

(12) PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. AU 199948150 B2
(10) Patent No. 759380

(54) Title
Method for separation on cation exchangers

(51)⁷ International Patent Classification(s)
B01J 039/18 B01J 039/04

(21) Application No: **199948150**

(22) Application Date: **1999.06.16**

(87) WIPO No: **WO99/65607**

(30) Priority Data

(31) Number	(32) Date	(33) Country
9802214	1998.06.18	SE

(43) Publication Date : **2000.01.05**

(43) Publication Journal Date : **2000.03.16**

(44) Accepted Journal Date : **2003.04.10**

(71) Applicant(s)
Amersham Biosciences AB

(72) Inventor(s)
Rolf Berglund; Jan Bergstrom; Lennart Soderberg

(74) Agent/Attorney
DAVIES COLLISON CAVE, 1 Little Collins Street, MELBOURNE VIC 3000

(56) Related Art
WO 97/29825

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : B01J 39/18, 39/04		A1	(11) International Publication Number: WO 99/65607
			(43) International Publication Date: 23 December 1999 (23.12.99)
(21) International Application Number: PCT/SE99/01082		(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 16 June 1999 (16.06.99)			
(30) Priority Data: 9802214-8 18 June 1998 (18.06.98) SE		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant (for all designated States except US): AMERSHAM PHARMACIA BIOTECH AB [SE/SE]; Björkgatan 30, S-751 84 Uppsala (SE).			
(72) Inventors; and (75) Inventors/Applicants (for US only): BERGLUND, Rolf [SE/SE]; Rabarbergatan 23, S-754 49 Uppsala (SE). BERGSTRÖM, Jan [SE/SE]; Rönjningsvägen 3, S-740 22 Bälinge (SE). SÖDERBERG, Lennart [SE/SE]; Krusenbergs, Ekshagarna, S-755 98 Uppsala (SE).			
(74) Agents: ROLLINS, Anthony, J. et al.; Nycomed Amersham plc, Patent Group, White Lion Road, Amersham, Bucks HP7 9LL (GB).			
(54) Title: METHOD FOR SEPARATION ON CATION EXCHANGERS			
(57) Abstract <p>A process for separation of a compound from a solution by cation exchange. One characterizing feature is that cation exchanger used carries a number of ligands $X-Y(Z)_n$ and requires an elevated ionic strength for elution a bound compound compared to standard cation exchangers. In the ligand a) X is $-O-$, $-S(R_1)-$ or $-N(R_1)(R_2)-$; b) Y may be: 1) a 5 or 6 membered cyclic structure containing double bonds, preferably an aromatic group, or 2) an alicyclic C_5-C_6 group or an aliphatic, straight or branched, C_1-C_3 group with the possibility that the chain joining X with Z may be interrupted by 1 or 2 ether oxygen or thioether sulphur atoms and with the proviso that X is not $-O-$ or $-S-$; c) Z is cation exchanging group with a negative total charge or a protonated form thereof; and d) n is an integer > 0. The method is further characterized in that the binding is taking place at a pH at which the ligand has a net charge.</p>			



METHOD FOR SEPARATION ON CATION EXCHANGERS

Technical field

The present invention relates to a kind of cation exchangers for which we have discovered new properties. In the invention the new properties is utilized
5 for the separation of complex mixtures of compounds by adsorption/binding of one or more compounds of the mixtures to the cation exchanger. Compounds of interest are typically positively charged. They often have a complex structure, for instance by being a protein.

Some of the cation exchangers as such are novel.

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Background of the invention

The expression "separation" in the context of the instant invention means removal or separation of one or more compounds from a mixture of dissolved substances. Separation may be utilized in connection with isolation, purification,
15 tion, concentration, analysis etc of the compounds in question. Separation by cation exchange on a cation exchanger occurs because the compounds of an applied mixture interact differently with the cation exchanger. The interaction may vary from no interaction to very strong interaction.

The term "protein" comprises compounds containing peptide bonds between
20 amino acids. In other words the term comprises oligopeptides and polypeptides as well as lipoproteins, glycoproteins, proteoglycans, etc.

The terms "adsorb" and "bound" to a cation exchanger include that the compound is retarded when allowed to pass through the cation exchanger.

25 An ion exchanger consists of an insoluble and a more or less swellable matrix, to which charged groups (ligands) have been covalently bound. The counterions of these charged groups may be exchanged for other ions of the same type of charge.

Ion exchangers with negatively charged groups (ligands) have positively
30 charged counter-ions (cations) and are therefore called cation exchangers. Presence of negatively charged groups (ligands) is a fundamental feature of cation exchangers. The kind and the amount of ligand determine the affinity and selectivity of the ion exchanger for a positively charged compound. The capacity is predominantly determined by the total number of groups and their
35 availability. The most common cation exchanging groups are sulphonate ($-\text{SO}_3^-$ / $-\text{SO}_3\text{H}$), carboxylate ($-\text{COO}^-$ / $-\text{COOH}$), phosphate ($-\text{O}-\text{PO}_3^{2-}$ / $-\text{O}-\text{PO}_3\text{H}^-$ / $-\text{O}-$

PO₃H₂) and phosphonate (-PO₃²⁻ / -PO₃H⁻ / -PO₃H₂). The groups are usually bound via a spacer to the matrix (the free bond means binding to saturated or unsaturated carbon atom in a spacer or in a base matrix).

Cation exchangers in which the negatively charged group is sulphonate are usually classified as strong cation exchangers. The other cation exchangers are called weak cation exchangers (carboxylate, phosphate or phosphonate groups). Strong cation exchangers are completely ionized within a broad pH range, usually pH > 2. For weak cation exchangers the capacity of ion exchange and the degree of ionization vary with pH (usually within the interval pH 2-12).

The matrix may be based on inorganic compounds, synthetic resins, polysaccharides, etc. Different matrices may have different physical properties. Porosity, mechanical strength, rigidity, flow characteristics, swelling properties, the degree of non-specific adsorption, etc of the matrices may vary.

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For separation of substances on cation exchangers the following applies:

- a) If the conditions are selected in such a way that undesired substances in the sample are bound to the cation exchanger and the substance of interest is passed through, there is no need for a separate desorption/elution of the substance of interest. Re-use of the cation exchanger may require regeneration.
- b) If the conditions are selected in such a way that the substance of interest is bound to the cation exchanger, desorption/elution is required to release the substance from the cation exchanger. In this case the substance is recovered by the desorption step.

