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 (54) Title: CHROMATOGRAPHY PURIFICATION OF ANTIBODIES

(57) **Abrégé/Abstract:**

Methods, kits and apparatuses for chromatography purification of antibodies are provided. In some embodiments, antibodies are purified by mixed mode chromatography that does not comprise hydroxyapatite (HT) or fluorapatite (FT). The mixed mode chromatography step is then followed by a HT/FT chromatography step.

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(54) Title: CHROMATOGRAPHY PURIFICATION OF ANTIBODIES

(57) Abstract: Methods, kits and apparatuses for chromatography purification of antibodies are provided. In some embodiments, antibodies are purified by mixed mode chromatography that does not comprise hydroxyapatite (HT) or fluorapatite (FT). The mixed mode chromatography step is then followed by a HT/FT chromatography step.



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## CHROMATOGRAPHY PURIFICATION OF ANTIBODIES

[0001] <deleted>

### BACKGROUND

[0002] Mixed mode chromatography involves the use of solid phase chromatographic supports that  
5 employ multiple chemical mechanisms to adsorb proteins or other solutes. Examples include but are not  
limited to chromatographic supports that exploit combinations of two or more of the following  
mechanisms: anion exchange, cation exchange, hydrophobic interaction, hydrophilic interaction,  
hydrogen bonding, pi-pi bonding, and metal affinity. Mixed mode chromatography supports provide  
unique selectivities that cannot be reproduced by single mode chromatography methods such as ion  
10 exchange.

### SUMMARY

[0003] The present disclosure provides for methods of purifying antibodies from a solution  
containing antibodies. Various embodiments of the claimed invention relate to

- a. contacting a first solution containing antibodies to a mixed mode  
15 chromatography matrix, wherein the matrix comprises at least one hydrophobic moiety and at  
least one acidic moiety and does not include sulfur, thereby binding at least a portion of the  
antibodies to the matrix;
- b. separating the matrix with bound antibodies from the first solution;
- c. eluting the bound antibodies by raising the pH of solution in contact with  
20 the matrix and bound antibodies, thereby producing a solution comprising eluted antibodies;
- d. contacting the solution comprising eluted antibodies of step c to a solid  
support matrix, the solid support matrix comprising hydroxyapatite (HT) or fluorapatite (FT),  
wherein at least a portion of the antibodies bind to the solid support matrix;
- e. separating the solid support matrix and bound antibodies from the second  
25 solution; and
- f. eluting the bound antibodies from the solid support matrix, thereby  
purifying the antibodies from the solution.

[0004] In some embodiments, the solution of eluted antibodies of step c is not substantially changed  
before step d.



- [0005] In some embodiments, the contacting step a comprises binding the antibodies to the mixed mode matrix at a pH below 7. In some embodiments, the matrix comprises charged moieties at a pH below 7.
- [0006] In some embodiments, the contacting step a comprises binding the antibodies to the matrix at a pH at or above 7.
- [0007] In some embodiments, the contacting step a comprises binding the antibodies to the matrix in the presence of at least 1 M salt. In some embodiments, the contacting step a comprises binding the antibodies to the matrix in the presence of 1 M salt or less. In some embodiments, for example, less than 0.5 M or less than 0.25 M is used.
- [0008] In some embodiments, the eluting step (c) comprises changing the salt concentration of the solution in contact with the matrix and bound antibodies, thereby eluting the antibodies.
- [0009] In some embodiments, the eluting step (c) comprises changing the pH of the solution in contact with the matrix and bound antibodies, thereby eluting the antibodies. In some embodiments, the pH is changed from a pH below pH 7 to a pH above pH 7.
- [0010] In some embodiments, the eluting step (f) comprises changing the salt concentration, thereby eluting the antibodies.
- [0011] In some embodiments, the mixed mode chromatography matrix comprises a mixed mode ligand comprising an alkyl acid comprising between 4-10 carbons.
- [0012] In some embodiments, the mixed mode ligand comprises hexanoic acid.
- [0013] In some embodiments, the mixed mode chromatography matrix comprises a mixed mode ligand comprising phenylalanine. In some embodiments, the amine of the phenylalanine is acetylated or otherwise capped.
- [0014] In some embodiments, wherein the mixed mode chromatography matrix comprises a mixed mode ligand comprises a t-butyl ether derivative of polymethacrylate.
- [0015] In some embodiments, the methods further comprise an anion exchange chromatography step, a cation exchange, hydrophobic interaction or an ultrafiltration step, precipitation step, crystallization step, or liquid:liquid phase partitioning.

[0016] In some embodiments, the eluting step c comprises increasing the pH of the solution in contact with the matrix and bound antibodies.

[0017] In some embodiments, the solid support matrix comprises ceramic hydroxyapatite (CHT). In some embodiments, the solid support matrix comprises ceramic fluoroxyapatite (CFT).

5 [0018] The present disclosure also provides an apparatus for purifying antibodies. In some embodiments, the apparatus comprises,

a first column tube and a second column tube, wherein the first and second column tubes comprise inlets and outlets, wherein the outlet of the first column tube connects to the inlet of the second column tube such that the first column tube is in fluid communication with the second column  
10 tube,

wherein the first column tube contains a mixed mode chromatography matrix, wherein the matrix does not include sulfur, and the second column tube contains a solid support matrix, the solid support matrix comprising hydroxyapatite (HT) or fluorapatite (FT).

[0019] In some embodiments, the mixed mode chromatography matrix comprises a mixed mode  
15 ligand comprising an alkyl acid comprising between 4-10 carbons.

[0020] In some embodiments, the mixed mode ligand comprises hexanoic acid.

[0021] In some embodiments, the mixed mode chromatography matrix comprises a mixed mode ligand comprising phenylalanine. In some embodiments, the amine of the phenylalanine is acetylated or otherwise capped.

20 [0022] In some embodiments, the mixed mode chromatography matrix comprises a mixed mode ligand comprises a t-butyl ether derivative of polymethacrylate.

[0023] In some embodiments, the solid support matrix comprises ceramic hydroxyapatite (CHT). In some embodiments, the solid support matrix comprises ceramic fluoroxyapatite (CFT).

[0024] The present disclosure also provides kits for purifying antibodies. Various embodiments of  
25 the claimed invention relate to a kit, a kit comprising,

a mixed mode chromatography matrix, wherein the matrix comprises at least one hydrophobic moiety and at least one acidic moiety and does not include sulfur; and

a solid support matrix, the solid support matrix comprising hydroxyapatite (HT) or fluorapatite (FT).



[0025] In some embodiments, the kits further comprise  
a first column tube or cartridge comprising the mixed mode chromatography matrix;  
and  
a second column tube or cartridge comprising the HT or FT solid support matrix.

5 [0026] In some embodiments, the first and second column tubes comprise inlets and outlets, wherein the outlet of the first column tube attachable to the inlet of the second column tube such that, when attached, the first column tube is in fluid communication with the second column tube.

[0027] In some embodiments, the mixed mode chromatography matrix comprises a mixed mode ligand comprising an alkyl acid comprising between 4-10 carbons.

10 [0028] In some embodiments, the mixed mode ligand comprises hexanoic acid.

[0029] In some embodiments, the mixed mode chromatography matrix comprises a mixed mode ligand comprising phenylalanine. In some embodiments, the amine of the phenylalanine is acetylated or otherwise capped.

[0030] In some embodiments, the mixed mode chromatography matrix comprises a mixed mode  
15 ligand comprises a t-butyl ether derivative of polymethacrylate.

[0031] In some embodiments, the solid support matrix comprises ceramic hydroxyapatite (CHT). In some embodiments, the solid support matrix comprises ceramic fluoroxyapatite (CFT).

[0031A] Various embodiments of the claimed invention relate to a method for purifying antibodies from a solution containing antibodies, the method comprising

- 20 a. contacting a first solution containing antibodies to a mixed mode chromatography matrix, wherein the mixed mode chromatography matrix comprises phenylalanine, an n-alkyl acid comprising 2-10 carbons, or a t-butyl ether derivative of polymethacrylate, thereby binding at least a portion of the antibodies to the matrix;
- b. separating the matrix with bound antibodies from the first solution;
- 25 c. eluting the bound antibodies by changing the pH or salt concentration of solution in contact with the matrix and bound antibodies, thereby producing a solution comprising eluted antibodies and purifying antibodies from a solution containing antibodies.

## DEFINITIONS

[0032] "Antibody" refers to an immunoglobulin, composite, or fragmentary form thereof. The term may include but is not limited to polyclonal or monoclonal antibodies of the classes IgA, IgD, IgE, IgG, and IgM, derived from human or other mammalian cell lines, including  
5 natural or genetically modified forms such as humanized, human, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, grafted, and in vitro generated antibodies.

"Antibody" may also include composite forms including but not limited to fusion proteins containing an immunoglobulin moiety. "Antibody" may also include antibody fragments such as Fab, F(ab')<sub>2</sub>, Fv, scFv, Fd, dAb, Fc and other compositions, whether or not they  
10 retain antigen-binding function.

[0033] "Mixed mode chromatography support" refers to a chromatographic solid phase that substantially involves a combination of two or more chemical mechanisms. Examples of chemical mechanisms that can be combined in mixed mode supports include but are not limited to cation exchange, anion exchange, hydrophobic interaction, hydrophilic interaction,  
15 hydrogen bonding, pi-pi bonding, and metal affinity. The solid phase can be a porous particle, nonporous particle, membrane, or monolith.

[0034] "Hydroxyapatite" refers to a mixed mode support comprising an insoluble hydroxylated mineral of calcium phosphate with the structural formula  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ . Its dominant modes of interaction are phosphoryl cation exchange and calcium metal affinity.  
20 Hydroxapatite is commercially available in various forms, including but not limited to ceramic, crystalline and composite forms. Composite forms contain hydroxyapatite microcrystals entrapped within the pores of agarose or other beads.

[0035] "Fluorapatite" refers to a mixed mode support comprising an insoluble fluoridated mineral of calcium phosphate with the structural formula  $\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$ . Its dominant modes  
25 of interaction are phosphoryl cation exchange and calcium metal affinity. Fluorapatite is commercially available in various forms, including but not limited to ceramic and crystalline composite forms.

[0036] "Ceramic" hydroxyapatite (CHT) or "ceramic" fluorapatite (CFT) refer to forms of the respective minerals in which nanocrystals are agglomerated into particles and fused at  
30 high temperature to create stable ceramic microspheres suitable for chromatography applications. Commercial examples of ceramic hydroxyapatite include, but are not limited to CHT Type I and CHT Type II. Commercial examples of fluorapatite include, but are not



limited to CFT Type I and CFT Type II. Unless specified, CHT and CFT refer to roughly spherical particles of any average diameter, including but not limited to about 10, 20, 40, and 80 microns. The choice of hydroxyapatite or fluorapatite, the type, and average particle diameter can be determined by the skilled artisan.

5

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0037] Figure 1 illustrates a chromatogram from antibody capture from cell culture filtrate on UNOsphere Phenylalanine. The conductivity profile demonstrates that sample loading can be achieved at conductivity values corresponding more or less with physiological  
10 conditions, such as encountered in cell culture supernatants, and illustrates that antibody remains bound even when a wash with 1M NaCl is applied. The conductivity profile, in conjunction with the UV profile, further illustrates that the antibody can be eluted in a pH gradient at low conductivity to facilitate application of the eluted IgG to CHT or to an ion exchanger.

15 [0038] Figure 2 is a chromatogram illustrating polishing purification of antibody eluted from UNOsphere Phenylalanine in Figure 1 on hydroxyapatite. The reagents and conditions were as follows: CHT type 1, 40  $\mu\text{m}$ . 1 mL 5 mm x 5 cm, 300 cm/hr. Buffer A: 10mM phosphate, pH 6.7. Buffer B: 10 mM phosphate, 1.5M NaCl, pH 6.7. Buffer C: 500 mM sodium phosphate, pH 7.0. EQ column with buffer A. The IgG eluate from the capture step  
20 on UNOsphere Phenylalanine was loaded onto the hydroxyapatite column, washed with buffer A, and eluted in a 30 CV linear gradient to buffer B. The column was cleaned with buffer C.

[0039] Figure 3 is a Flamingo stained, reduced SDS-PAGE gel loaded as discussed in the Examples.

25 [0040] Figure 4 is a chromatogram showing binding and elution of purified immunoglobulin from a non-acetylated phenylalanine UNOsphere resin as discussed in the Examples.

[0041] Figure 5 is a chromatogram showing binding and elution of purified immunoglobulin from an acetylated phenylalanine UNOsphere resin as discussed in the  
30 Examples.



- [0042] Figure 6 is a chromatogram showing binding and elution being practiced in hydrophobic mode with a protein A purified monoclonal human IgG1. The column used comprised 1 mL UNOsphere Phenylalanine 5 mm x 5 cm, 300 cm/hr. .. The column was equilibrated with buffer A (4M NaCl, 0.01M citate, 0.01M phosphate, pH 4.5) and 100 $\mu$ L of the filtered unequilibrated sample was injected. The column was washed with ~2.5 column volumes (CV) of buffer A, and then eluted with 15CV linear gradient from 100% A to 100% B (Buffer B. 0.01M citrate, 0.01M phosphate, pH 7.5). The column was washed with B until equilibrium was reached with buffer B.
- [0043] Figure 7 is a chromatogram showing antibody capture from cell culture filtrate on Macro Prep t-Butyl HIC resin as discussed in the Examples.
- [0044] Figure 8 illustrates a Coomassie brilliant blue stained, reduced, SDS PAGE gel loaded as discussed in the Examples.
- [0045] Figure 9 is a chromatogram showing binding and elution of purified immunoglobulin from an amino caproic acid UNOsphere resin as discussed in the Examples.
- [0046] Figure 10 is a chromatogram showing binding and elution of purified immunoglobulin from an acetylated amino caproic acid UNOsphere resin as discussed in the Examples.
- [0047] Figure 11 is a chromatogram showing binding and elution of purified immunoglobulin from a hexanoic acid UNOsphere resin as discussed in the Examples.
- [0048] Figure 12 is a chromatogram showing binding and elution of purified immunoglobulin from a hexanoic acid agarose resin as discussed in the Examples.
- [0049] Figure 13 is a chromatogram showing binding and elution of purified immunoglobulin from a hexanoic acid sepharose resin as discussed in the Examples.
- [0050] Figure 14 is a chromatogram showing binding and elution of purified immunoglobulin from a phenyl derivative of UNOsphere diol resin containing carboxyl groups as discussed in the Examples.
- [0051] Figure 15 is a chromatogram showing binding and elution of purified immunoglobulin from a Macro Prep t-Butyl HIC resin as discussed in the Examples.

## DETAILED DESCRIPTION OF THE INVENTION

*I. Introduction*

[0052] The present invention provides for purification of antibodies and other proteins using a variety of mixed mode chromatography reagents. A number of mixed mode ligands are described, including for example phenylalanine, alkyl acids, and t-butyl ether derivatives of polymethacrylate. It will be appreciated from the discussion herein that other types of mixed mode ligands can also be used to effectively purify antibodies and other proteins.

[0053] One aspect of the invention is the combination of a mixed mode chromatography step with a chromatography step using hydroxyapatite (HT) or fluorapatite (FT). This second step is useful for various "polishing" aspects, resulting in a highly purified final product without significant aggregates or cellular contaminants. Further, when used to purify antibodies, the combination of the mixed mode chromatography (not comprising HT/FT) with subsequent chromatography on HT or FT allows for a highly purified product that is produced at a fraction of the cost of the current industry standard for antibody purification, which involves antibody capture by affinity chromatography using Protein A chromatography media.

