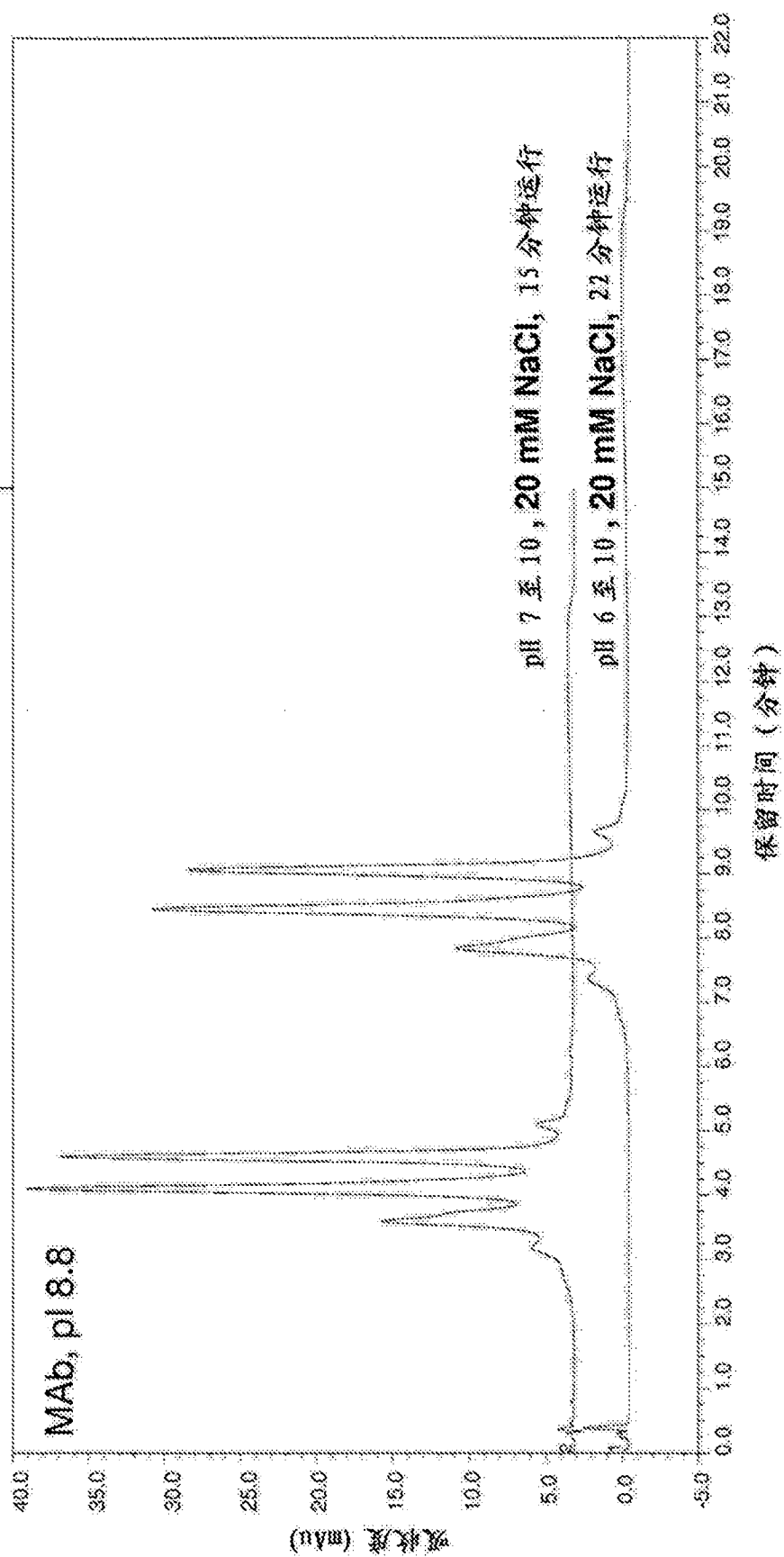


图 22

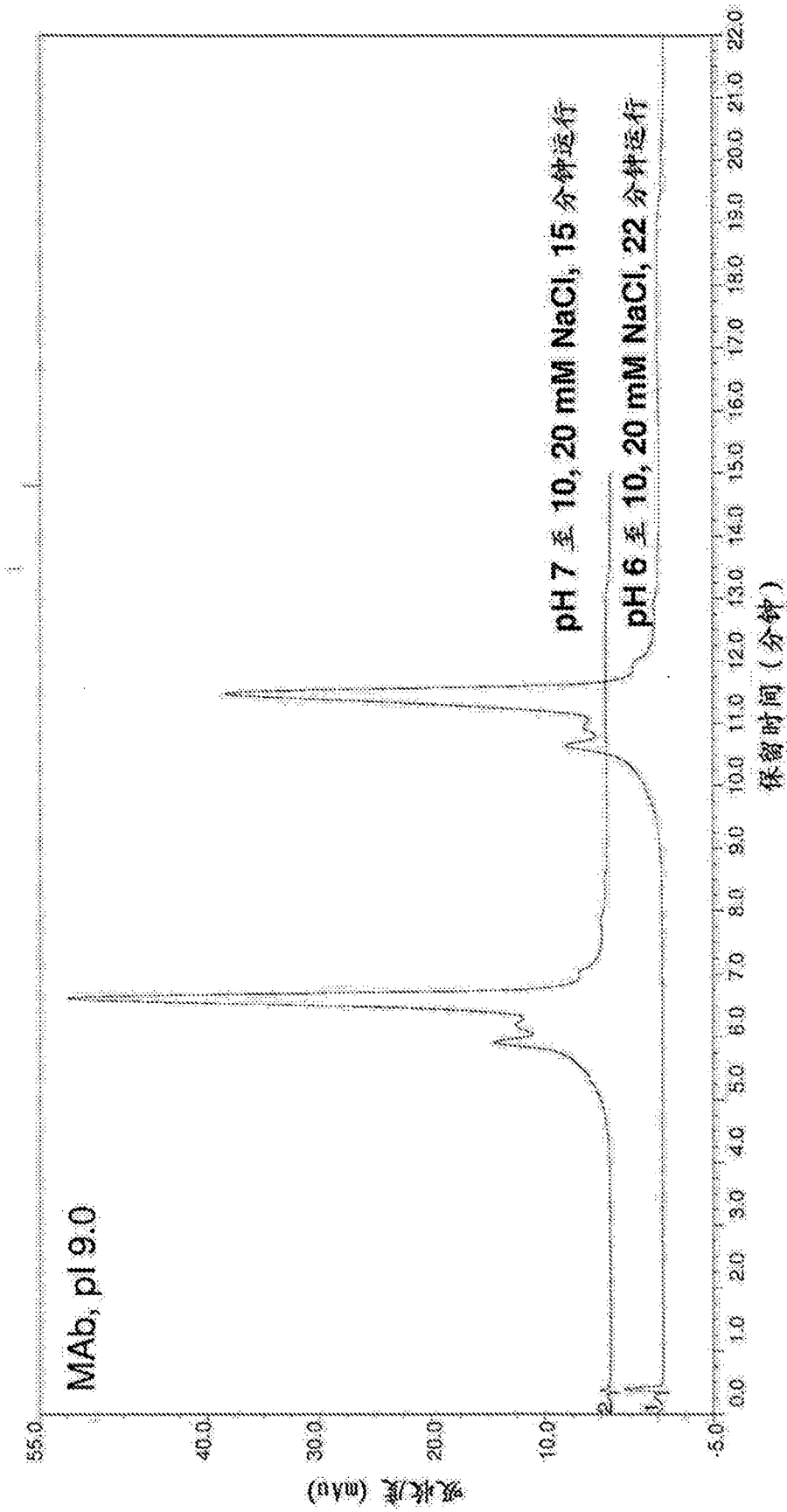


图 23

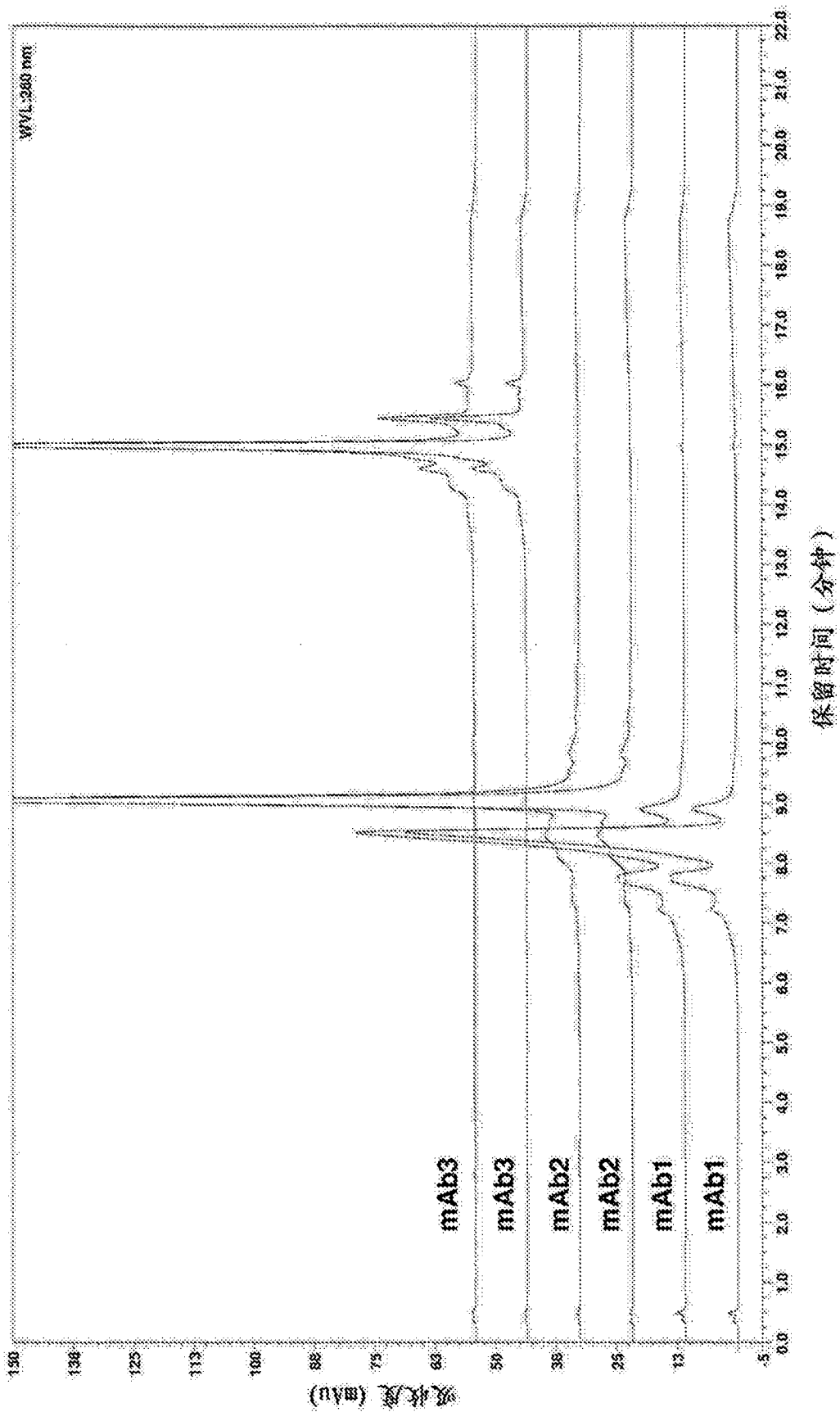


图 24