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Separation using cation exchangers may be achieved by:

- (a) Batchwise processes, e.g. by having the cation exchanger dispersed in a liquid containing the substance to be adsorbed/bound,
- (b) Column processes (e.g. ion exchange chromatography) in which the cation exchanger is in form of a monolithic bed or in form of particles that define an expanded/fluidised bed or a packed bed. A liquid containing the substance(s) to be adsorbed/bound is allowed to pass through the bed, and
- (c) Membrane processes etc.

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Separation by cation exchange to purify a substance is often combined with other techniques distinguishing compounds based on differences in size and form (gel filtration), in biospecific affinity (bioaffinity chromatography), in ability for hydrophobic interaction etc.

- 5 Purification of a compound is usually initiated by extraction. The resultant crude extract typically has a large volume and such a high ionic strength that it is not possible to adsorb on conventional ion exchangers without dilution, which further will increase the volume (conventional cation exchangers don't have sufficient adsorption ability at a high ionic strength). Large volumes will
10 thus have to be handled and therefore relevant investments must be made in space demanding and expensive equipment and also use of highly purified water.

Related Art

- 15 Dipolar adsorbents prepared by coupling of sulphanilic acid using epichlorohydrin has been described (ligand + spacer = $-\text{CH}_2\text{CHOHCH}_2\text{N}^+\text{H}_2\text{C}_6\text{H}_4\text{SO}_3^-$) (Porat et al., J. Chromatog. 51 (1970) 479-489; and Ohkubo et al., J. Chromatog. A, 779 (1997), 113-122). These two articles do not disclose the new features discovered by us. The articles do not disclose a method in which the
20 ligand is negatively charged and the target substance to be removed is positively charged.

- 2,4,6-trihalo-1,3,5-triazine has been utilized to bind different compounds $\text{RHNR}'\text{X}$ to carriers inter alia to cellulose. R has been hydrogen, aryl or alkyl, R' alkylene or arylene and X carboxy, sulphonyl, phosphate, phosphonate,
25 boronate, etc. (See Behrend et al., WPI Abstract Accession No. 86-312313 (= DD-A-237844). This coupling methodology gives structures that are unstable to hydrolysis.

- EP 326233 discloses a cation exchanger in which there is a hydrophobic base matrix to which cation exchanging groups are attached. The hydrophobicity of this type of cation exchangers makes them unsuitable for separation
30 of biomolecules such as proteins.

- An anion exchanger with improved properties relative to previously known anion exchangers has recently been described in WO-A-9729825. The cation exchangers used in the instant invention are a particularly interesting complement to the anion exchangers according to WO-A-9729825. The ion ex-
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changing ligands of the instant invention and in WO-A-9729825 are complex in different ways reflecting the complexity of protein interactions.

The article by Skoog et al in Principles of Instrumental Analysis, 4th ed., Chapter 26F, (1992) 654-658 discloses various principles for constructing and using ion exchangers.

WO 9808603 discloses separation media of the general structure M-SP1-L in which M is a base matrix, SP1 is a spacer and L is a ligand comprising a mono- or bicyclic aromatic or heteroaromatic moiety that may be substituted. In one variant L is X-A-SUB where X is -O-, -S- or -NH- and A is the aromatic or heteroaromatic moiety that is substituted. The substituent on A may be an acidic group. The separation media are suggested for the isolation of proteins, in particular immunoglobulins. The publication does not disclose the properties we have discovered and contains no disclosure of a separation method utilizing these properties.

US 5,652,348 discloses ion exchange resins in which the hydrophobicity/hydrophilicity of the resin including the ligand is changed upon change in pH. The hydrophobicity may be increased by the introduction of hydrophobic non-ionizable ligands. Adsorption/desorption is controlled by altering the hydrophobicity/hydrophilicity of the matrix including ligand.

Detailed description of the invention

The instant invention is based on our discovery that cation exchangers carrying ligands of a certain kind of structure may exhibit unexpected strong interactions with positively charged compounds of complex structure, for instance macromolecules such as proteins and many other biomolecules. This is reflected in our finding that this kind of cation exchange-

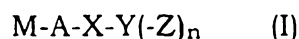


ers often require an abnormally high ionic strength for elution of adsorbed proteins.

The use of these cation exchangers in separations provides improvements and is an important aspect of the instant invention.

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The cation exchangers concerned comprise a base matrix, preferably a polymer matrix, with one or more cation exchanging ligands covalently bound to the base matrix. The cation exchanger is characterized by formula (I):



10 wherein

- a) X-Y(-Z)_n is the ligand (which shall be capable of having a negatively net charge (anionic) when the adsorption is taking place according to the invention).
- b) n is an integer ≥ 1 , typically 1, 2 or 3. For n = 2 or more, Z may be different in one and the same ligand.

15 c) M is the matrix and contains additional charged ligands, which may be the same as or different from -A-X-Y(-Z)_n. The matrix may also contain other positively charged ligands and/or other negatively charged ligands.

d) A is a spacer and is selected according to the general guidelines for covalently binding of ligands, intended for cation exchange, to matrices. See below.

20

e) X is -O-, -S(R₁)- or -N(R₁)(R₂)-. R₁ and R₂ are H, a free electron pair, A-X-Y(-Z)_n, Y(-Z)_n or a straight, cyclic or branched aliphatic C₁-C₆ group. R₁ and R₂ are preferably saturated groups (alkyl) which for C₂₋₆ alkyl may have 1 to 4 of its hydrogen atoms replaced by OH groups or lower alkoxy. At most one of R₁ and R₂ can be a free electron pair. On the sulphur atom there may be one or two groups A-X-Y(-Z)_n or Y(-Z)_n and on the nitrogen atom one, two or three groups A-X-Y(-Z)_n or Y(-Z)_n.

25

f) Y may be:

- 1) a 5- or 6-membered cyclic structure containing double bonds, preferably an aromatic group, or
- 2) an alicyclic C₅-C₆ group or an aliphatic, straight or branched, preferably saturated, C₁-C₈ group with at least 1 or 2 carbon atoms in the chain joining X with Z, with the proviso that X is not -O- or -S-.

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g) Z is a cation exchanging group, preferably selected among the negatively charged groups mentioned above (for instance carboxylate, sulphonate, sulphate, phosphonate, phosphate, sulphate).