[0054] In some embodiments, the mixed mode step results in an eluate that can be added directly to the hydroxyapatite (HT) or fluorapatite (FT) chromatography media, thereby reducing the need for any sample manipulation during the purification process. This aspect is particularly valuable for large scale commercial purification of proteins, but is also convenient for smaller laboratory scale preparations.

[0055] Any antibody preparation can be used in the present invention, including unpurified or partially purified antibodies from natural, synthetic, or recombinant sources. Unpurified antibody preparations can come from various sources including, but not limited to, plasma, serum, ascites, milk, plant extracts, bacterial lysates, yeast lysates, or conditioned cell culture media. Partially purified preparations can come from unpurified preparations that have been processed by at least one chromatography, precipitation, other fractionation step, or any combination of the foregoing. In some embodiments, the antibodies have not been purified by protein A affinity prior to purification as described herein.



## **II. Mixed-mode chromatography**

### **A. Ligands**

[0056] The present invention provides for purification of antibodies using mixed mode chromatography, wherein the chromatography solid phase is not HT or FT, optionally  
5 combined with other purification steps that can include, for example, HT or FT chromatography.

[0057] Various mixed mode chromatography media are available commercially, any of which can be used to practice of this invention. Commercially available examples include but are not limited to MEP-Hypercel™, Capto-MMCTM, Capto-Adhere™, and ABx™.

10 [0058] In some embodiments, the mixed-mode chromatography support exploits a combination of anion exchange and hydrophobic interaction functionalities. Commercial examples of such supports include, but are not limited to, MEP-Hypercel™ and Capto Adhere™.

15 [0059] In some embodiments, the mixed-mode chromatography support exploits a combination of cation exchange and hydrophilic interaction functionalities. Commercial examples of such supports include, but are not limited to, Capto-MMCTM.

[0060] In some embodiments, the mixed-mode chromatography support exploits a combination of cation exchange, anion exchange, and hydrophobic interaction functionalities. Commercial examples of such supports include, but are not limited to, ABx™.

20 [0061] In some embodiments, the mixed-mode chromatography support exploits a combination of anion exchange and hydrophobic interaction functionalities with potential for hydrogen bonding and pi-pi bonding. Commercial examples of such supports include, but are not limited to, Capto-Adhere™.

25 [0062] In some embodiments, the mixed-mode chromatography support exploits a combination of cation exchange and hydrophobic interaction functionalities with potential for hydrogen bonding and pi-pi bonding. Commercial examples of such supports include, but are not limited to, Capto-MMCTM.

[0063] Other non-commercial mixed mode media include, for example, mixed-mode chromatography support exploiting a combination of cation or anion exchange with  
30 hydrophobic interaction functionalities in the same ligand, or in a combination of ligands.

Some examples of such ligands are described in, e.g., U.S. Patent Nos. 7,008,542; 6,498,236; and 5,945,520.

[0064] In some embodiments, mixed-mode chromatography supports exploiting a combination of cation exchange with hydrophobic interaction functionalities can be used.

5 For example, ligands comprising at least one acidic moiety such as a carboxyl group and also comprising at least one hydrophobic moiety such as a phenyl ring or an aliphatic hydrocarbon chain can be used.

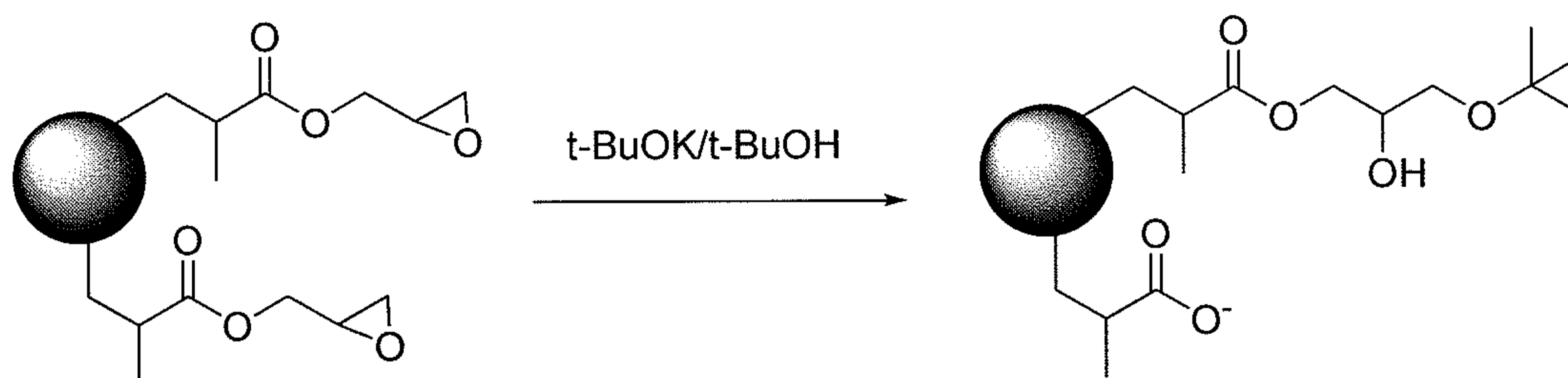
[0065] In some embodiments, phenylalanine is covalently linked to a solid support. For example, the phenylalanine can be covalently linked to the solid support via the amine of  
10 phenylalanine. Phenylalanine can be linked to a solid support, for example, by nucleophilic replacement of a leaving group on the solid support, or by other chemistries known to those skilled in the art.

[0066] The secondary amino on the phenylalanine can be "capped" with an additional moiety to form a secondary amine, thereby preventing or reducing the formation of cationic ammonium (and therefore formation of a zwitterion) at the pH at which the chromatography is performed. In some embodiments, the amine is capped with an acetyl group. Those of  
15 skill in the art will appreciate there are a number of ways to acetylate the amine. In some embodiments, the beads are dried and then exposed to acetyl chloride. In some embodiments the beads are submitted to solvent exchange with acetone, rather than drying, and then  
20 exposed to acetyl chloride.

[0067] In another embodiment, a t-butyl ether derivative of a polymethacrylate is used as the mixed mode ligand. An example of this type of derivative is Macro-Prep t-butyl HIC™, which is commercially available from Bio-Rad, Inc. (Hercules, CA). T-butyl ether derivatives can be formed from polymeric beads comprising (1) glycidyl methacrylate  
25 groups. For example, a fraction of the ester groups on the polymer backbone can be hydrolyzed to carboxylic acid groups while the reaction of t-butoxide proceeds with the epoxide. This is illustrated in the following diagram.



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[0068] Macro-Prep t-Butyl HIC typically contains from 131-266 micromoles carboxyl groups/ml and 25-45 micromoles t-butyl groups/ml resin, though those of skill in the art will appreciate that the total amount and ratio of these two groups can be varied as desired.

5 [0069] In yet other embodiments, an alkyl acid (e.g., a carboxylic acid) is used as a mixed mode ligand. In some embodiments, the alkyl acid is an n-alkyl acid having between 4-10, 4-6, 4-8, 6-8, or 5-7 carbons, e.g., 2, 3, 4, 5, 6, 7, 8 carbons. As discussed in more detail in the examples, hexanoic acid can be used. Alternatively, in some embodiments, acetic acid, butanoic acid, octanoic acid, or decanoic acid are used.

10 [0070] Alkyl acids can be linked to a hydroxy-functionalized solid matrix. Halogenated alkyl acids can react directly with the hydroxy-functionalized solid matrix. For example, a bromoalkyl acid, such as 6-bromohexanoic acid, can be coupled to UNOsphere Diol. Reactions can be performed, e.g., with 1 M NaOH in the presence of excess bromoacid.

15 [0071] In yet another embodiment, Tosoh HIC media, which contains backbone carboxylate, similar to Macro-Prep t-Butyl HIC resin, is treated with NaOH, to form backbone carboxylates, producing a mixed mode similar to Macro-Prep t-Butyl HIC.

[0072] In some embodiments, the mixed mode matrix (i.e., the ligand or solid support) does not include sulfur.

### *Chromatography conditions*

20 [0073] As will be appreciated in the art, load, wash and elution conditions for use in the mixed mode chromatography of the invention will depend on the specific chromatography media/ligands used. Optimally, the antibodies are bound to the mixed mode resin, with only a pH adjustment, and without any adjustment of the antibody cell culture filtrate to minimize conductivity of the sample, in order to optimally bind antibody to the mixed mode resin.

25 [0074] In some embodiments, loading (i.e., binding the antibodies to the matrix), and optionally washing, is performed at a pH below 7, e.g., between 3-7, 4-7, 3-5, 4-5, 4-6, etc. A pH below 7 is of particular use where cationic/hydrophobic mixed mode ligands are



employed. In some embodiments the matrix comprises charged moieties (e.g., negatively charged or positively charged) at a pH below 7.

[0075] In some embodiments, loading (i.e., binding the antibodies to the matrix), and optionally washing, is performed at a pH above 7, e.g., between 7-8, 7-9, etc. A pH at or  
5 above 7 is of particular use where anionic/hydrophobic mixed mode ligands are employed.

[0076] In some embodiments, loading (i.e., binding the antibodies to the matrix), and optionally washing, is performed in the presence of at least 1 M salt (e.g., sodium). In other embodiments, loading (i.e., binding the antibodies to the matrix), and optionally washing is performed in the presence of less than 1 M salt.

10 [0077] In embodiments employing mixed-mode chromatography supports exploiting a combination of cation exchange with hydrophilic interaction functionalities, it can be useful to load a sample onto a column, and subsequently wash the column, at a low pH (e.g., pH 3-5, e.g., 4-5). The desired protein (e.g., antibody) is then eluted by raising the pH, for example, above about pH 6. In some embodiments, the protein is eluted by raising the  
15 pH from the wash condition to a pH of at least 6.5, 7, or more, e.g., to about pH 7.2. In some of these embodiments, the salt concentrations are not substantially changed during the elution step. In some of these embodiments, the salt is maintained at a low concentration (e.g., below about 50 mM, e.g., below 20 mM, e.g., between 0-20 mM, e.g., 10 mM). Exemplary salts and buffer include, e.g., sodium phosphate, sodium acetate, potassium phosphate or  
20 potassium acetate. There are other acidic buffers that can be used for the binding to the mixed mode resin at low pH, including succinate and MES, both of which are compatible with subsequent application to CHT. Citrate could be used, but would have to be removed before application of eluted IgG to CHT.

[0078] Elution conditions resulting in a nearly neutral pH (e.g., pH 6-8, e.g., about pH 7)  
25 and low salt (e.g., 0-50 mM) are of particular use because the solution comprising the desired eluted protein can be subsequently loaded onto a CHT or CFT column or other solid support without substantially changing the solution. This allows for elimination of intervening steps such as dialysis or ultrafiltration that may otherwise be used before a second round of chromatography or other purification. "Substantially change" refers to no or less than 5%  
30 change in pH, salt conditions, and/or conductivity.



### ***B. Support Matrices***

[0079] A variety of support matrices can be used according to the present invention. Generally, the support matrix will be a hydrophilic polymer that allows for linkage of the ligand, optionally via a spacer.

5 [0080] In some embodiments, the base matrix is hydrophilic and in the form of a polymer, e.g. a polymer that is insoluble and more or less swellable in water. Suitable polymers are polyhydroxy polymers, e.g. based on polysaccharides, such as agarose, dextran, cellulose, starch, pullulan, etc. and completely synthetic polymers, such as polyacrylic amide, polymethacrylic amide, poly(hydroxyalkylvinyl ethers), poly(hydroxyalkylacrylates) and  
10 polymethacrylates (e.g. polyglycidylmethacrylate), polyvinyl alcohols and polymers based on styrenes and divinylbenzenes, and copolymers in which two or more of the monomers corresponding to the above-mentioned polymers are included. Polymers, which are soluble in water, may be derivatized to become insoluble, e.g. by cross-linking and by coupling to an insoluble body via adsorption or covalent binding. Hydrophilic groups can be introduced on  
15 hydrophobic polymers (e.g. on copolymers of monovinyl and divinylbenzenes) by polymerisation of monomers exhibiting groups which can be converted to OH, or by hydrophilization of the final polymer, e.g. by adsorption of suitable compounds, such as hydrophilic polymers. In some embodiments, the support is an UNOsphere™ support, a polymer produced from water-soluble hydrophilic monomers (Bio-Rad, Hercules, CA).  
20 Alternatively, the matrix is agarose (GE Sepharose or Sterogene Superflow and Ultraflow).

### ***III. HT/FT chromatography step***

[0081] In some embodiments of the invention, HT or FT chromatography is performed on the antibodies after a previous mixed mode chromatography step that did not involve HT or  
25 FT. In some embodiments, the HT or FT is a ceramic HT or ceramic FT. Commercial examples of ceramic hydroxyapatite include, but are not limited to CHT Type I and CHT Type II. Commercial examples of fluorapatite include, but are not limited to CFT Type I and CFT Type II. Unless specified, CHT and CFT refer to roughly spherical particles of any average diameter, including but not limited to about 10, 20, 40, and 80 microns. The choice  
30 of hydroxyapatite or fluorapatite, the type, and average particle diameter can be determined by the skilled artisan. Other forms of HT are commercially available from, e.g., Bio-Rad, Pall



Corp. and American International Chemical, Inc. A composite gel form of HT referred to as an "ultrogel" is commercially available from Pall Corp.

[0082] A variety of different loading, washing and elution conditions can be used, as desired. In some embodiments, the initial loading conditions are adapted such that the protein (e.g., antibody) eluted from the initial non-HT, non-FT mixed mode step is applied  
5 directly to the HT/FT column.

[0083] Elution can be achieved, for example, by changing the salt conditions in the liquid phase. For example, the salt and/or conductivity of the liquid phase is increased (linearly or step-wise) to a point that which the antibody elutes. Exemplary washing conditions include,  
10 e.g., 10mM phosphate, pH 6.7, with elution achieved by increasing the salt concentration (step-wise or in a linear fashion) (e.g., to 10 mM phosphate, 1.5M NaCl, pH 6.7).

#### *IV. General Chromatography Information*

[0084] In preparation for contacting the antibody preparation with the mixed mode support,  
15 the chemical environment inside the column is typically equilibrated. This is commonly accomplished by flowing an equilibration buffer through the column to establish the appropriate pH; conductivity; identity, molecular weight, and concentration of nonionic organic polymer (discussed further below); and other pertinent variables.

[0085] In some embodiments, the antibody preparation is also equilibrated to conditions  
20 compatible with the column equilibration buffer before the invention can be practiced. This generally consists of adjusting the pH of the antibody preparation

[0086] In some embodiments, after the column and antibody preparation is equilibrated, the antibody preparation may be contacted with the column. The antibody preparation may be applied at a linear flow velocity in the range of, for example, about 50-300 cm/hr.  
25 Appropriate flow velocity can be determined by the skilled artisan.

[0087] In some embodiments, the invention is practiced in a packed bed column, a fluidized/expanded bed column and/or a batch operation where the mixed mode support is mixed with the antibody preparation for a certain time.

[0088] In some embodiments, a mixed mode chromatography support is packed in a  
30 column.



[0089] In some embodiments, the mixed mode support is packed in a column of at least 5 mm internal diameter and a height of at least 25 mm. Such embodiments **are** useful, e.g., for evaluating the effects of various conditions on a particular antibody.

[0090] Another embodiment employs the mixed mode support, packed in a column of any  
5 dimension required to support preparative applications. Column diameter may range from less than 1 cm to more than 1 meter, and column height may range from less than 1 cm to more than 30 cm depending on the requirements of a particular application. Appropriate column dimensions can be determined by the skilled artisan.