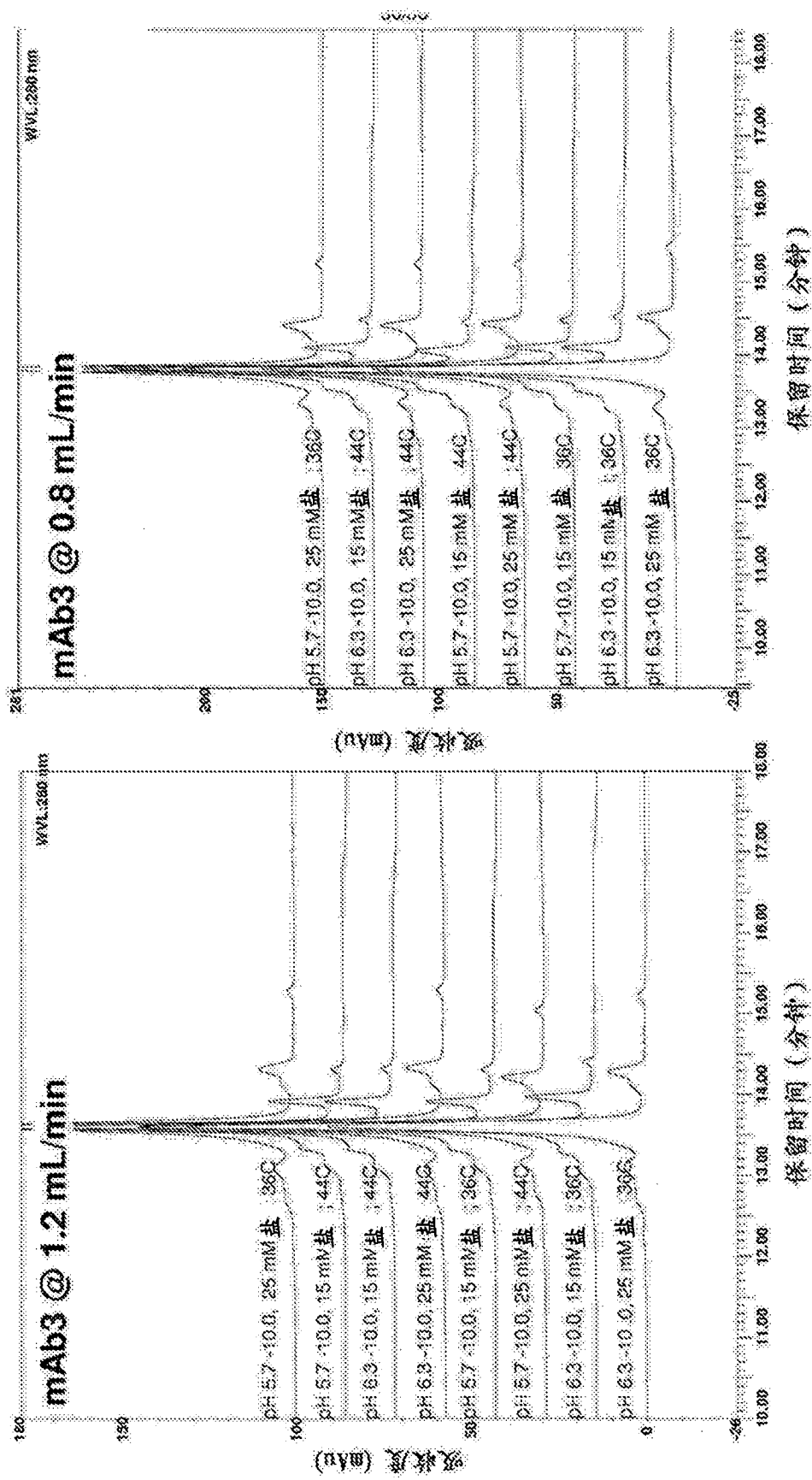


图 25

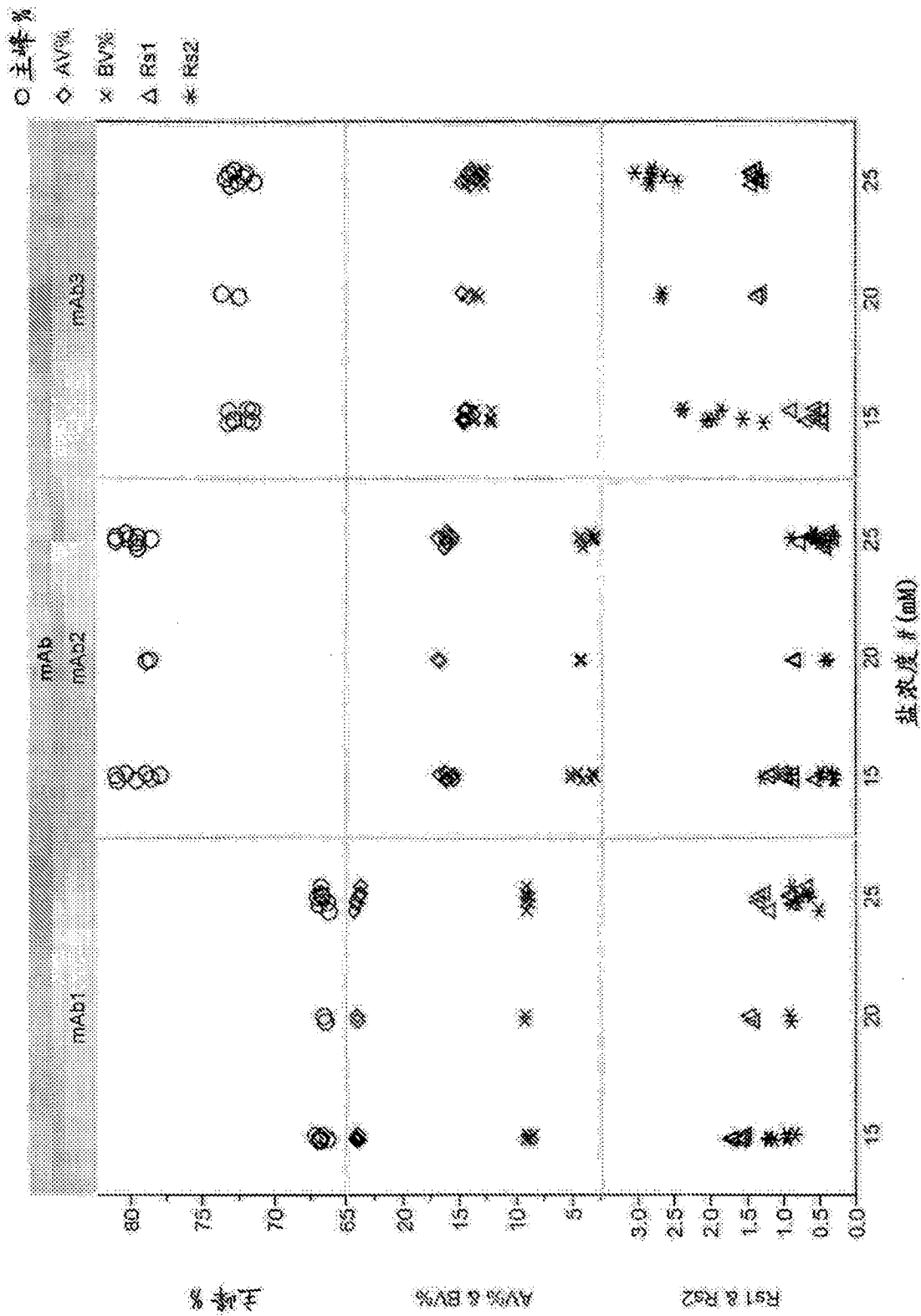


图 26

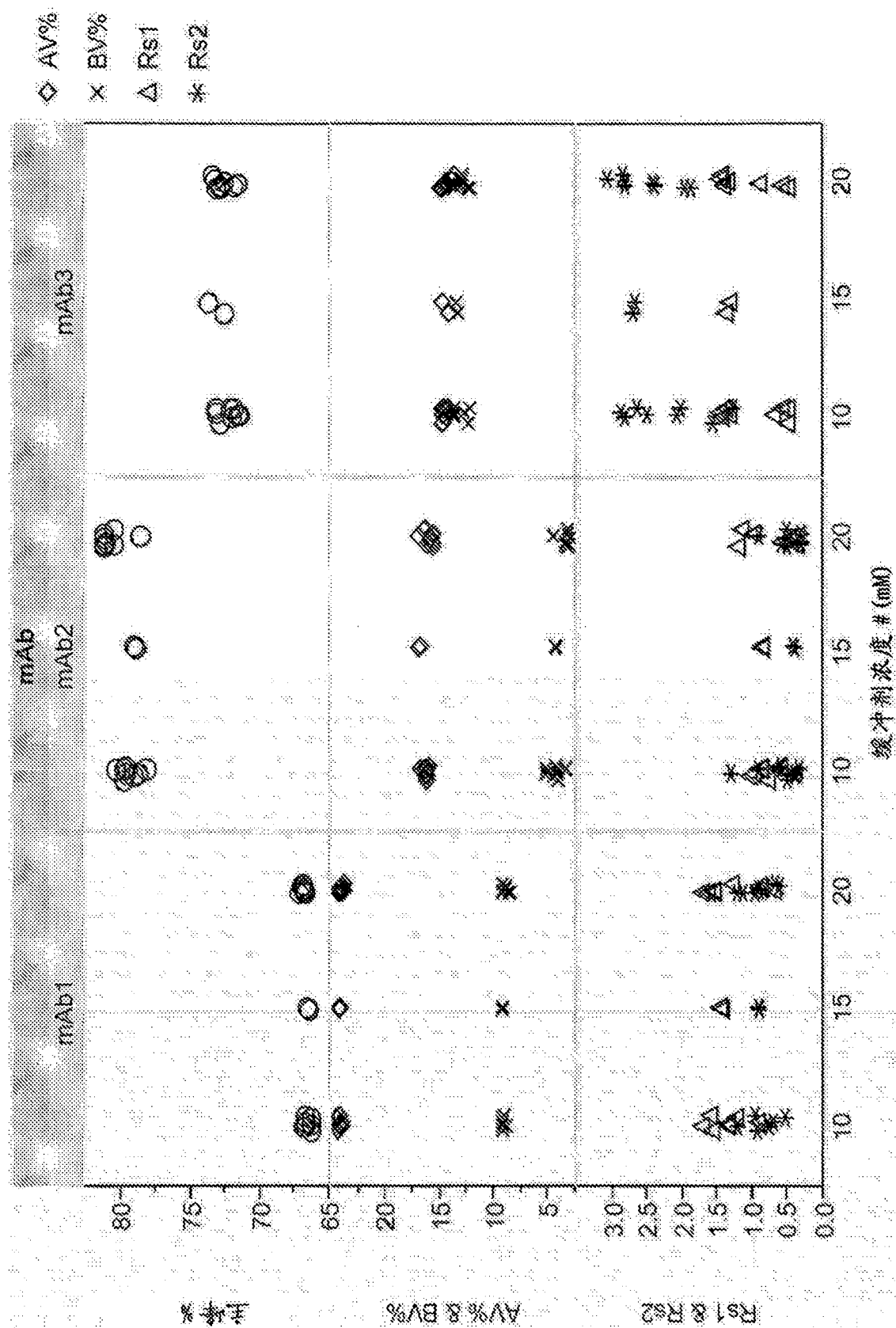