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- 6 -

Cyclopentyl and cyclohexyl are the most important alicyclic groups that can be used as Y.

Depending on the number of carbon atoms, alicyclic, aliphatic and aromatic structures may be substituted by 1, 2, 3, 4, 5 or 6 OR groups. RO is OH or lower alkoxy or any other alkoxy group not disturbing the advantageous properties utilized in the invention.

Chains in aliphatic groups (Y as well as R, R₁ and R₂) may be interrupted by a thioether sulphur or an ether oxygen (-S- and -O-, respectively), e.g. in one or two positions. In the aliphatic groups referred to (Y, R, R₁ and R₂) only one atom selected among sulphur, oxygen and nitrogen should bind to one and the same carbon atom.

By lower alkoxy and lower alkyl are meant C₁₋₁₀, preferably C₁₋₆ alkoxy/alkyl.

In an embodiment of the invention there is provided a cation exchanger suitable for use in a process for the separation of positively charged compounds, wherein the cation exchanger has the formula (I) below



in which formula

- a) M is a hydrophilic matrix;
- b) A is a spacer, and
- c) X-Y(-Z)_n is a cation exchanging ligand; wherein
- d) X is -O-, -S(R₁)- or -N(R₁)(R₂)-, wherein R₁ and R₂ are selected among H, a free electron pair, A-X-Y(-Z)_n, Y(-Z)_n and straight or branched aliphatic C₁₋₆ groups, which for C₂₋₆ alkyl may have 1-4 of its hydrogen atoms replaced by OH groups in which a hydrogen may be replaced by a lower alkyl, with the proviso that only one of R₁ and R₂ can be a free electron pair;



- 6A -

e) Y may be:

- 1) a 5- or 6-membered cyclic structure containing double bonds, or
- 2) an alicyclic C₅-C₆ group or an aliphatic, straight or branched C₁-C₈

5 interrupted by one or two ether oxygen or thioether sulphur atoms and with the proviso that X is not -O- or -S-; and

f) Z is a cation-exchanging group.

10 In another embodiment of the invention there is provided a process for separation of positively charged compounds, primarily biomolecules and particularly amphoteric ones such as proteins, from a solution, comprising the steps of contacting the solution with a cation exchanger under conditions permitting binding of one or more of the positively charged compounds to the cation exchanger, and, if desired, eluting bound compounds, wherein

15 (i) the ionic strength in the solution during binding is >15 mS/cm, and the ionic strength in the solution during elution exceeds the ionic strength applied when binding,

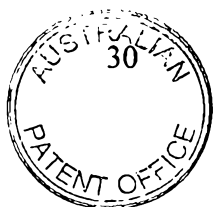
and

(ii) the cation exchanger has the formula (I)

20
$$M-A-X-Y(-Z)_n \quad (I)$$

in which formula

- a) M is a hydrophilic matrix;
- b) A is a spacer, and
- c) X-Y(-Z)_n is a cation exchanging ligand; wherein
- 25 d) X is -O-, -S(R₁)- or -N(R₁)(R₂)-, wherein R₁ and R₂ are selected among H, a free electron pair, A-X-Y(-Z)_n, Y(-Z)_n and straight or branched aliphatic, C₁-C₆ groups, which for C₂₋₆ alkyl may have 1-4 of its hydrogen atoms replaced by OH groups in which a hydrogen may be replaced by a lower alkyl, with the proviso that only one of R₁ and R₂ can be a free electron pair;



- 6B -

e) Y may be:

- 1) a 5- or 6-membered cyclic structure containing double bonds, or
- 2) an alicyclic C_5-C_6 group or an aliphatic, straight or branched C_1-C_8 group with the possibility that the chain joining X with Z may be interrupted by one or two ether oxygen or thioether sulphur atoms and with the proviso that X is not -O- or -S-; and

f) Z is a cation-exchanging group;

and in that the binding between the cation exchanger and the positively charged compound(s) is allowed to proceed at a pH value within the range of 2-14 at which ligands

10 -X-Y(-Z)_n have a negative net charge.

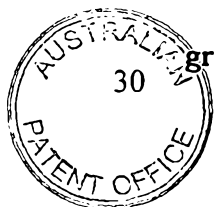
Advantageous embodiments of the cation exchanger

Advantageous ligands (-X-Y(-Z)_n) have X equal to -O- or -N(R₁)(R₂)- with at least one of R₁ and R₂ being equal to a free electron pair or H while the other of
 15 R₁ and R₂ is H or a straight, branched or cyclic aliphatic group according to above, for instance alkyl.

Advantageous variants with X = -N(R₁)(R₂)- may arise if there is/are 1-4, such as 1-3, lower alkoxy groups or OH (= R'O) at a distance of 2 or 3 carbon atoms from the nitrogen atom in -N(R₁)(R₂)-. These R'O groups can be placed in Y, in R₁ or R₂ or in the spacer A. R'O is preferably OH. Particularly advanta-
 20 geous variants (-X-Y(Z)_n) arise if one of R₁ and R₂ is a free electron pair or H, such as in a ligand in which X = -N[C(CH₂OH)₃]-, Y = -CH₂CH₂CH₂- or -CH₂CHOHCH₂-, n is 1, and Z is -SO₃⁻.

Another group of advantageous ligands are those in which Y equals an aromatic group, typically a benzene ring (substituted or unsubstituted phenyl-
 25 ene). X and Y may in this case be ortho, meta or para to each other. Especially important variants are ligands derived from hydroxybenzoic acid, sulphasalicylic acid or hydroquinone sulphonate, particularly if coupling to the spacer has occurred via any of the ring bound hydroxy groups of the compounds.

If Y is an aliphatic group it is preferably a C₂-C₈ group, such as a C₂-C₆ group, preferably a C₂-C₄ group, which groups may be substituted by OR



groups according to above. In case Y equals a C_1 group a preferred embodiment is $X = -N(R_1)(R_2)-$, where one of R_1 or R_2 is a free electron pair or hydrogen and the other is an alkyl according to above with 1, 2 or 3 OR at a distance of two carbon atoms from N with RO being as defined above.