[0091] After use, the mixed mode column may optionally be cleaned, sanitized, and stored  
10 in an appropriate agent, and optionally, re-used.

[0092] As desired, the solutions used in one or more of the load, wash or elution steps during the mixed mode and/or HT or FT chromatography step comprise a nonionic organic polymer. Optionally, nonionic polymers are only used during the HT/FT step and not during the prior mixed mode step. Examples of nonionic organic polymers include, but are not  
15 limited to polyethylene glycol (PEG), polypropylene glycol, cellulose, dextran, starch, and polyvinylpyrrolidone.

[0093] The presence of a nonionic organic polymer enhances binding capacity of antibody on HT or FT, thereby enabling higher levels of productivity to be achieved, and expanding the range of methods that may be considered. The presence of nonionic organic polymer also  
20 preferentially enhances the retention of antibody on HT or FT, in comparison to most contaminating proteins, thereby enabling novel selectivity for improved removal of non-antibody proteins. Further, the presence of nonionic organic polymer preferentially enhances retention of aggregated antibody and other very large molecules on HT or FT, in comparison to non-aggregated antibody, thereby enabling novel selectivity and superior separation  
25 performance for large-contaminant removal. PEG provides a general model for behavior of soluble nonionic organic polymers within the context of the invention. Thus, while PEG is discussed in the following text, one should recognize that the information applies equally to other nonionic polymers, including but not limited to those specifically listed herein.

[0094] The invention may be practiced with PEG with an average polymer molecular  
30 weight ranging from about 100 to about 10,000 Daltons. Exemplary PEG includes PEG having an average molecular weight of, e.g., 200, 300, 400, 500, 900, 1000, 1400, 2000, 3300, 4500, 8000, 10000, 14000, etc. In some embodiments, the PEG has an average weight



between 400-1000, 200-1000, 400-2000, or 1000-5000. A wide variety of different PEGs are available from, e.g., Aldrich.

**[0095]** PEG or other organic polymers can be linear or branched polymers.

**[0096]** Lower molecular weight PEGs will generally require a higher concentration to  
5 achieve an effect similar to higher molecular weight PEGs.

**[0097]** Lower concentrations of a given molecular weight of PEG are generally used to enhance the binding of larger antibodies and fusion proteins compared to concentrations of PEG resulting in the same amount of enhanced binding of smaller proteins. For example, IgM, with an approximate molecular weight of about 960kD, will generally require a lower  
10 concentration of PEG to achieve a certain degree of binding enhancement than IgG, with an approximate molecular weight of 160kD. Retention of aggregates, complexes, and other large molecule contaminants will generally be enhanced to a greater degree than the unaggregated forms of the proteins from which they are derived.

**[0098]** Lower concentrations of PEG will be generally required to enhance the binding of  
15 molecules that are strongly retained by the mixed mode chromatography support, compared to the concentration for PEG to achieve the same enhanced binding for molecules that are weakly retained.

**[0099]** The effects described in the two preceding paragraphs will generally be compound: the retention of large molecules that are strongly retained in the absence of nonionic organic  
20 polymer will be enhanced more by application of the invention than molecules that are smaller and weakly retained, smaller and strongly retained, or larger and weakly retained.

**[0100]** In some embodiments, PEG with an average molecular weight of about 6,000 Daltons is employed in a concentration range from 0.0-7.5% to separate intact IgG from aggregated forms.

**[0101]** In some embodiments, PEG with an average molecular weight of about 2,000 Daltons is employed in a concentration range from 0.0-15.0% to separate intact IgG from aggregated forms.

**[0102]** The identity, appropriate average molecular weight, and concentration of the organic polymer to practice the invention can be determined by the skilled artisan.



## V. *Optional additional steps*

[0103] The present invention may be combined with other purification methods to achieve higher levels of purification. Examples include, but are not limited to, other methods commonly used for purification of antibodies, such as protein A and other forms of affinity chromatography, anion exchange chromatography, cation exchange chromatography, hydrophobic interaction chromatography, immobilized metal affinity chromatography, and additional mixed mode chromatography methods. Other options, include, but are not limited to precipitation, crystallization, and/or liquid partitioning methods.

## 10 VI. *Apparatus*

[0104] The present invention provides apparatuses that are useful for carrying out the methods of the invention. For example, the present invention provides an apparatus for purifying antibodies, the apparatus comprising, a first column tube (or cartridge) and a second column tube (or cartridge), wherein the first and second column tubes comprise inlets and outlets, wherein the outlet of the first column tube connects to the inlet of the second column tube (either directly or indirectly through, e.g., a connector tube) such that the first column tube is in fluid communication with the second column tube, wherein the first column tube contains a mixed mode chromatography matrix and the second column tube contains a solid support matrix, the solid support matrix comprising hydroxyapatite (HT) or fluorapatite (FT). An apparatus with this configuration optionally allows for uninterrupted flow from the initial mixed mode chromatography column through top the HT/FT column. Optionally, various inlets and outlets can be included to allow for changing conditions (e.g., wash and elution solutions can be added via the inlets, test samples can be collected from outlets).

[0105] The apparatus can include any mixed mode support or ligands without limitation, e.g., as discussed above with respect to the method.

## VII. *Kits*

[0106] Kits for use in purification of proteins (e.g., antibodies) are also provided. In some embodiments, the kits comprise: a mixed mode chromatography matrix; and/or a solid support matrix, the solid support matrix comprising hydroxyapatite (HT) or fluorapatite (FT).



The above described matrices can be provided in the form of a reagent that can be packed into column(s) or can be packaged such that they can be packed in a separate column. Optionally, the kits will include separate columns to be packed at a time chosen by the user.

5 [0107] In some embodiments, the kits comprise a first column tube or cartridge comprising the mixed mode chromatography matrix; and a second column tube or cartridge comprising the solid support matrix. In some embodiments, the first and second column tubes comprise inlets and outlets, wherein the outlet of the first column tube can be connected to the inlet of the second column tube such that the first column tube is in fluid communication with the second column tube when connected the two tubes are connected.

10 [0108] In addition, the kits may include instructional materials containing directions (i.e., protocols) for the practice of the methods of this invention. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (e.g.,  
15 magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

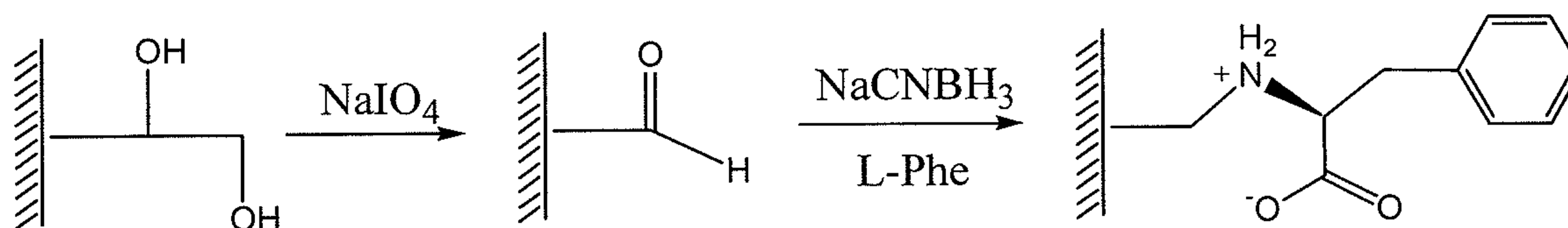
## EXAMPLES

### *Example 1: Production Of Phenylalanine Ligand Column*

20 [0109] UNOsphere chromatography media are based on water-soluble, hydrophilic acrylamido and vinylic monomers that are polymerized in the presence of porogens, to yield spherical particles (US 6,423,666). UNOsphere Diol is a polymer produced with pendent diol groups, which can be used to produce a variety of functional media for use in lab-scale and process chromatography. UNOsphere Phenylalanine mixed mode resin was made from  
25 UNOsphere Diol base bead in two steps: (a) oxidation of UNOsphere Diol to UNOsphere Aldehyde; and (b) coupling of phenylalanine onto UNOsphere matrix via reductive amination. The amino group of phenylalanine becomes secondary after the reductive amination reaction. If needed, this secondary amino group can be acetylated to eliminate its protonation at acidic pHs. Coupling level of phenylalanine ligand is determined by acid-base  
30 titration.

[0110] A general scheme of the procedure is shown below:





### Detailed Procedures

- 5 [0111] Oxidation of UNOsphere Diol to UNOsphere Aldehyde. Wash 40 ml settled UNOsphere Diol resin with 400 ml x 4 deionized water in a column. Transfer washed resin cake into a 250 ml polyethylene bottle. Add 100 ml of 0.2 M NaIO<sub>4</sub> solution into the bottle and mix the content head-to-head at ambient temperature with a rotator. After 1 hr of mixing, wash the resin with 400 ml x 4 deionized water in a column.
- 10 [0112] Coupling of L-Phenylalanine onto UNOsphere Aldehyde via reductive amination. Dissolve 0.49 g of L-Phenylalanine in 25 ml of PBS at 70 °C. Let the L-Phenylalanine solution cool to ambient temperature. Suspend 10 ml of UNOsphere Aldehyde resin cake in the L-Phenylalanine solution. Add 125 mg of NaCNBH<sub>3</sub> into the suspension and gently mix the suspension head-to-head overnight at ambient temperature with a rotator. Wash resin with 100 ml x 4 deionized water in a column.
- 15 [0113] Determine ligand content. L-Phenylalanine ligand content can be determined using an acid-base titration method. Acidify 10 ml UNOsphere L-Phenylalanine resin with 1.0 N HCl, and then wash the resin with 100 ml x 4 deionized water. Titrate the acidified resin with 0.10 N NaOH in a 0.20 ml increment until pH reaches 11.4. Repeat this process for 10 ml UNOsphere Diol resin. Use volume difference of titrant, 0.10 N NaOH, for these two resins to calculate ligand content. The ligand content is about 0.043 mmol of L-Phenylalanine per ml of resin.
- 20 [0114] Acetylation of secondary amino group. Wash 10 ml UNOsphere L-Phenylalanine resin from (c) with 100 ml x 4 deionized water and 50 ml MeOH. Dry the resin in a vacuum oven at 60 °C under house vacuum overnight. Cool resin to ambient temperature and suspend it into 25 ml acetone (dried over molecular sieves). Cool the suspension to -10 °C and add slowly 78 µl diisopropylethylamine, followed by 32 µl acetyl chloride. Gently stir the reaction mixture at -5 to -10 °C for 35 minutes. Wash the resin with 50 ml acetone, 50 ml
- 30 MeOH, and 100 ml x 3 deionized water.

***Example 2: Phenylalanine Ligand - Cation Exchange Mode***

[0115] One mode of the invention can be referred to as “cation exchange mode” because the phenylalanine column is operated by exploiting the carboxylic acid group as a cation exchanger (though the hydrophobic portion of the phenylalanine continues to contribute to antibody interaction). The hydrophobic nature of the ligand allows good binding capacity (IgG uptake) by the media in the presence of higher amounts of salt (via a hydrophobic effect) than would usually be tolerated by traditional hydrophilic cation exchangers.

[0116] This specific example has been performed under the following conditions: 1 mL of phenylalanine UNOsphere was packed in a 5 mm X 5 cm column and equilibrated with 5 column volumes (CV) 0.05M acetate buffer at pH 4.5, at a linear flow rate of 300 cm/hr. 15 mL of clarified cell culture supernatant containing monoclonal chimeric IgG at about 1 mg/mL was titrated to pH 4.7 by gradual addition of 0.1M acetic acid, then applied to the column. The column was washed with equilibration buffer until the UV profile returned to baseline. It was then washed with 5CV of 0.05M acetate, 1M NaCl, pH 4.5, then restored to equilibration buffer. The column was then eluted with a 15CV linear gradient to 10 mM sodium phosphate buffer, pH 7.2. Results of the elution are shown in Figure 1.

[0117] After initial fractionation on phenylalanine UNOsphere in either cation exchange or hydrophobic interaction mode, the eluted IgG was loaded onto hydroxyapatite (CHT type 1 40 micron) and eluted in a sodium chloride gradient as described in numerous recent Bio-Rad publications. This increases the purity of the antibody, particularly removing antibody aggregates, DNA, endotoxin, and virus. Conditions for the CHT chromatography are as follows.

CHT type 1, 40  $\mu$ m. 1 mL 5 mm x 5 cm, 300 cm/hr.

Buffer A: 10mM sodium phosphate, pH 6.7.

25 Buffer B: 10 mM sodium phosphate, 1.5M NaCl, pH 6.7.

Buffer C: 500 mM sodium phosphate, pH 7.0.

[0118] The column was equilibrated with buffer A. The antibody eluate from the UNOsphere phenylalanine chromatography was loaded and then washed with buffer A. Elution was achieved with a 30 CV linear gradient to buffer B. The column was then cleaned with buffer C. A chromatogram of the elution is shown in Figure 2.



[0119] Samples at various stages of purification were loaded and analyzed on SDS-PAGE to monitor the purification process (Figure 3).

[0120] Lane 4 represents the unpurified cell culture supernatant that was applied to the Phenylalanine column.

5 [0121] Lane 9 represents material that did not bind to the column when it was applied to the column. This would be material that corresponds to the peak running from about 15-45 mL on Figure 1. The absence of a band corresponding to antibody heavy chain indicates that antibody is effectively bound by the column. A band corresponding roughly with antibody light chain suggests that the column supports selective removal of light chain.

10 [0122] Lane 5 represents later-eluting unbound material corresponding to the peak that occurs from about 45-53 mL on Figure 1. This material is dominated by a band corresponding roughly with antibody light chain, and demonstrates that the column has a weak affinity for light chain, sufficient to retard its passage through the column, but not sufficient to bind it. This demonstrates that the column can be used for separation of free  
15 light chain and intact antibody, and suggests potential applications for purification of antibody fragments.

[0123] Lane 10 represents material eluted from phenylalanine resin in a high salt wash step, corresponding to about 53-65 mL on Figure 1. The presence of heavy chain suggests some loss of antibody, but the dominance of light chain indicates that most of the loss is not intact  
20 antibody.

[0124] Lane 1 represents the leading shoulder on the elution peak, corresponding to about 80-85 mL on the Figure. It contains a trace of light chain but no significant concentration of intact antibody.

[0125] Lane 6 represents the main elution peak from phenylalanine resin, corresponding to  
25 about 85-92 mL on Figure 1. It is dominated by the two peaks representing antibody heavy and light chain in proportions indicating intact antibody, which dominates the composition of the fraction overall. In combination with the chromatogram (Figure 1), this shows that the column is effective in removing the majority of contaminants. The difference in composition between the two elution fractions (Lanes 5 and 6) suggest that elution conditions could be  
30 optimized to provide even higher purity. The column fraction corresponding to Lane 6 was carried forward to the CHT (hydroxyapatite step).

[0126] Lane 11 represents material that did not bind to the CHT column. This corresponds to the part of the chromatogram from about 205-220 mL (Figure 2). Antibody content is nil, indicating good elimination of contaminants.

5 [0127] Lane 2 represents the primary elution peak from CHT, corresponding to about 232-240 mL. This peak typically corresponds with non-aggregated antibody, suggesting the utility of the combined techniques (phenylalanine and CHT) for supporting a high degree of purification, selective fractionation of antibody fragments from intact antibody, and selective fractionation of antibody aggregates from (intact) non-aggregated antibody.

10 [0128] Lane 7 represents the trailing peak from CHT, corresponding to about 245-260 mL. This peak typically corresponds with antibody aggregates when antibodies are fractionated under these conditions.