图 27A

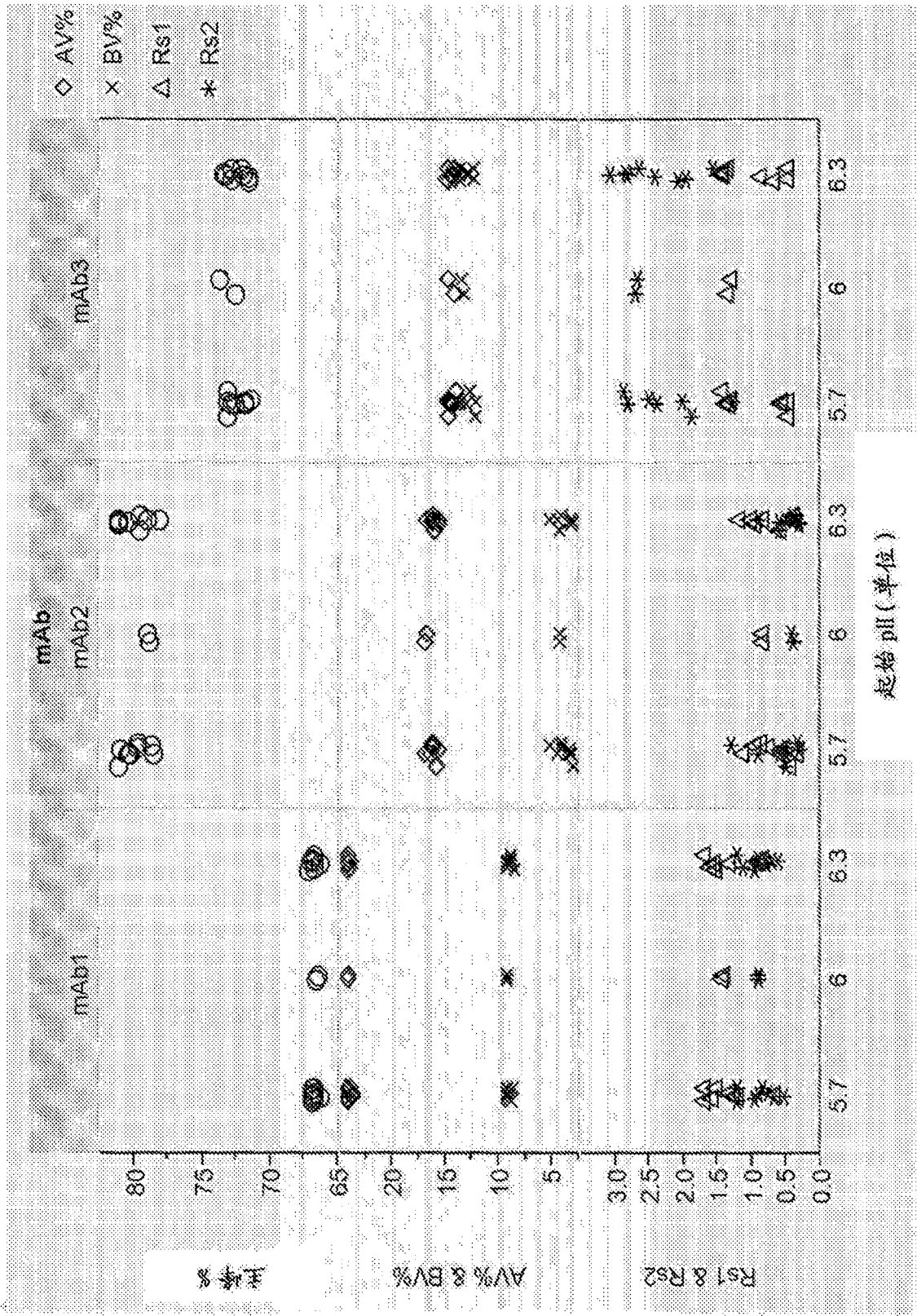


图 27B

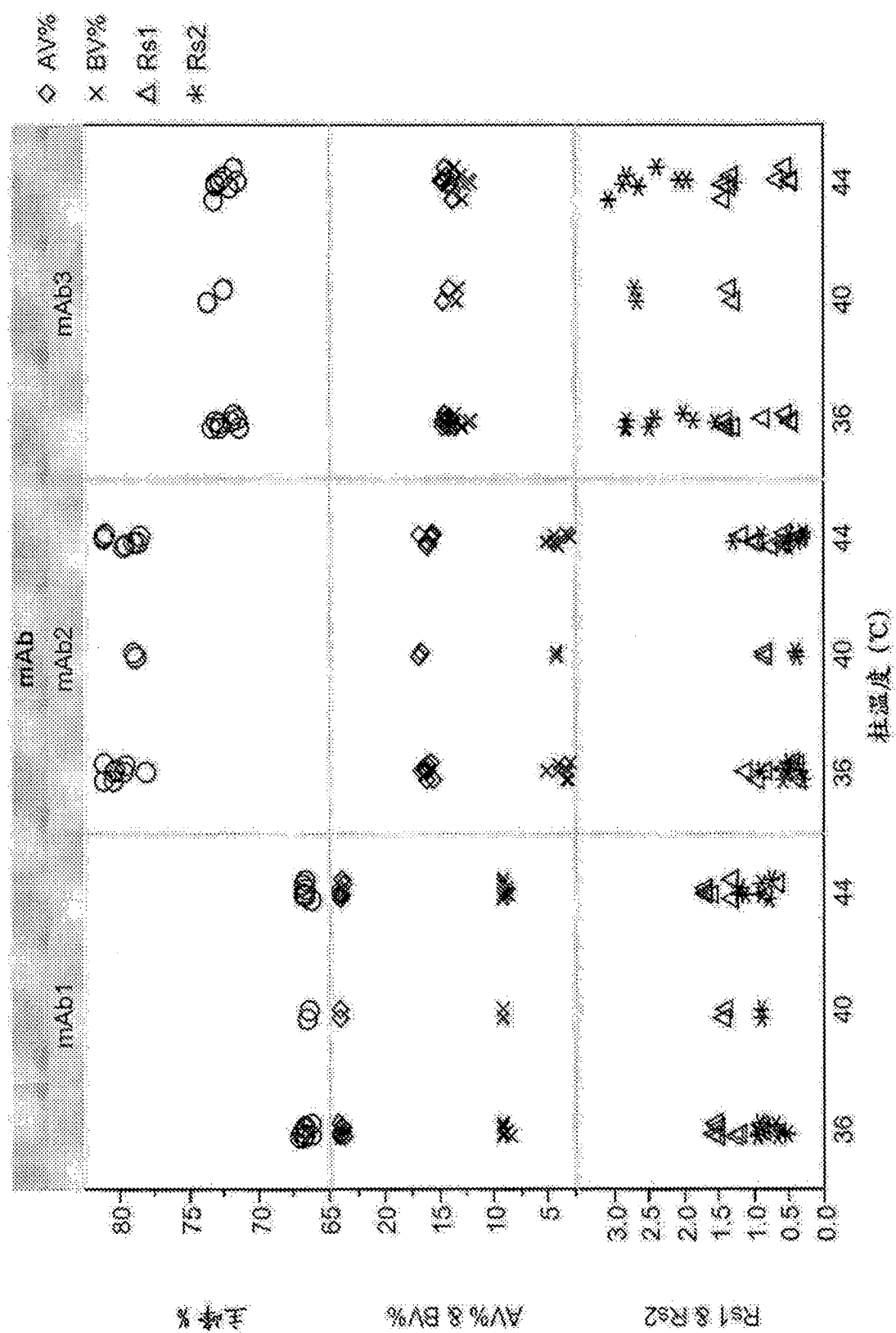


图 27C

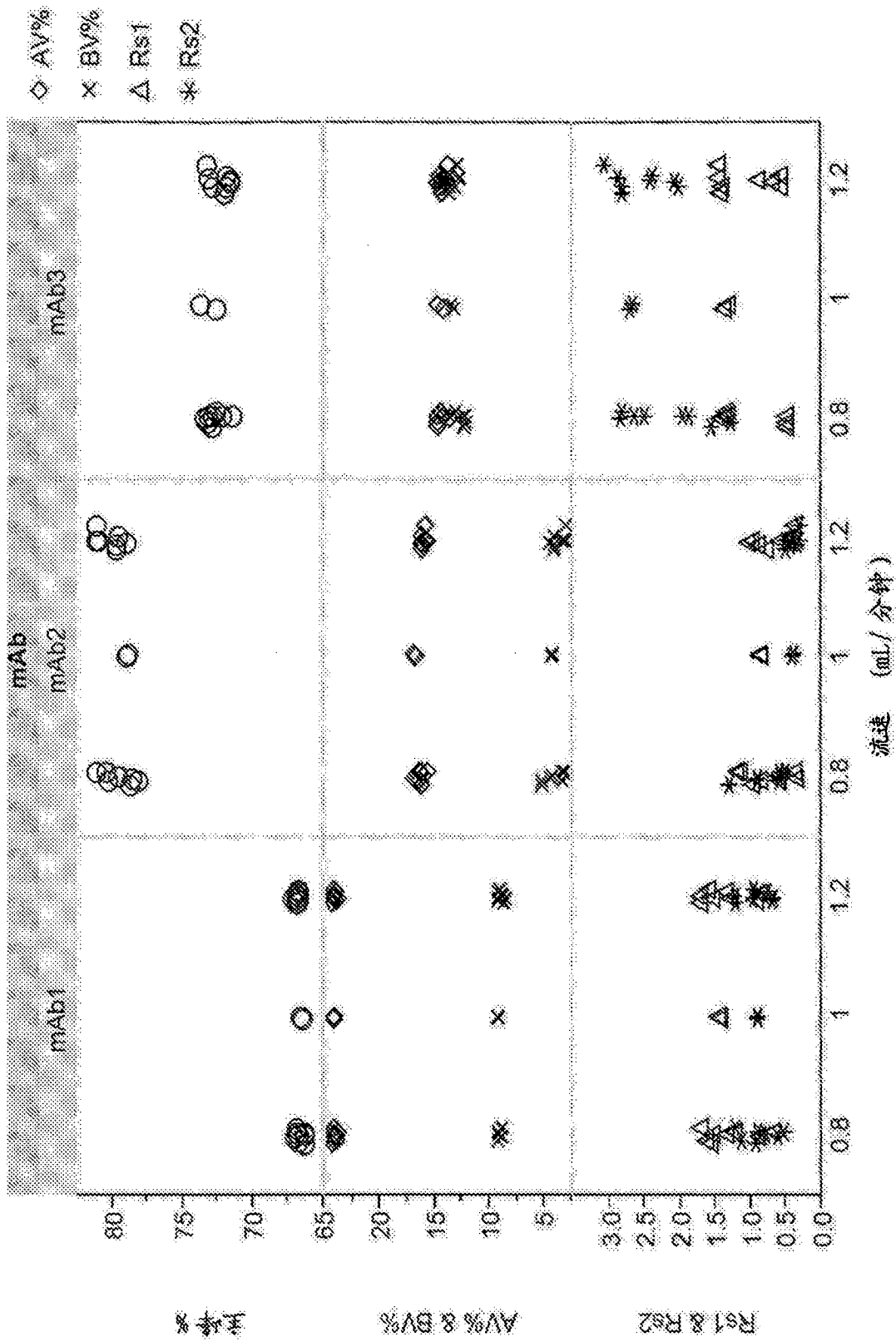


图 27D

Abstract

The present invention provides methods for analyzing compositions of polypeptides such as antibodies by ionic strength-mediated pH gradient ion exchange chromatography. In some aspects, the methods use a combination of pH gradients and ionic strength gradients to separate the polypeptide from charge variants of the polypeptide. In some aspects, the methods use a stable ionic strength to optimize the pH gradient separation window to separate the polypeptide from charge variants. Such methods are useful for analyzing polypeptide, *e.g.* antibodies, with a pI greater than 9 or a pI less than 7. In some aspects, the invention provides a multiproduct method for the analysis of polypeptides of varying pI's.