5

The cation exchangers to be used in the invention exhibit a higher, preferably more than 25% higher, such as more than 40% and even more than 100% higher maximum elution ionic strength in the pH interval 2-12 for one or more proteins selected among ribonuclease, chymotrypsinogen A, cytochrome C, lysozyme, wheat-germ lectin and β -lactoglobulin, compared with the maximum elution ion strength required for the same proteins on a reference ion exchanger where the ion exchanging group is sulphonate ($-SO_3^-$) and the base matrix, the spacer and the degree of substitution are the same but where the groups X and Y are absent.

15

The most important aspect of the present invention is a process for separation of positively charged compounds from a solution. The process comprises that the solution is contacted with a cation exchanger to bind one or more of the positively charged compounds to the cation exchanger, and also, if desired, to elute/desorb bound compounds. The compounds concerned are primarily biomolecules and particularly amphoteric ones, such as proteins.

The characterizing features of the process is (a) that the cation exchanger is according to Formula (I) and have higher maximum elution ionic strength in relation to a reference sulphonate cation exchanger in the same manner as described above, and (b) that the binding between the cation exchanger and a positively charged compound is allowed to occur at a pH value within the interval 2-14 where a significant part of the ligands ($-X-Y(-Z)_n$) have a negative net charge, preferentially all of them. For ligands in which $n = 2$ or more, it might be preferred to run the adsorption step at a pH where the ligands have a negative net charge but not the maximal net charge ($n = 2$ or more).

The selection of this kind of cation exchangers means that the binding often may occur at a higher ionic strength than the elution ionic strength required when eluting the compound at the same pH value from a reference ion exchanger according to above. The comparison is made with pH and other conditions being the same.

The cation exchanger of the invention may be selected so that the ionic strength, when binding occurs, may be at least 25% higher, such as at least 40% higher than when using the corresponding reference ion exchanger according to above (as measured at the same pH). If required binding may also
5 be performed at more than 100% higher ionic strength than when using the corresponding reference ion exchanger according to above (as measured at the same pH).

In absolute figures this may involve the binding being performed at an ionic strength exceeding 15 or 20 mS/cm, such as exceeding 30 mS/cm and in
10 some cases exceeding 40 mS/cm. The applicable figures in a particular case will depend on the selection of ligand and compound to be eluted.

An interesting application of this embodiment of the invention is large-scale processes in which large volumes of a crude product having a high ionic strength are to be applied on the cation exchanger. In general, diluting is re-
15 quired in order to enable binding of the compound of interest to the conventional ion exchanger. By use of the process of the invention the need of diluting is often reduced.

The elution step in the process of the invention, may, for amphoteric compounds and other compounds that may be positively charged, such as proteins, primarily be performed according to four main alternatives

- (a) Changing pH (rising or lowering) in the elution liquid,
- (b) Increasing the ionic strength or
- (c) Combining a change of pH and ionic strength, or
- 25 (d) Including a ligand analogue in the liquid used for eluting.

In principle any condition/methodology alone or in combination that neutralizes the interaction causing binding may be utilized for desorption.

In alternative c) the change of ionic strength may be downwards or upwards depending on the effect the pH change has on the interactions causing binding.
30 ing.

In alternative (d) the ligand analogue may be combined with any one of alternatives a-c.

The most advantageous alternatives typically neutralize the interaction causing binding without requiring an increase in the ionic strength. In this way the
35 eluate will contain the released substance and will have a lowered salt concentration compared to the sample originally applied. The requirements of de-

salting may thus be reduced. From this perspective, elution by changing the pH of the eluting liquid (alternatives a) combined with a lowering of ionic strength is particularly preferred among alternatives (a)-(c). The alternative with a ligand analogue may give similar advantages.

- 5 If elution/desorption does not involve a change in pH, elution/desorption of the ion exchanger usually has to be performed at an ionic strength which is higher than the corresponding elution ionic strength required for a corresponding reference ion exchanger according to above (as measured at the same pH). The ionic strength in this step may thus be at least 25% higher
10 such as at least 40% higher than for the reference cation exchanger. In some extreme cases an ionic strength may be required that is more than 100% higher than for the corresponding reference ion exchanger (as measured at the same pH).
- 15 Another preferred embodiment involves selecting the cation exchanger according to Formula I so that elution can be performed by use of such a ionic strength gradient that the elution interval becomes broader than the interval required at the same pH value for the reference ion exchanger according to above. This will normally be accomplished by selecting the cation exchanger of
20 formula I according to the preferred modes described above. The interval referred to may start at the lowest ionic strength applied for elution and ends at the ionic strength at the maximal peak height for a compound of interest. In the alternative the interval may be the interval defined by elution ionic strength for peak maximum for two different compounds appearing during
25 elution. This means greater possibilities to separate compounds, which are related to each other with reference to charge, and/or to improve yield of these compounds. In a preferred embodiment the elution interval, within which the current compound(s) is eluted, is at least 20%, preferably at least 40%, broader than the corresponding ionic strength interval with the reference ion
30 exchanger according to above (as measured, at in other respects, the same conditions). In many cases the ionic strength interval may be further broadened and become at least 50%, at least 75% or at least 100% broader than the corresponding ionic strength interval with the reference ion exchanger according to above (as measured, at in other respects, the same conditions).
- 35 Considering the whole interval, within which the current compounds can be eluted, usually an interval with a range of up to approximately 10 mS/cm or

somewhat wider has previously been utilized. According to the present invention the interval can be expanded to a range of at least 15, preferably at least 20 or even more preferably at least 30 mS/cm by adjusting the choice of the cation exchanger defined by Formula I. The applicable figures in a particular case is dependent on the ligand and the compound(s) to be separated.

The change in ionic strength and/or pH may be done as a stepwise gradient (containing one, two or more steps) or as a continuous gradient. The application of stepwise or continuous gradients is also applicable to other changes in conditions that are used to neutralize the binding of the compound(s) of interest to the ligand.

Furthermore, the different process aspects can be utilized in any suitable combination.

Other preferred embodiments of the different process aspects of the invention are defined by the preferred embodiments of the cation exchanger.

The base matrix in the cation exchangers according to the invention is preferably hydrophilic and in the form of a polymer, which is insoluble and more or less swellable in water. Hydrophobic polymers that have been derivatized to become hydrophilic are included in this definition. 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 polymethacrylates (e.g. polyglycidylmethacrylate), polyvinylalcohols 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 hydrophobic polymers (e.g. on copolymers of monovinyl and divinylbenzene) by polymerization 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.