15 [0129] Lane 12 represents the cleaning peak from CHT, corresponding to about 26-264 mL on Figure 2. The fraction is dominated by contaminants but appears to contain a small amount of antibody. Antibody occurring in this fraction is typically revealed to be highly aggregated.

[0130] Lanes 3 and 8 are blank.

### ***Example 3: Effect of capping of Phenylalanine Ligand***

20 [0131] Purified immunoglobulin binding and elution was examined for UNOsphere Phenylalanine where the amino of the phenylalanine was, or was not, acetylated. Figures 4-5 shows that only a very small amount of IgG bound and eluted from non-acetylated UNOsphere Phenylalanine (Figure 4) compared with that bound and eluted from acetylated UNOsphere Phenylalanine (Figure 5) The top chromatogram in Figure 5 represents UNOsphere Phenylalanine first solvent exchanged from water to acetone and then treated  
25 with acetyl chloride. The bottom chromatogram in Figure 5 represents UNOsphere Phenylalanine in which the beads were vacuum dried and then acetylated with acetyl chloride.



***Example 4: Phenylalanine Ligand - Hydrophobic Interaction Mode***

[0132] Another mode of the invention can be referred to as “hydrophobic interaction mode” because the phenylalanine column is operated by exploiting the phenyl group as a hydrophobic interaction group. This is illustrated in Figure 6. In this experiment, the  
5 (Protein A-purified) antibody was loaded onto the UNOsphere Phenylalanine column in the presence of 4M NaCl. As the salt was washed out of the column (red trace), a pH gradient was initiated from 4.5-7.5 (light grey trace), which eluted the bound antibody (blue trace).

***Example 5: T-Butyl Ester Derivative Of Polymethacrylate***

10 [0133] Macro-Prep epoxide base polymer is a polymethacrylate polymer produced from glycidyl methacrylate and diethylene glycol dimethacrylate crosslinker. Macro-Prep epoxide base bead does not contain a hydrophobic group; the hydrophobic group is introduced into the polymer in a post-polymerization reaction. Macro-Prep t-Butyl HIC resin is a mixed mode resin, produced by reaction of t-butanol and potassium t-butoxide with Macro-Prep  
15 Epoxide. During the t-butyl derivatization reaction, a small fraction of the ester groups on the polymer backbone are hydrolyzed to carboxylic acid groups, while t-butanol reacts with the pendent epoxide groups. Macro-Prep t-Butyl HIC typically contains from 131-266 micromoles carboxyl groups/ml and 25-45 micromoles t-butyl groups/ml resin. Unlike the integrated mixed mode ligand approach that we demonstrated using phenylalanine, the  
20 carboxyl groups are separated from the hydrophobic groups in Macro-Prep t-Butyl HIC. Macro-Prep t-Butyl HIC polymer is sufficiently porous to bind larger proteins, including immunoglobulins.

[0134] A monoclonal antibody cell culture filtrate was purified on the Macro Prep t-Butyl HIC resin under the following conditions. Chromatography conditions: T-Butyl HR 10/10 (8  
25 mL of Macro-Prep t-Butyl HIC resin was packed in a 10mm X 10cm chromatography column). The column was equilibrated with 25 mM acetate, pH 4.5, ~0.3mS/cm 4 mL/min (300 cm/hr). To prepare the sample, it was diluted 1:1 with 25 mM acetate, titrated to pH 4.75, conductivity at about 11 mS/cm. A 100 mL sample was loaded and the column was subsequently washed with 25 mM acetate, pH 4.5. The flow rate was then reduced to 150  
30 cm/hr (2 mL/min) and antibody was eluted with ten column volumes in a linear gradient to 10mM citrate, 10mM phosphate, pH ~7.5. (cond. about 4.6mS/cm). Following elution, the column was stripped with 1M NaCl, then 1 M NaOH.

[0135] A chromatogram illustrating chromatography of monoclonal antibody cell culture filtrate on Macro-Prep t-Butyl HIC is shown in Figure 7. Fractions from the chromatograph were analyzed by SDS PAGE (Figure 8). The antibody was efficiently captured on Macro-Prep t-Butyl HIC (no antibody bands in the unbound or wash fractions). Antibody eluted by pH gradient was well-purified and concentrated from the cell culture filtrate, without non-specific losses (no antibody detected in column strip fractions). The polyacrylamide gel electrophoresis pattern of the fractions (Figure 8) is similar to that observed for the eluate from the phenylalanine column described above. These results on Macro-Prep t-Butyl HIC demonstrate that an integrated mixed mode ligand is not required for our mixed mode/CHT antibody purification method.

10 [0136] Based on our work described in examples above, chromatographic polishing of the pH-eluted antibody fraction from Macro-Prep t-Butyl HIC can be chromatographed on CHT, similar to the antibody pool eluted from phenylalanine mixed mode media, to further remove minor protein contaminants, antibody aggregates, DNA, and endotoxin.

15 *Example 6: Hexanoic Acid Ligand*

[0137] Alkyl acids and amino alkyl acids were compared for their ability to purify antibodies. Aminocaproic acid was coupled to UNOsphere Diol by reductive amination (similar reaction to the phenylalanine coupling). No IgG binding was detected in our purified IgG uptake assay (Figure 9). Acetylation of this material demonstrated only a slight uptake and release of purified IgG in our assay (Figure 10). By contrast, coupling bromohexanoic acid to UNOsphere Diol provides IgG binding with a sharply eluted peak upon pH shift (Figure 11). When bromohexanoic acid was coupled similarly to agarose (Sterogene Ultraflow, Figure 12) or to GE agarose (Sepharose™ 4B, Figure 13), Sterogene agarose gave a profile very similar to that of UNOsphere hexanoic acid, while Sepharose™ 4B showed much higher IgG binding with pH-based elution.

25 [0138] There are subtle differences between aminocaproic acid and bromohexanoic acid [-HN(CH<sub>2</sub>)<sub>5</sub>COOH] vs. [-(CH<sub>2</sub>)<sub>5</sub>COOH], when coupled to hydroxyl groups on polymers, which result in significant differences in IgG binding properties. This effect was not anticipated, based on previous work. Lihme (US6,498,236), used a variety of activated spacer groups including epichlorohydrin, allyl glycidyl ether, bis-epoxides and glutaric dialdehyde. Burton (US5,652,348) used a variety of activated groups for coupling amines. Some produced uncharged amide linkages, but others including epoxide,

30



cyanogen bromide, tosyl, divinylsulfone produced a secondary amine derivative which would give a positive charge at acidic pH's. Without intending to be bound by any theory of operation, we hypothesize the difference is due to protonation of the secondary amine nitrogen, forming a zwitterion at low pH, which would repel the (positively charged) IgG molecule from the bead at acidic pH's, conditions where the carboxyl group is largely protonated (and uncharged). Acetylation or other "capping" of the amine nitrogen would eliminate the ability to protonate the amine to form a zwitterion, for both aminocaproic acid and phenylalanine.

***Example 7: Additional mixed mode matrix***

10 [0139] An UNOsphere Diol polymer with a carboxyl group in the backbone of the polymer was produced, then derivatized this bead with phenyl groups, to create a material with separate hydrophobic and ionic groups, somewhat like the Macro-Prep t-Butyl HIC polymer. Figure 14 is a purified IgG binding chromatogram for this material. The elution profile of the IgG is slightly different from that of media containing a single ligand comprised of both the weak acid and hydrophobic group. For this 15 media, where the acid and hydrophobic groups are not necessarily in close proximity, there is a broader elution profile for IgG, beginning even before the pH gradient trace rises. For comparison, the IgG binding/elution profile for Macro-Prep t-Butyl HIC is shown in Figure 15.

[0140] Many modifications and variations of this invention can be made without departing from its scope, as will be apparent to those skilled in the art. The specific embodiments described herein are 20 offered by way of example only and are not meant to be limiting in any way. It is intended that the specification and examples be considered as exemplary only, with the scope of the invention being indicated by the following claims.

WHAT IS CLAIMED IS:

1. A method for purifying antibodies from a solution containing antibodies, the method comprising
  - a. contacting a first solution containing antibodies to a mixed mode chromatography matrix, wherein the matrix comprises at least one hydrophobic moiety and at least one acidic moiety and does not include sulfur, thereby binding at least a portion of the antibodies to the matrix;
  - b. separating the matrix with bound antibodies from the first solution;
  - c. eluting the bound antibodies by raising the pH of solution in contact with the matrix and bound antibodies, thereby producing a solution comprising eluted antibodies;
  - d. contacting the solution comprising eluted antibodies of step c to a solid support matrix, the solid support matrix comprising hydroxyapatite (HT) or fluorapatite (FT), wherein at least a portion of the antibodies bind to the solid support matrix;
  - e. separating the solid support matrix and bound antibodies from the second solution; and
  - f. eluting the bound antibodies from the solid support matrix, thereby purifying the antibodies from the solution.
2. The method of claim 1, wherein the contacting step a comprises binding the antibodies to the matrix at a pH below 7.
3. The method of claim 2, wherein the matrix comprises charged moieties at a pH below 7.
4. The method of claim 1, wherein the contacting step a comprises binding the antibodies to the matrix in the presence of at least 1 M salt.
5. The method of claim 1, wherein the contacting step a comprises binding the antibodies to the matrix in the presence of 1 M or less salt.



6. The method of claim 1, wherein the eluting step (c) comprises raising the pH from below pH 7 to a pH above pH 7.

7. The method of claim 1, wherein the mixed mode chromatography matrix comprises a mixed mode ligand comprising an alkyl acid comprising between 4-10 carbons.

8. The method of claim 1, wherein the mixed mode chromatography matrix comprises a mixed mode ligand comprising hexanoic acid.

9. The method of claim 1, wherein the mixed mode chromatography matrix comprises a mixed mode ligand comprising phenylalanine.

10. The method of claim 9, wherein the amine of phenylalanine is acetylated.

11. The method of claim 1, wherein the mixed mode chromatography matrix comprises a mixed mode ligand comprises a t-butyl ester derivative of polymethacrylate.

12. The method of claim 1, further comprising an anion exchange chromatography step, a cation exchange chromatography step, a hydrophobic chromatography step, an ultrafiltration step, precipitation step, crystallization step, or liquid:liquid phase partitioning.

13. The method of claim 1, wherein the eluting step c comprises increasing the pH of the solution in contact with the matrix and bound antibodies.

14. The method of claim 1, wherein the solid support matrix comprises ceramic hydroxyapatite (CHT).

15. The method of claim 1, wherein the solid support matrix comprises ceramic fluorapatite (CFT).

16. A kit for purifying antibodies, the kit comprising,

a mixed mode chromatography matrix, wherein the matrix comprises at least one hydrophobic moiety and at least one acidic moiety and does not include sulfur; and

a solid support matrix, the solid support matrix comprising hydroxyapatite (HT) or fluorapatite (FT).

17. The kit of claim 16, wherein the mixed mode chromatography matrix comprises a mixed mode ligand comprising an alkyl acid comprising between 4-10 carbons.

18. The kit of claim 16, wherein the mixed mode chromatography matrix comprises a mixed mode ligand comprising hexanoic acid.

19. The kit of claim 16, wherein the mixed mode chromatography matrix comprises a mixed mode ligand comprising phenylalanine.

20. The kit of claim 19, wherein the amine of phenylalanine is acetylated.

21. The kit of claim 16, wherein the mixed mode chromatography matrix comprises a mixed mode ligand comprises a t-butyl ester derivative of polymethacrylate.

22. The kit of claim 16, wherein the solid support matrix comprises ceramic hydroxyapatite (CHT).

23. The kit of claim 16, wherein the solid support matrix comprises ceramic fluoroxyapatite (CFT).

24. A method for purifying antibodies from a solution containing antibodies, the method comprising

a. contacting a first solution containing antibodies to a mixed mode chromatography matrix, wherein the mixed mode chromatography matrix comprises phenylalanine, an n-alkyl acid comprising 2-10 carbons, or a t-butyl ether derivative of polymethacrylate, thereby binding at least a portion of the antibodies to the matrix;

b. separating the matrix with bound antibodies from the first solution;



c. eluting the bound antibodies by changing the pH or salt concentration of solution in contact with the matrix and bound antibodies, thereby producing a solution comprising eluted antibodies and purifying antibodies from a solution containing antibodies.

25. The method of claim 24, wherein the mixed mode chromatography matrix comprises phenylalanine.

26. The method of claim 25, wherein the amine of phenylalanine is acetylated.

27. The method of claim 24, wherein the mixed mode chromatography matrix comprises an n-alkyl acid comprising 2-10 carbons.

28. The method of claim 27, wherein the n-alkyl acid is selected from the group consisting of butanoic acid, hexanoic acid, octanoic acid, and decanoic acid.

29. The method of claim 24, wherein the mixed mode chromatography matrix comprises a t-butyl ether derivative of polymethacrylate.

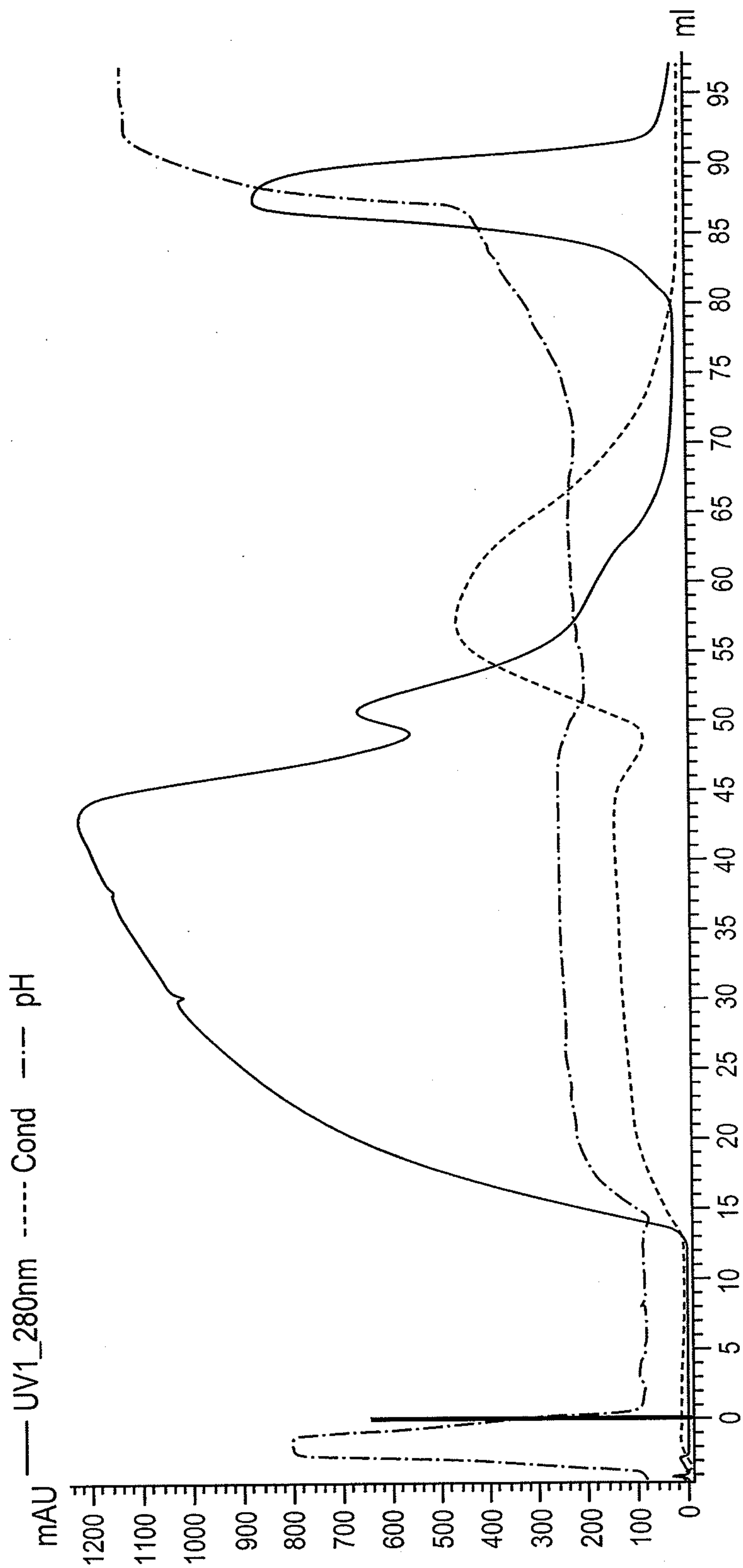


FIG. 1



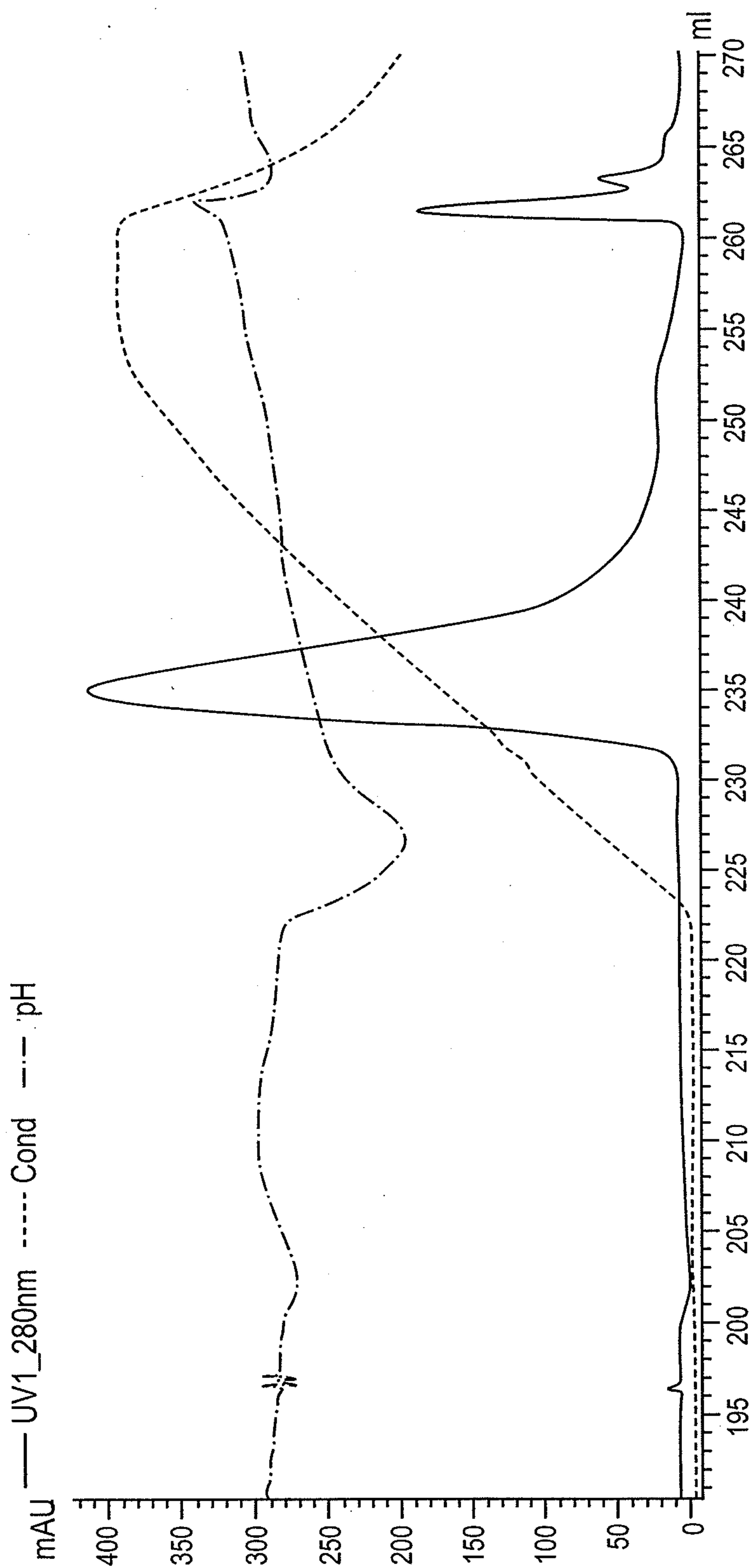
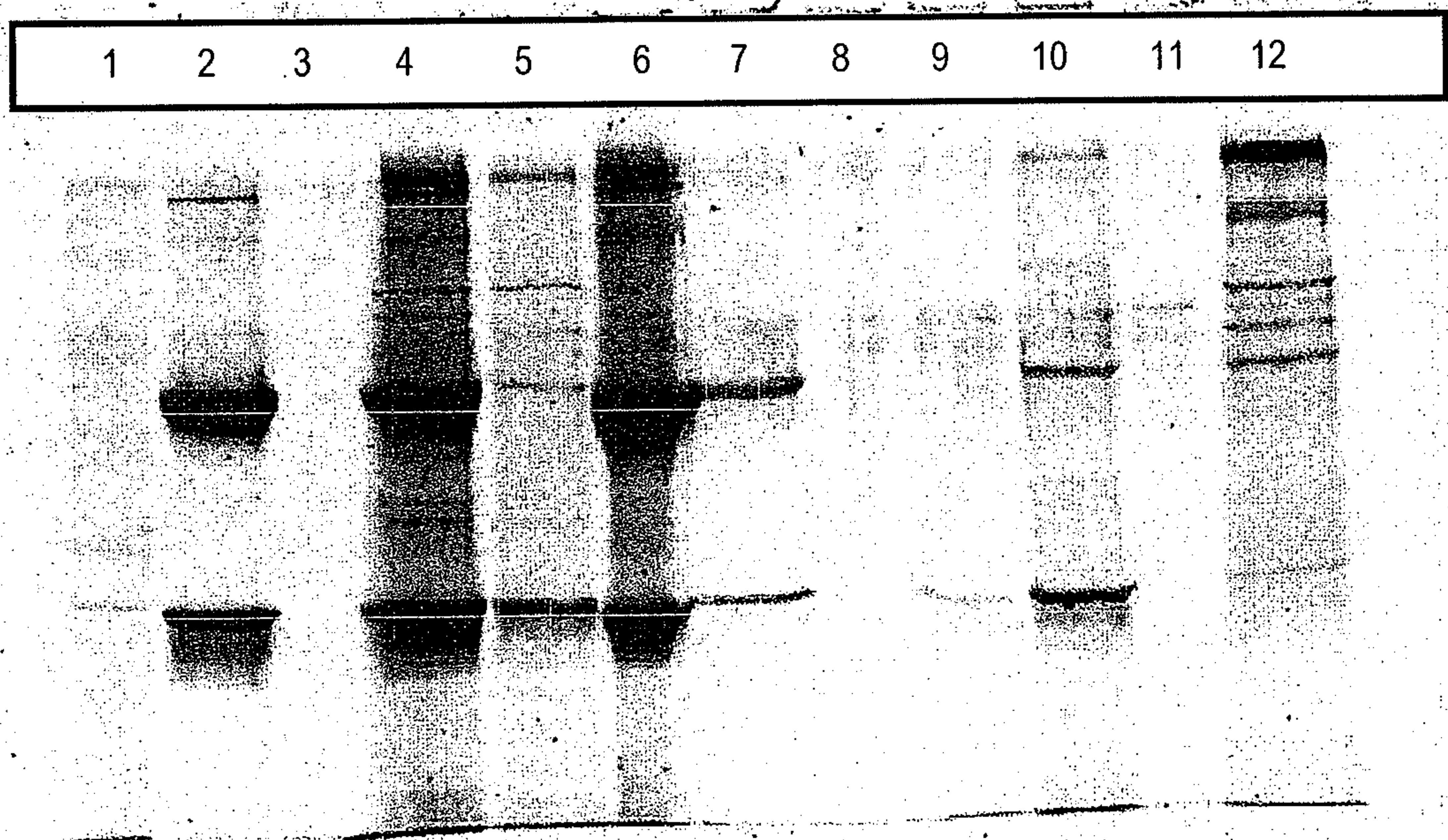


FIG. 2



**FIG. 3**



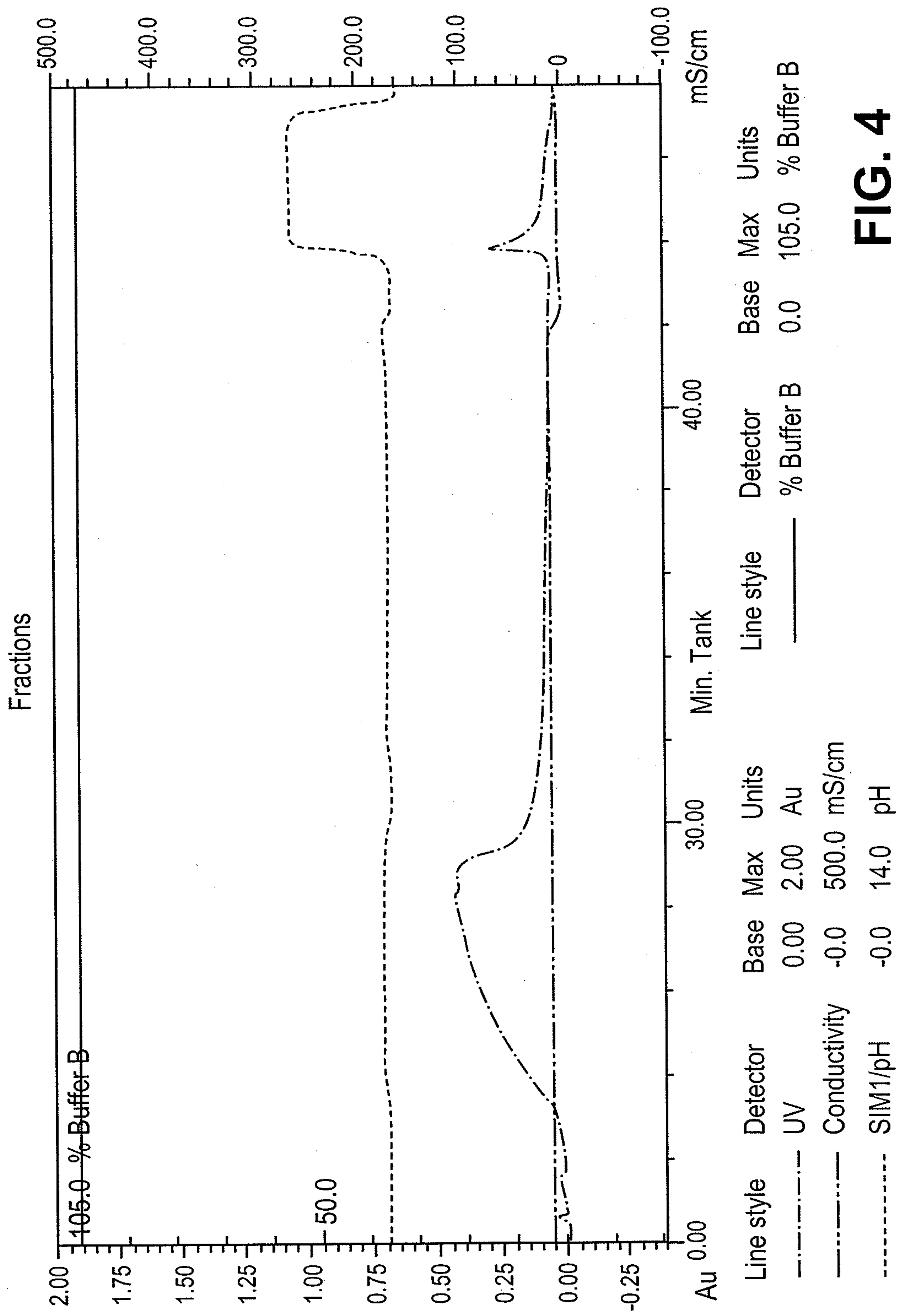
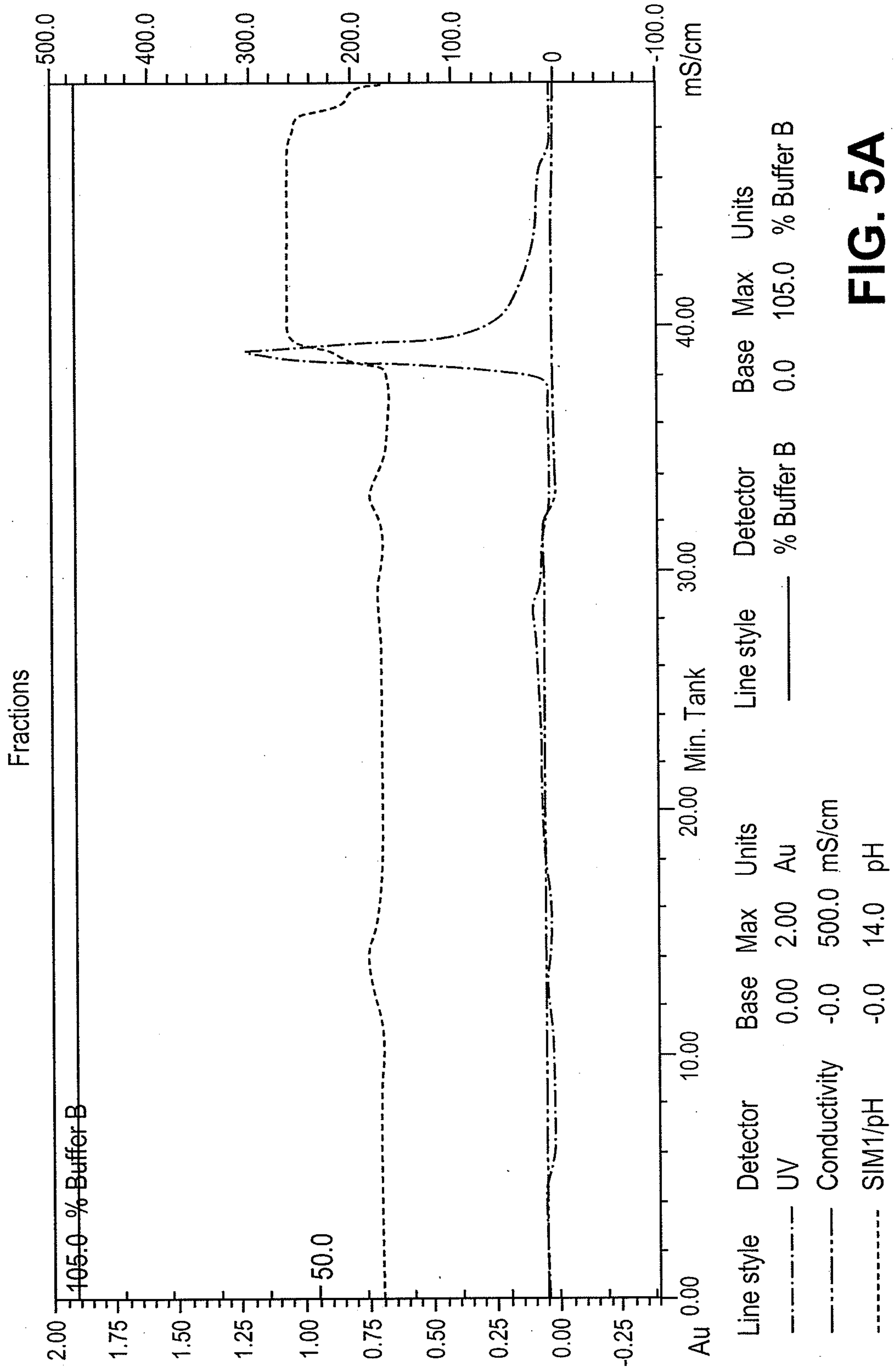
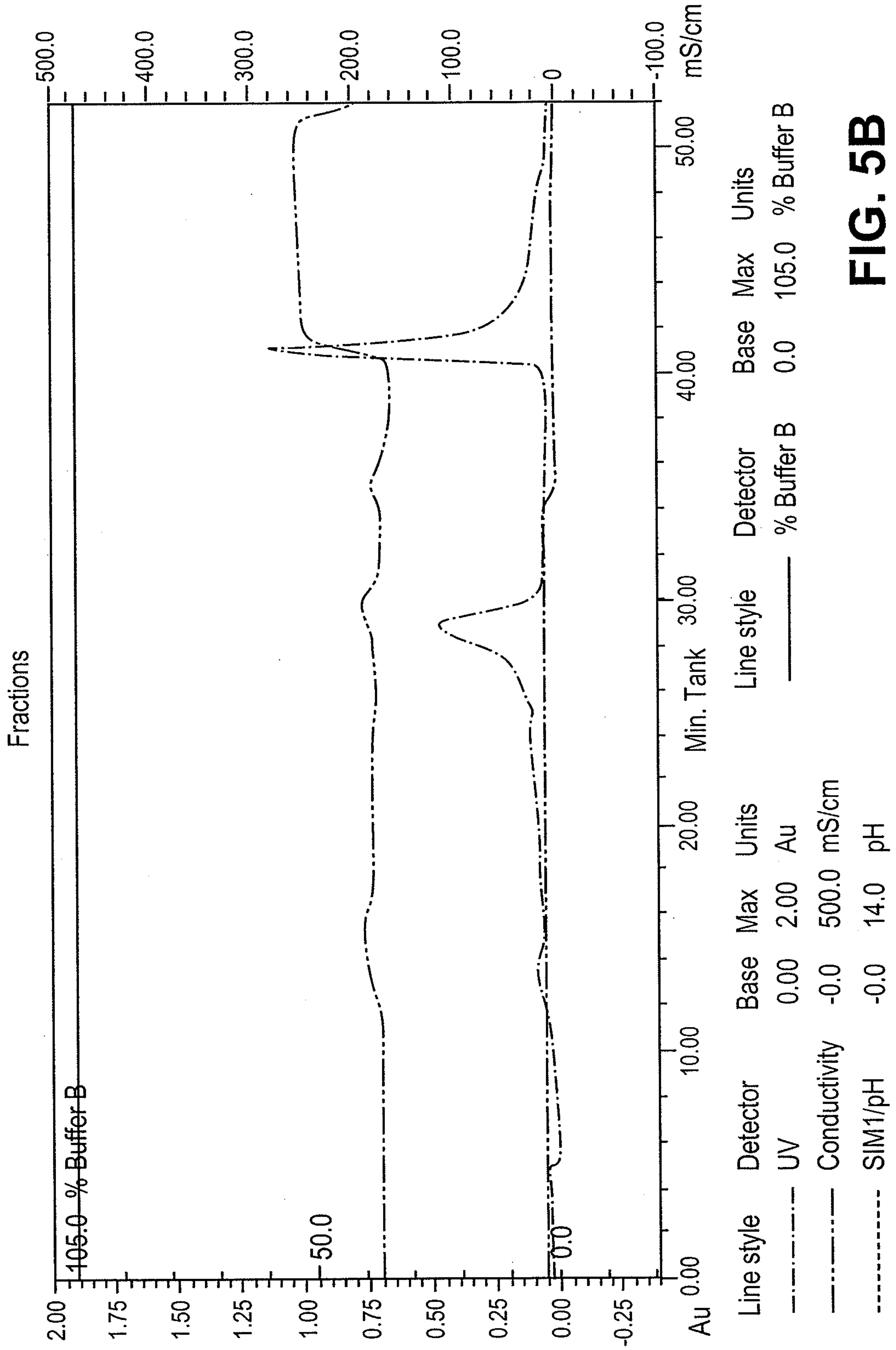