The matrix can also be based on inorganic material, such as silica. Preferred matrices lack hydrolytically unstable groups, such as silan, ester and amide groups.

The matrix is often porous.

- 5 The term "hydrophilic matrix" in practice means that the accessible surface of the matrix is hydrophilic in the sense that is penetrated by aqueous liquids. Typically the accessible surfaces on a hydrophilic base matrix expose a plurality of polar groups for instance comprising oxygen and/or nitrogen atoms. Examples of such polar groups are hydroxy, amino, carboxy, ester, ether of lower
10 alkyls (such as $(-\text{CH}_2\text{CH}_2\text{O})_n\text{H}$ where n is an integer).

For covalent coupling of the ion exchange ligands of the invention to a matrix, traditional coupling chemistry may be used including also techniques to be developed in the future. This means that the spacer A, joining the ion ex-
15 change ligand $-\text{X}-\text{Y}(-\text{Z})_n$ with the matrix M in the cation exchanger, may be of the same type as in traditional ion exchangers. The demands for hydrolytic stability require that spacers should be built of groups that are stable against hydrolysis. They may for instance contain groups selected among straight, branched, cyclic saturated and unsaturated and aromatic hydrocarbon groups
20 (e.g. with up to 1-10 carbon atoms), ether groups $(-\text{O}-)$, thioether $(-\text{S}-)$ amide $(-\text{CONH}-)$ and other groups with corresponding or acceptable hydrolytic stability. Typically the spacer binds to X via a sp^3 -hybridized or aromatic carbon atom.

Examples of common structure elements, which alone or in combination with each other or with other structures may work as spacers are:

- 25 $-\text{O}-\text{CH}_2-$; $-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2-$; $-\text{O}-\text{CH}_2-\text{CH}_2-\text{CH}_2-$; $-\text{O}-\text{CH}_2-\text{CH}(\text{CH}_3)-$; $-\text{S}-\text{CH}_2-$; $-\text{CO}-\text{NH}-\text{CH}_2$; and $-\text{NH}-\text{CO}-\text{CH}_2-$; $-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2-$; $-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2-$ (= the spacer in the sulphonate reference ion exchanger of the patent examples).

- Typical stable spacers have only one atom selected from oxygen, sulphur
30 and nitrogen bound to the same carbon atom.

The level of ligands in the cation exchangers used in the invention is usually selected in the interval of 0.001-4 mmol/ml, preferably 0.01-1 mmol/ml matrix swollen with water. Possible and preferred ranges are determined by
35 the structure of the matrix. Thus, the level of ligand is usually within the range of 0.1-0.3 mmol/ml swelled matrix (gel) for agarose based matrices. For dex-

tran based matrices the interval in question may be extended up to 0.5-0.6 mmole/ml swelled matrix (gel).

The invention will now be further illustrated by a number of non-limiting
5 examples.

EXPERIMENTAL PART

I. THE SYNTHESIS OF ION EXCHANGERS.

10 Alkylation, standard method for production of allyl hydroxypropyl Sepharose HP (ion exchanger according to the invention):

Cross-linked agarose (30 μ m particles) produced by a reaction between epichlorohydrin and agarose in the presence of NaOH (Porath et al., (J. Chromatog. 60 (1971) 167-77 and US 3,959,251) was reacted with allylglycidylether
15 with NaOH as a base to an allyl level ($\text{CH}_2=\text{CHCH}_2\text{OCH}_2\text{CHOHCH}_2-$) of 0.03 - 0.30 mmole/ml).

A. Synthesis of four variants of reference S-ion exchangers (ligand sulphate).

20 Starting from cross-linked allylated agarose according to above (allyl group levels of 0.033, 0.111, and 0.200 mmole/ml) three different S Sepharose ion exchangers were produced with ion exchange capacities of 0.031, 0.094 and 0.140 mmole/ml. 25 g of cross-linked allylated agarose was washed with water and loaded together with 30 ml of distilled water and 1.8 g of sodium acetate
25 in a 100 ml beaker provided with a hanging magnetic stirrer, and then bromine water was added to remaining yellow colour. The gel was washed on a glass filter with distilled water (> 5 bed volumes), and was suctioned dried for 15-30 seconds. Then the brominated gel was loaded together with 30 ml of distilled water and 8 g of sodium sulphite into a three-necked 100 ml Bellco
30 flask with a hanging magnetic stirrer. PH was adjusted to about 10-11. The reaction was proceeded over night (23 hours) at 50°C.

The reaction mixture was neutralized with acetic acid. The gel was then washed with distilled water (> 10 bed volumes) and for possible storage distilled water containing 23% (w/w) ethanol was used.

35 To obtain a S-ion exchanger with an ion exchange capacity of 0.220 mmole/ml the ion exchanger with a capacity of 0.140 mmole/ml was allylated

once more (to an allyl level of 0.30 mmole/ml) with subsequent sulphite coupling according to above.

B. Production of a phosphate ion exchanger.

- 5 20 g of cross-linked allylated agarose according to A above was washed with water and loaded together with 80 - 100 ml of distilled water and 4.6 g of sodium acetate into a 100 ml Bellco flask provided with a hanging magnetic stirrer. Bromine water was then added to a remaining yellow colour. The gel was washed in a glass funnel with distilled water (> 5 bed volumes). The gel was
10 suctioned dried for 15-30 seconds.

The gel was suctioned dried and loaded together with 30 ml of distilled water, 43.1 g (0.2476 mole) of dipotassium hydrogen phosphate and 9.90 g of sodium hydroxide into a three-necked 25 ml Bellco flask with a hanging magnetic stirrer. The reaction was proceeded over night 16-22 hours at 40°C. The
15 reaction was stopped by lowering the pH to 7.0 with conc. HCl and then the gel was washed on a glass filter with distilled water (> 10 bed volumes).

Inflection point titration under conditions of equilibrium directly on the substituted matrix with NaOH solution gave a level of ligand of 0.04 mmole/g of dry aspirated gel.

20

C. Synthesis of a cation exchanger based on hydroxybenzoic acid.

- 10 g of cross-linked allylated agarose according to A above (level of allyl groups of 0.21 mmole/ml gel) was washed with water and loaded into a 100 ml Bellco flask provided with a hanging magnetic stirrer together with 80-100
25 ml of distilled water and 2.3 g of sodium sulphate as a buffer. Finally, bromine water was added to a remaining yellow colour. Washing was performed with distilled water (> 5 bed volumes).