FIG. 4



**FIG. 5A**





**FIG. 5B**

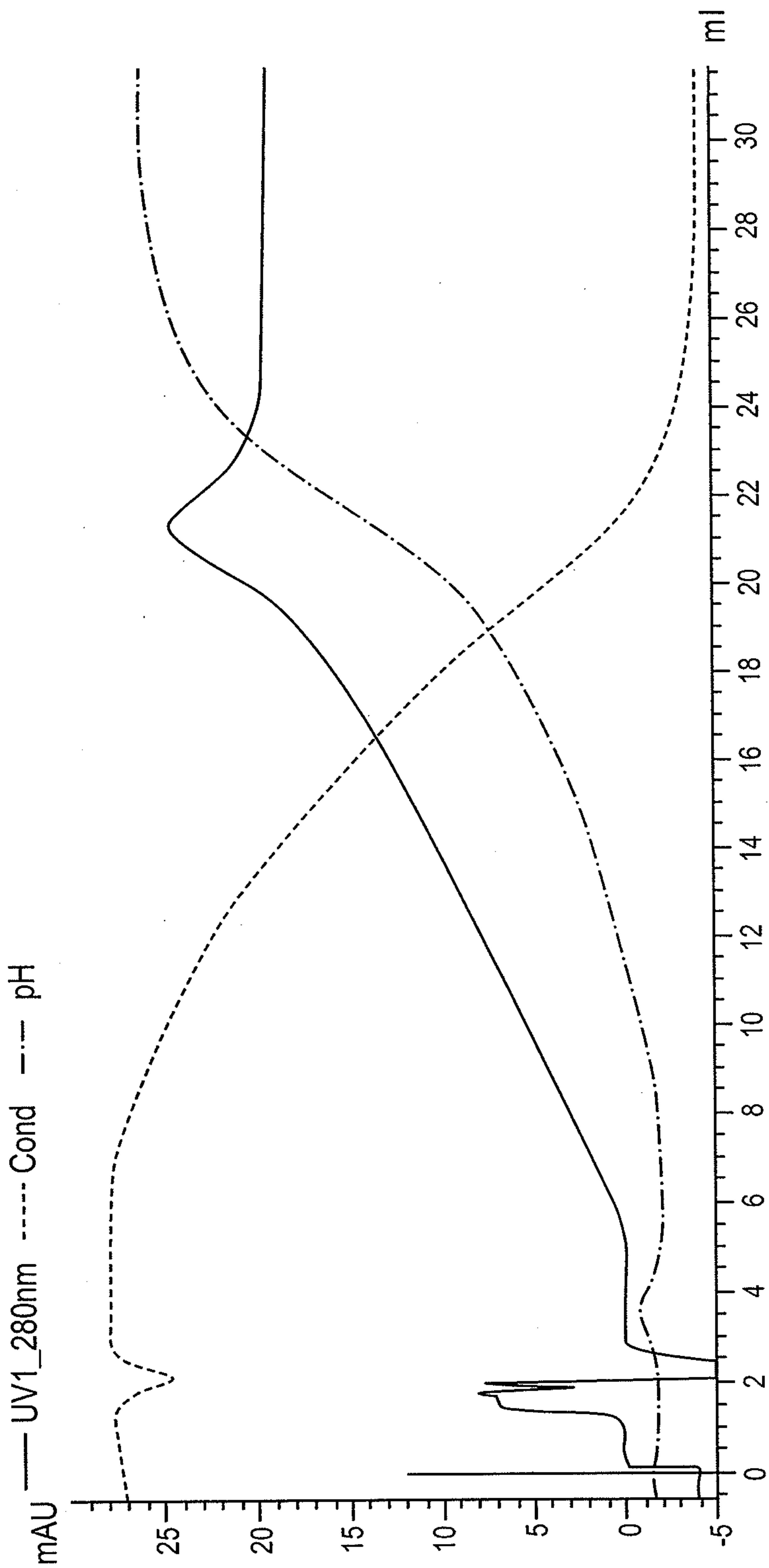


FIG. 6



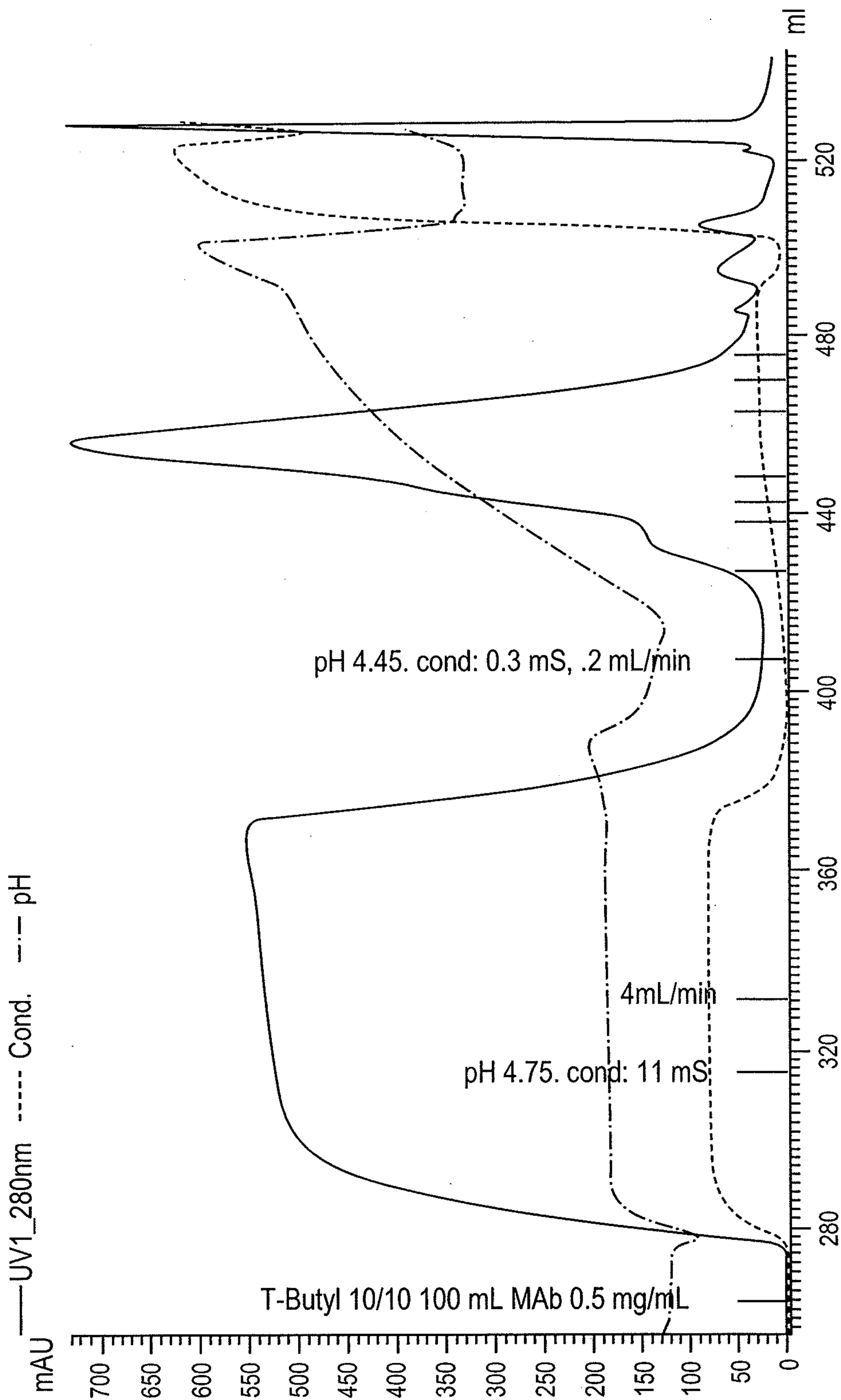
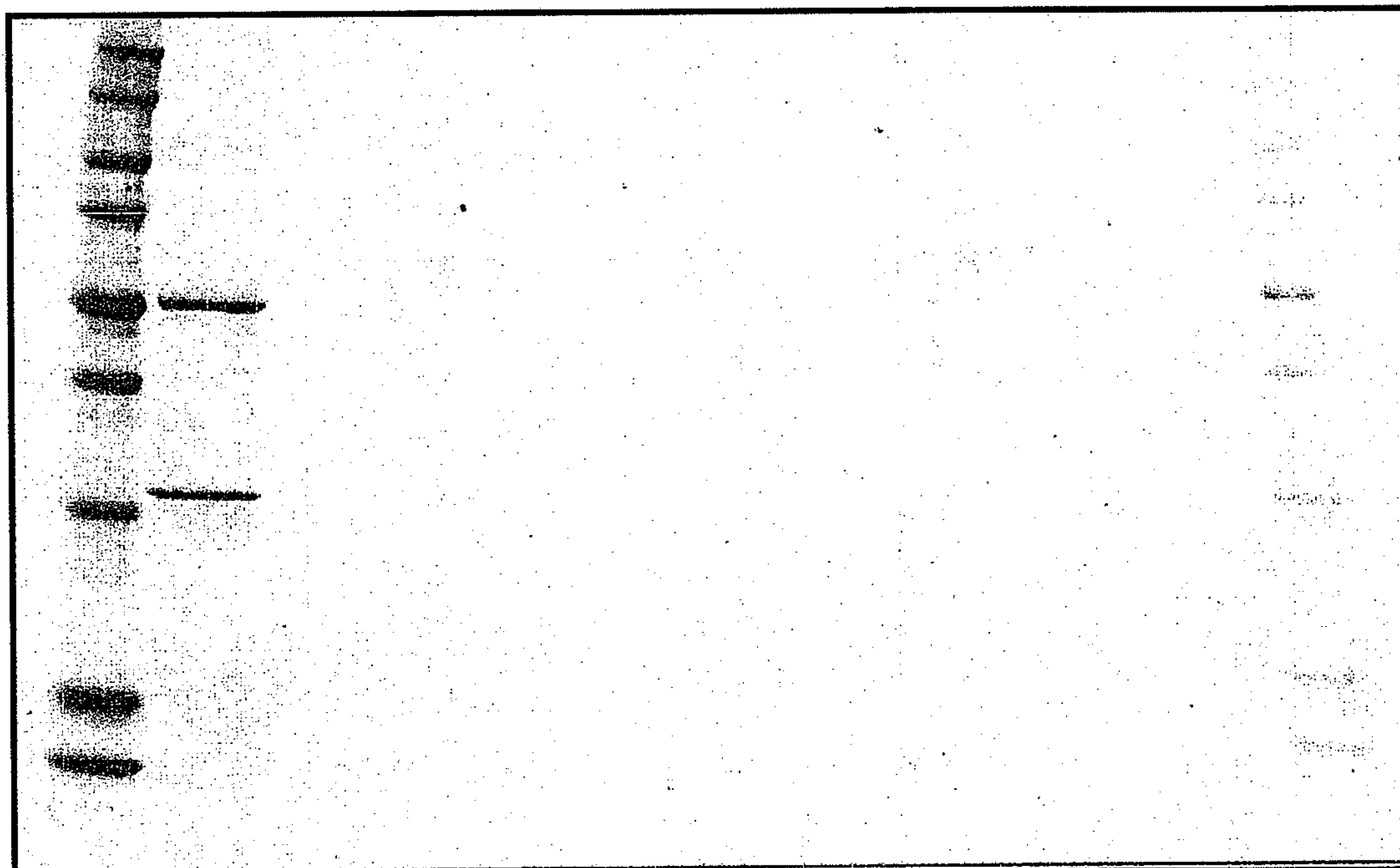