The gel was suctioned dried for 15-30 seconds, washed with distilled water and then loaded into a solution of 15.36 g (0.1112 mole) of 4-hydroxybenzoic
30 acid and 14.68 g (> 0.2224 mole) of potassium hydroxide in 20 ml of distilled water. First lye and water were mixed. The reaction was run in a three-necked 25 ml Bellco flask with a hanging magnetic stirrer bar. The reaction was proceeded over night, 16-22 hours at 40°C.

- The reaction was stopped by taking up the gel on a glass filter funnel and
35 washed with a few bed volumes distilled water and then the gel was suspended in distilled water and the pH value was adjusted with acetic acid to about 6.

Then the gel was washed with distilled water (> 10 bed volumes). For possible storage distilled water containing 23% (w/w) ethanol was used.

Inflection point titration under conditions of equilibrium directly on the substituted matrix with NaOH solution gave a level of ligand of 0.11 mmole/g dry
5 aspirated gel.

D. Synthesis of a cation exchanger based on sulphanilic acid.

10 g of water washed cross-linked allylated agarose according to A above (allyl group level of 0.21 mmole/ml gel) was loaded into a 100 ml Bellco flask
10 provided with a hanging magnetic stirrer together with 80-100 ml of distilled water and 2.3 g of sodium sulphate as a buffer. Bromine water was thereafter added to a remaining yellow colour. Washing was performed with distilled water (> 5 bed volumes).

The gel was suctioned dried for 15-30 seconds and then loaded into a solution of 19.26 g (0.1112 mole) of 4-sulphanilic acid and 7.34 g (>> 0.1112 mole)
15 of potassium hydroxide (>85%) in 20 ml of distilled water. First lye and water were mixed. The reaction was run in a three-necked 25 ml Bellco flask with a hanging magnetic stirrer. The reaction was performed during 16-22 hours at 40°C.

20 The reaction was stopped by taking up the gel on a glass filter funnel and washed with a few bed volumes of distilled water and then the gel was suspended in distilled water and the pH value was adjusted with 1 M HCl to pH about 6.

Then the gel was washed with distilled water (> 10 bed volumes). For possible
25 storage distilled water containing 23% (w/w) ethanol was used.

A level of ligand of 0.12 mmole/ml packed gel was measured by adsorbing TRIS on gel packed in a column at pH 6, whereupon the excess was washed off and adsorbed TRIS was eluted with a sodium chloride solution. The amount of TRIS in the eluate, being equivalent to the amount of negative groups on the
30 gel, was determined by inflectionpoint titration with sodium hydroxide.

E. Synthesis of a cation exchanger based on sulphosalicylic acid.

20 g of water washed cross-linked allylated agarose according to A above (allyl group level of 0.21 mmole/ml gel) was loaded into a 100 ml Bellco flask
35 provided with a hanging magnetic stirrer together with 80-100 ml of distilled water and 4.6 g of sodium sulphate as a buffer. Bromine water was thereafter

added to a remaining yellow colour. Washing was performed with distilled water (> 5 bed volumes).

The gel was suctioned dried for 15-30 seconds and then loaded into a solution of 56.54 g (0.2224 mole) of 5-sulphosalicylic acid-dihydrate and 44.04 g (0.6672 mole) of potassium hydroxide (>85%) in 40 ml of distilled water and 0.1 g of sodium borohydride. The reaction was run in a three-necked 100 ml Bellco flask with a hanging magnetic stirrer. The reaction was proceeded over night, 16-22 hours, at 40°C and under nitrogen gas.

The reaction was stopped by taking up the gel on a glass filter funnel and washed with a few bed volumes distilled water and then the gel was suspended in distilled water and the pH value was adjusted with 1 M acetic acid to 7.5.

Then the gel was washed with distilled water (> 10 bed volumes). For possible storage distilled water containing 23% (w/w) ethanol was used.

Inflection point titration directly on the substituted matrix with NaOH solution gave a level of ligand of 0.034 mmole/g dry aspirated gel (ion exchange cap. 0.068 mmole/g).

F. Synthesis of a cation exchanger based on dihydroxybenzene sulphonic acid.

20 20 g of water washed cross-linked allylated agarose according to A above (allyl group level of 0.21 mmole/ml gel) was loaded into a 100 ml Bellco flask provided with a hanging magnetic stirrer together with 80-100 ml distilled water and 4.6 g of sodium sulphate as a buffer. Bromine water was thereafter added to a remaining yellow colour. Washing was performed with distilled water (> 5 bed volumes).

The gel was suctioned dried for 15-30 seconds and then loaded into a solution of 56.54 g (0.2476 mole) of dihydroxybenzene sulphonic acid-potassium salt and 13.9 g of potassium hydroxide (> 85%) in 30 ml of distilled water and 0.1 g of sodium boron hydride. The reaction was run in a three-necked 100 ml Bellco flask with a hanging magnetic stirrer. The reaction was proceeded over night, 16-22 hours, at 40°C.

The reaction was stopped by taking up the gel on a glass filter funnel and washed with a few bed volumes of distilled water and then the gel was suspended in distilled water and the pH value was adjusted with 1 M acetic acid to pH about 6.

Then the gel was washed with distilled water (> 10 bed volumes). For possible storage distilled water containing 23% (w/w) ethanol was used.

Inflection point titration according to above, of to the gel adsorbed and then displaced TRIS, gave an ion exchange capacity of 0.10 mmole/ml packed gel.

5

G. Synthesis of a TAPS-ion exchanger. Coupling of N-[tris-(hydroxymethyl)methyl]-3-aminopropane sulphonic acid as a ligand (TAPS).

20 g of water washed cross-linked allylated agarose according to A above
10 (allyl group level of 0.21 mmole/ml gel) was loaded into a 100 ml Bellco flask provided with a hanging magnetic stirrer together with 80-100 ml of distilled water and 4.6 g of sodium sulphate as a buffer. Bromine water was thereafter added to a remaining yellow colour. Washing was performed with distilled water (> 5 bed volumes).