FIG. 7

Polyacrylamide gel electrophoresis analysis of Macro-Prep t-Butyl H1C fractions

Gel 1: 1 2 3 4 5 6 7 8 9 10 11 12 13



Gel 2: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

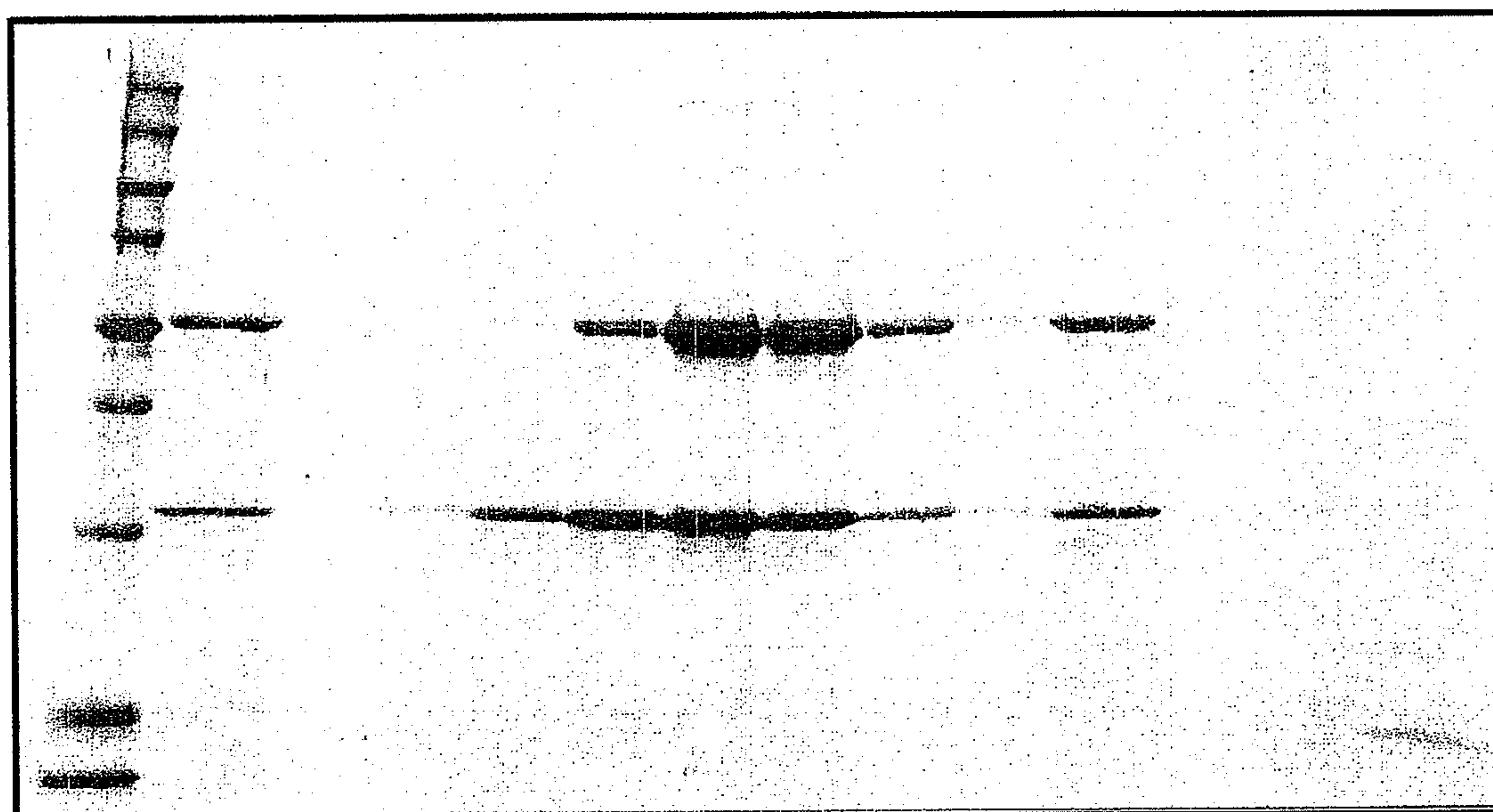


FIG. 8



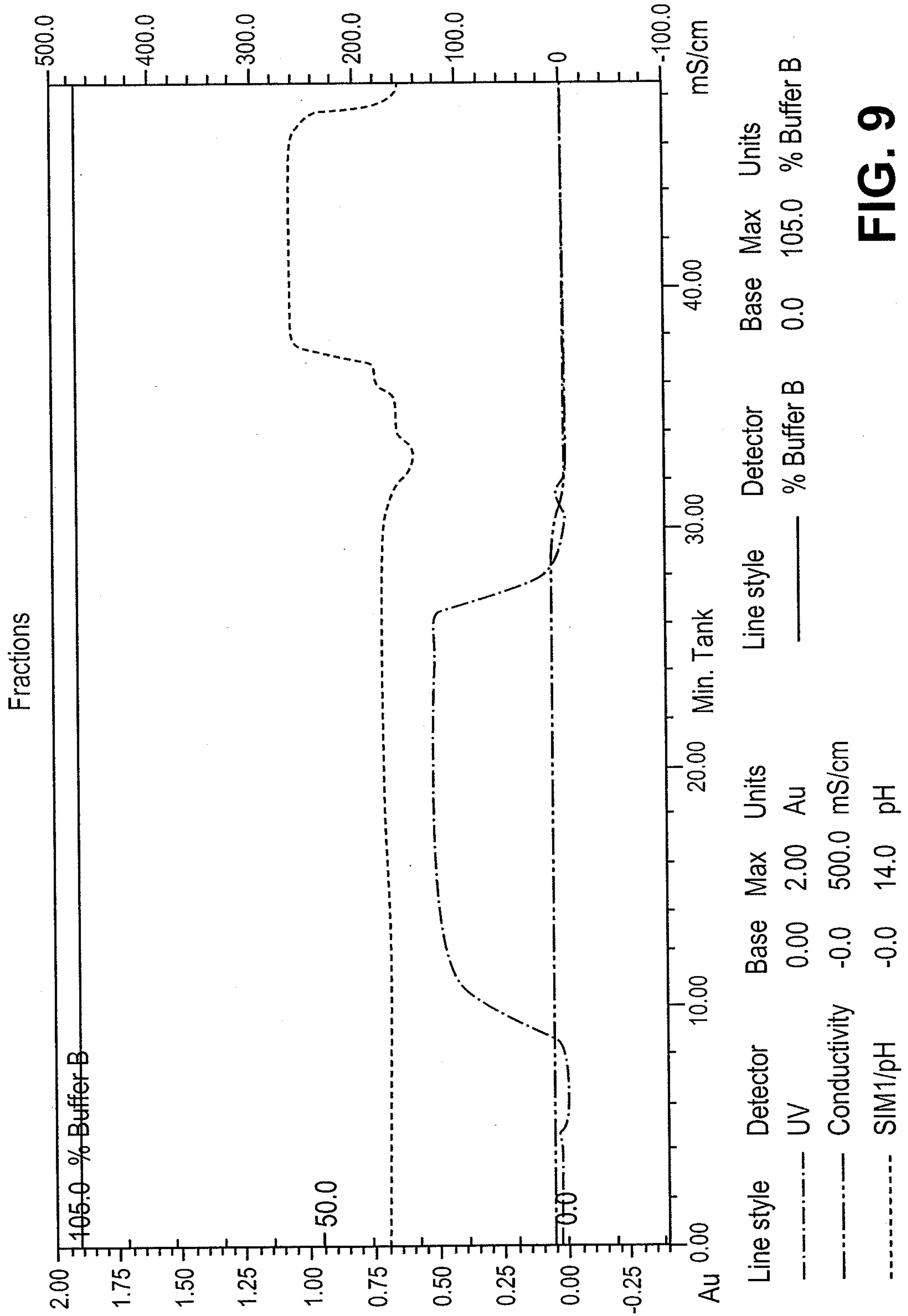
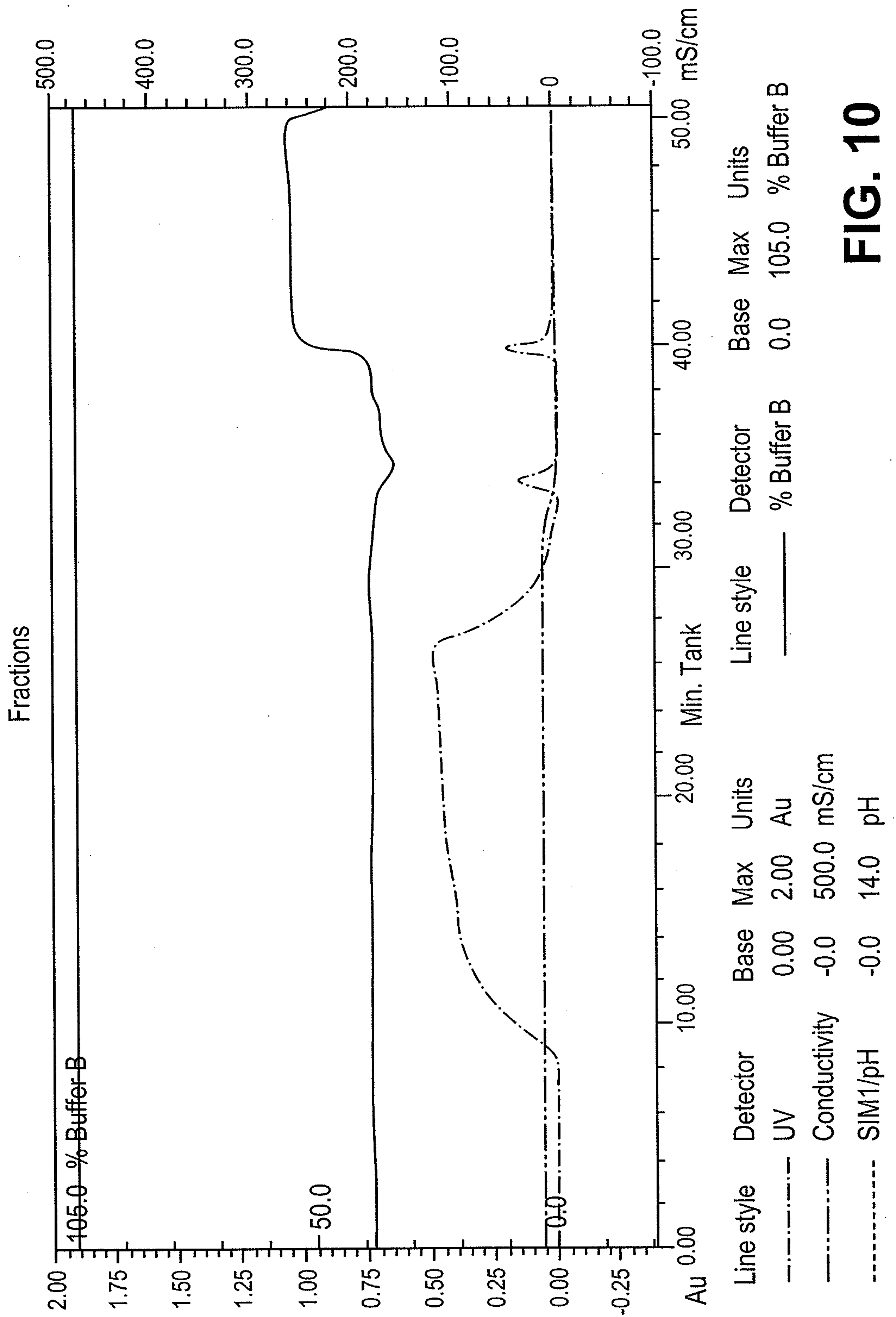