15 The gel was suctioned dried for 15-30 seconds and loaded together with 30 ml of distilled water, 10 g (0.25 mole) sodium hydroxide and 60.25 g (0.24765 mole) of N-[Tris(hydroxymethyl)methyl]-3-amino-propane-sulphonic acid into a three-necked 100 ml Bellco flask with a hanging magnetic stirrer. The reaction was performed over night, 16-22 hours, at 40°C.

20 The reaction was stopped by lowering the pH to 7.0 with conc. HCl, and then the gel was washed with distilled water (> 10 bed volumes).

Inflection point titration under conditions of equilibrium directly on the substituted matrix with NaOH solution gave a level of ligand of 0.10 ± 0.02 mmole/g dry aspirated gel. The pKa for the amino group was determined to
25 about 8.4.

II. RESULTS FROM CHROMATOGRAPHY OF PROTEINS

Figure 1: *Elution ionic strengths – comparison of different cation exchange ligands at pH 8.*

30 A-D. S-ligand (0.03, 0.09, 0.14 and 0.22 mmole/ml);

E. Phosphate (0.04 mmole/g);

F. BPR-butane-modified hydroquinone sulphonate (0.10 mmole/ml);

G. Sulphopropyl-modified hydroquinone sulphonate (0.25 mmole/ml);

H. Hydroquinone sulphonate (0.10 mmole/ml);

35 I. Sulphosalicylic acid (0.034 mmole/g);

J. TAPS (0.10 mmole/g);

- K. Sulphanilic acid (0.12 mmole/ml);
- L. 4-hydroxybenzoic acid (0.11 mmole/g);

Figure 2: *Elution ionic strengths – comparison of different cation exchange ligands at pH 4.*

- A-D. S-ligand (0.03, 0.09, 0.14 and 0.22 mmole/ml);
- E. Phosphate (0.04 mmole/g);
- F. BPR-butane-modified hydroquinone sulphonate (0.10 mmole/ml);
- G. Sulphopropyl-modified hydroquinone sulphonate (0.25 mmole/ml);
- 10 H. Hydroquinone sulphonate (0.10 mmole/ml);
- I. Sulphosalicylic acid (0.03 mmole/ml);
- J. TAPS (0.10 mmole/g);
- K. Sulphanilic acid (0.12 mmole/ml);
- L. 4-hydroxybenzoic acid (0.11 mmole/g);

15

Common to figure 1 and 2: The level of ligand of each prepared ion exchanger is indicated in parenthesis. Weight indications (g) relate to vacuum drained gel on a glass filter funnel.

20 Chromatography (proteins). Comparison of different cation exchange ligands coupled to cross-linked agarose gel according to above.

Ion exchange gels according to the descriptions for synthesis above were packed in a HR 10/2 column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) to a gel height of 2-2.5 cm at back pressure of about 1 bar.

- 25 The chromatographic evaluation was performed under the conditions and samples indicated below in a FPLC system from Amersham Pharmacia Biotech AB, Uppsala, Sweden.

At pH 8

30

- | | |
|--------------|--|
| Sample: | Ribonuclease A 1 mg/ml, Chymotrypsinogen A 1 mg/ml,
Cytochrome C 1 mg/ml, Lysozyme 1 mg/ml, |
| Loop volume: | 100 µl |
| Buffer A: | HEPES-NaOH 20 mM, pH 8.0 |
| 35 Buffer B: | HEPES-NaOH 20 mM, NaCl 0.5 or 1.0 M, pH 8.0 |
| Flow: | 0.5 ml/min |

Gradient slope: 0.05 M/ml
 UV detector: 280 nm 0.05 AU
 Conductometer Max = 100 mS/cm

5 At pH 4

Sample: Wheat-germ lectin (3 peaks) 1 mg/ml and β -lactoglobulin
 1 mg/ml,
 Loop volume: 100 μ l
 Buffer A: Formic acid-NaOH 20 mM, pH 8.0
 10 Buffer B: Formic acid-NaOH 20 mM, LiCl 0.75 or 1.0 M, pH 8.0
 Flow: 0.5 ml/min
 Gradient slope: 0.075 M/ml
 UV detector: 280 nm 0.05 AU
 Conductometer Max = 100 mS/cm

15

Results:

Figure 1 depicts the elution ionic strength in mS/cm at pH 8.0 for prepared ion exchangers for the studied model proteins. Ion exchangers, which under existing conditions exhibit a ligand with a negative net charge and an available
 20 functional group according to the invention have a different ability to separate the studied model proteins from each other by cation exchange chromatography. One difference is a significant increased dynamics, i.e. the elution ionic strength values for the sample substances included in the separation extend over a wider ionic strength interval. Another difference is that higher or con-
 25 siderably very much higher elution ionic strengths are required to elute all the used sample substances. These effects appear more or less simultaneously.

Figure 2 shows results from several chromatography runs at pH 4. At this low pH value the ligands of the example have no or reduced negative net charge. The TAPS-ligand is an example of a ligand where the net charge is zero
 30 and to which none of the sample substances adhere.

The sulphanilic acid group has a slightly positive net charge at pH 4. The amino group is completely charged (pKa just above 7) and the sulphonate group (pKa of approximately 3) starts to uncharge.

35 The result for the partially uncharged sulphonate group in the sulphanilic acid-ligand illustrates that the interaction is complex and that the result

maybe be difficult to interpret based on mechanistic terms. The carboxylic group of the 4-hydroxybenzoic acid-ligand is also uncharged at pH 4, while the hydroquinone sulphonate-ligand is charged at pH 4 and gives strong binding. Transforming a hydroxy group of the hydroquinone sulphate ligand to an ether group will dramatically change the binding capacity (for instance by (a) allylation and subsequent transformation of the double bound to a sulphonate group or (b) alkylation with BPR-butane (1,4-diglycidylbutylether)). This kind of modifications means that the aromatic group becomes sterically shielded while at the same time the phenolic hydroxyl becomes part of an ether bond. The result is that the sample substances will have an elution behaviour that is very much alike their behaviour on the reference ion exchanger (sulphonate-ligand).

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form or suggestion that that prior art forms part of the common general knowledge in Australia.

It would be appreciated by a person skilled in the art the numerous variations and/or modifications may be made to the invention as shown the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.