FIG. 9



**FIG. 10**



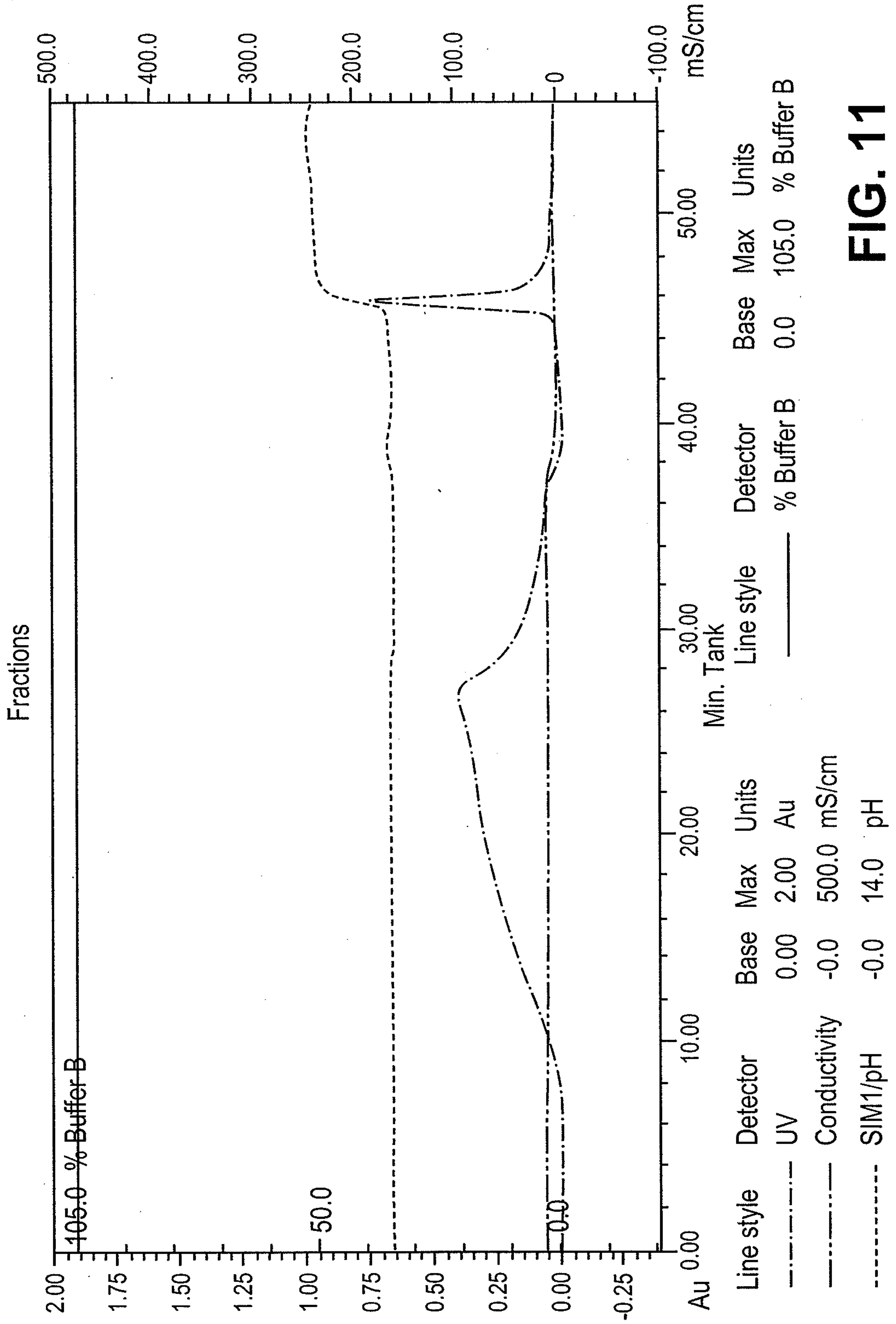


FIG. 11

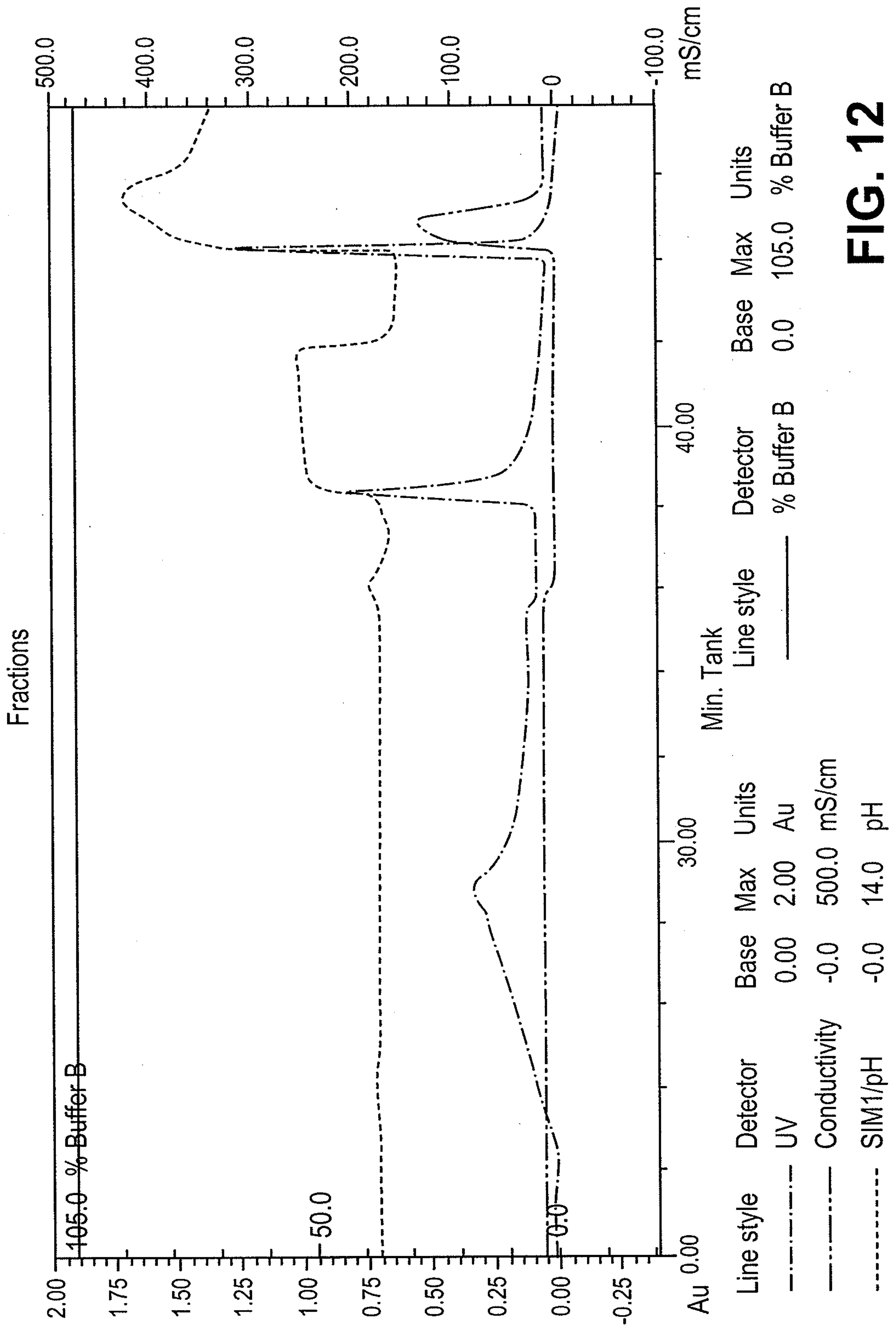


FIG. 12



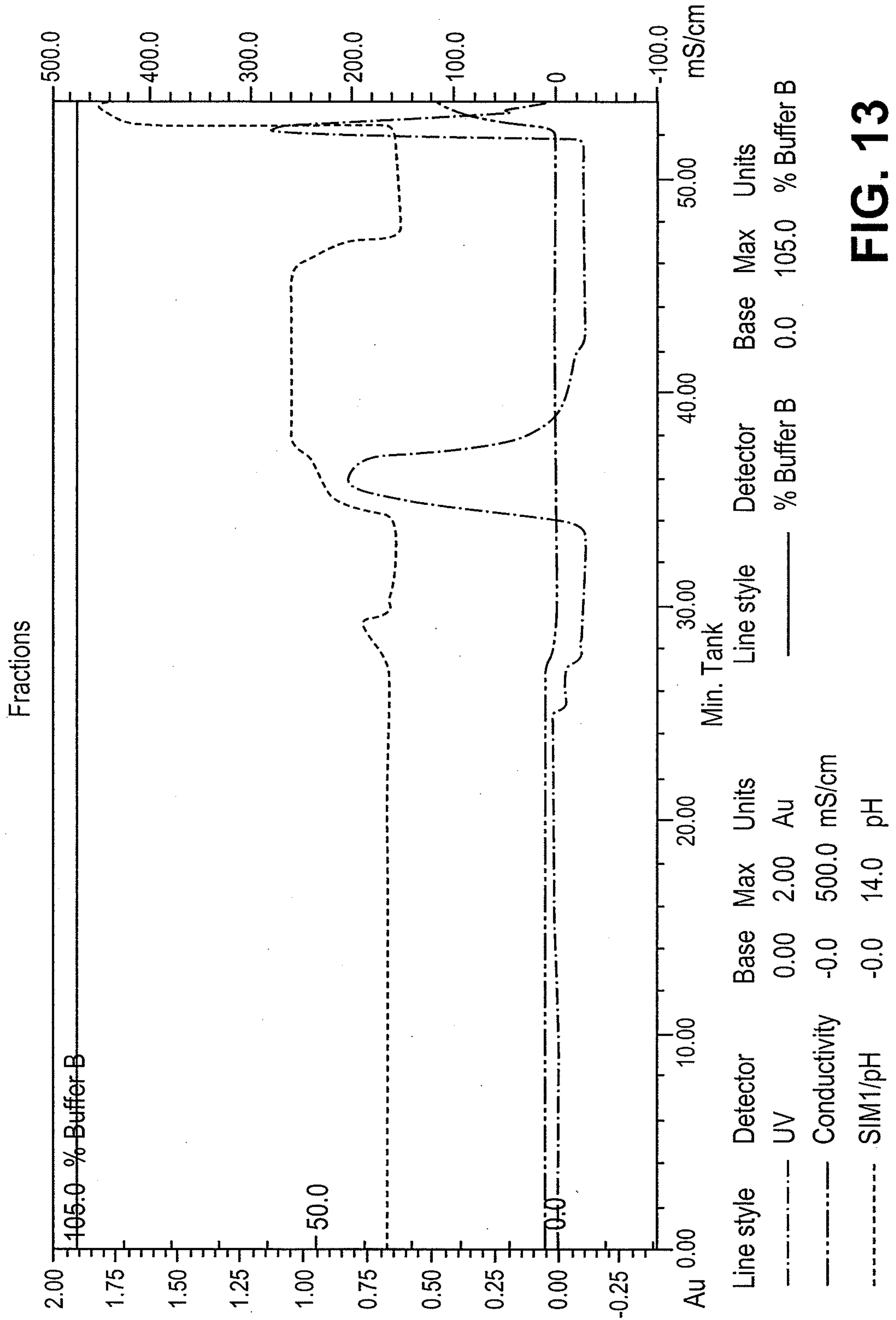


FIG. 13

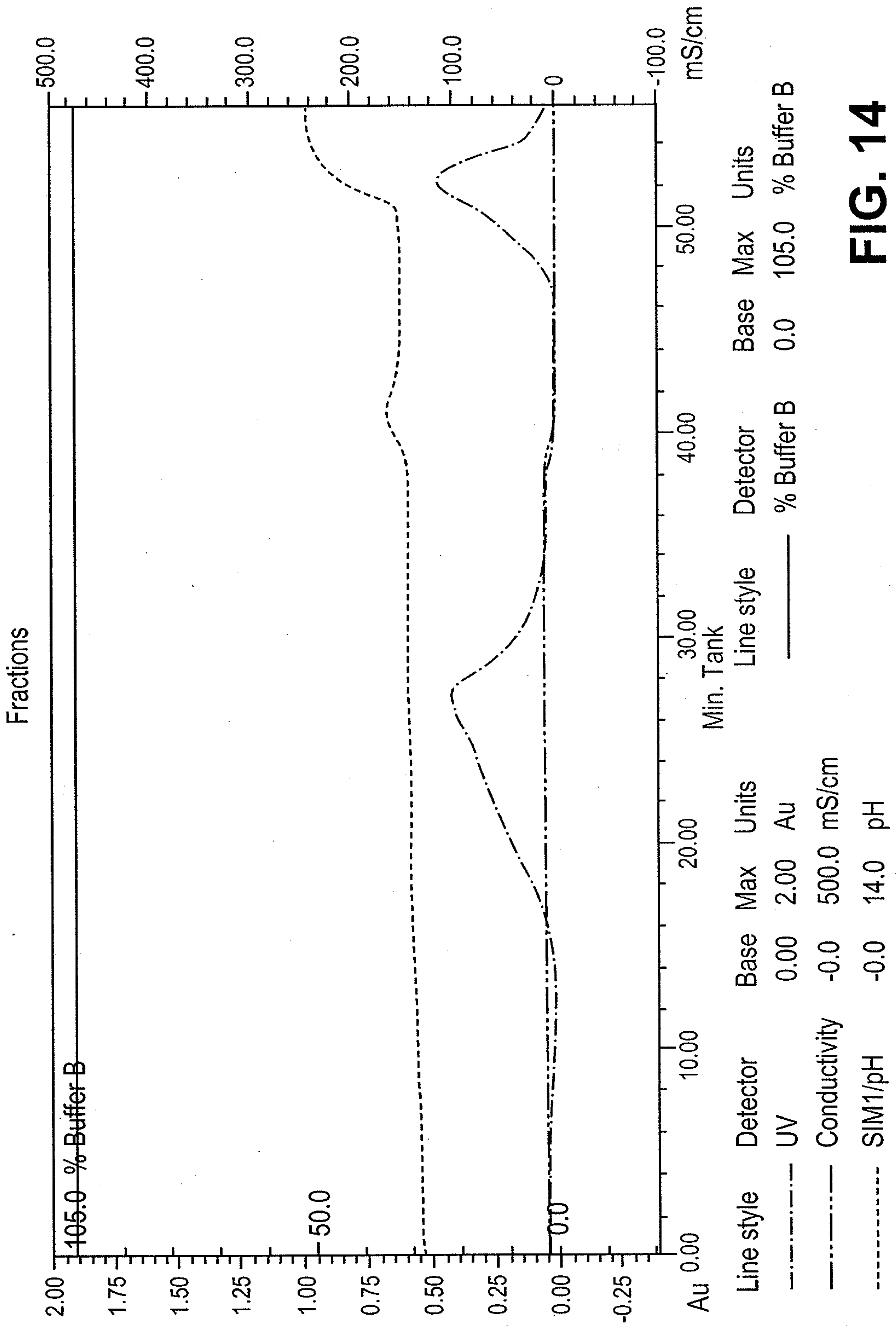


FIG. 14



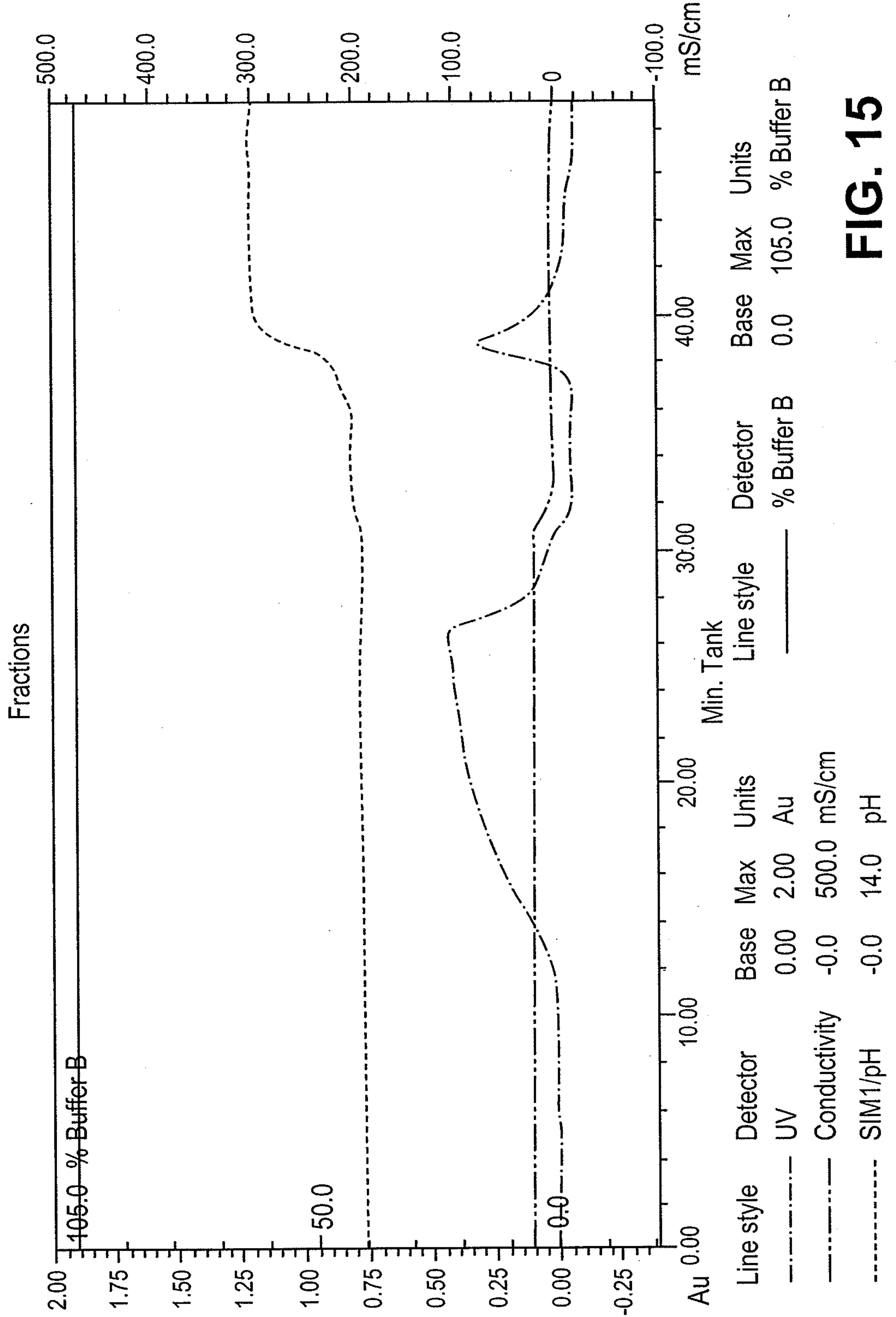


FIG. 15