- 20 -

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A process for separation of positively charged compounds from a solution
 5 comprising the steps of contacting the solution with a cation exchanger under conditions permitting binding of one of more of the positively charged compounds to the cation exchanger, and, if desired, eluting bound compounds, wherein

(i) the ionic strength in the solution during binding is >15 mS/cm, and
 the ionic strength in the solution during elution exceeds the ionic strength applied when
 10 binding,
 and

(ii) the cation exchanger has the formula (I)



in which formula

- 15 a) M is a hydrophilic matrix;
 b) A is a spacer, and
 c) $X-Y(-Z)_n$ is a cation exchanging ligand; wherein
 d) X is -O-, -S(R₁)- or -N(R₁)(R₂)-, wherein R₁ and R₂ are selected among H, a
 20 free electron pair, A-X-Y(-Z)_n, Y(-Z)_n and straight or branched aliphatic, C₁-C₆ groups, which for C₂₋₆ alkyl may have 1-4 of its hydrogen atoms replaced by OH groups in which a hydrogen may be replaced by a lower alkyl, with the proviso that only one of R₁ and R₂ can be a free electron pair;
 e) Y may be:
 25 1) a 5- or 6-membered cyclic structure containing double bonds, or
 2) an alicyclic C₅-C₆ group or an aliphatic, straight or branched C₁-C₈ group with the possibility that the chain joining X with Z may be interrupted by one or two ether oxygen or thioether sulphur atoms and with the proviso that X is not -O- or -S-; and
 f) Z is a cation-exchanging group;

and in that the binding between the cation exchanger and the positively charged



- 21 -

compound(s) is allowed to proceed at a pH value within the range of 2-14 at which ligands -X-Y(-Z)_n have a negative net charge.

2. The process of claim 1 wherein X is -O-, -S(R₁)- or -N(R₁)(R₂)-, wherein R₁ and R₂ are selected among H, a free electron pair, A-X-Y(-Z)_n, Y(-Z)_n and straight or branched saturated aliphatic C₁-C₆ groups, which for C₂₋₆ alkyl may have 1-4 of its hydrogen atoms replaced by OH groups in which a hydrogen may be replaced by a lower alkyl, with the proviso that only one of R₁ and R₂ can be a free electron pair.

3. The process of claim 1 or claim 2 in which Y is a 5- or 6-membered aromatic cyclic structure.

4. The process of claim 1 or claim 2 in which Y is an alicyclic C₅-C₆ group or a saturated aliphatic, straight or branched C₁-C₈ group with the possibility that the chain joining X with Z may be interrupted by one or two ether oxygen or thioether sulphur atoms and with the proviso that X is not -O- or -S-.

5. The process of any one of claims 1 to 4 wherein

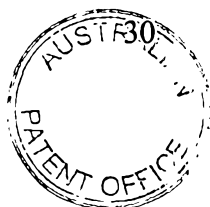
- a) the cation exchanger provides for binding at a higher ionic strength than the elution ionic strength required for eluting said one or more compounds at the same pH value from the reference ion exchanger defined in claim 1, and
- b) binding is allowed to occur at such a higher ionic strength.

6. The process according to claim 5 wherein

- a) the cation exchanger provides for binding at at least 25% higher ionic strength than the elution ionic solution.

7. The process of claim 6 wherein

- a) the cation exchanger provides for binding at at least 40% higher ionic strength than the elution ionic solution.



- 22 -

8. The process of claim 7 wherein

- a) the cation exchanger provides for binding at at least 100% higher ionic strength than the elution ionic solution.

5 9. The process of any one of claims 1 to 8, wherein the ionic strength in the solution during binding is >20 mS/cm.

10. The process of claim 9 wherein the ionic strength in the solution during binding is >30 mS/cm.

10

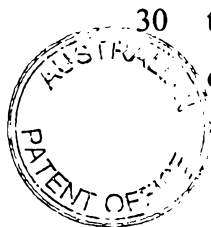
11. The process of claim 10 wherein the ionic strength in the solution during binding is >40 mS/cm.

12. The process of any one of claims 1 to 11, wherein the elution is carried out under
15 conditions neutralising the binding of the compound(s) to the ligand.

13. The process of any one of the claims 1 to 12, wherein the elution is carried out at a pH, which is separate from the pH at which binding has been performed.

20 14. The process of any one of the claims 1 to 13, wherein it can be used at a higher maximum elution ionic strength in the pH interval 2-14 for one or more proteins selected among ribonuclease, chymotrypsinogen A, cytochrome C, lysozyme, wheat-germ lectin and β -lactoglobulin, than the corresponding elution ionic strength measured at the same conditions on a reference ion exchanger on which the ion exchanging group (Z) is
25 sulphonate ($-\text{SO}_3^-$) and the base matrix, spacer and the degree of substitution is the same as in the cation exchanger of formula I but the groups X and Y are absent.

15. The process of any one of claims 1 to 14, wherein the elution is carried out by applying a change in ionic strength covering an interval which is at least 20% broader than
30 the corresponding interval for eluting the compound on the reference ion exchanger defined in claim 14.



- 23 -

16. The process of claim 15 wherein the elution is carried out by applying a change in ionic strength covering an interval which is at least 40% broader than the corresponding interval for eluting the compound on the reference ion exchanger defined in claim 14.

5

17. The process of any one of claims 1 to 16, wherein the elution is carried out by applying a change in ionic strength covering an interval which is at least 50% greater than the corresponding interval for the reference ion exchanger defined in claim 1 and measured at, in other respects, the same conditions.

10

18. The process of claim 17, wherein the elution is carried out by applying a change in ionic strength covering an interval which is at least 75% greater than the corresponding interval for the reference ion exchanger.

15

19. The process of claim 18, wherein the elution is carried out by applying a change in ionic strength covering an interval which is at least 100% greater than the corresponding interval for the reference ion exchanger.

20

20. The process of any one of claims 1 to 19, wherein the ionic strength interval used for elution of at least one of the compounds is >20 mS/cm.

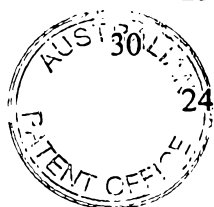
21. The process of claim 20, wherein the ionic strength interval used for elution of at least one of the compounds is >50 mS/cm.

25

22. The process of any one of claims 1 to 21, wherein 1-4 OR' groups are present in any one of the groups R_1 and R_2 with R' being selected among the same groups as R_1 and R_2 .

23. The process of claim 22, wherein R' is H.

24. The process of any one of claims 1 to 23, wherein X is $-N(R_1)(R_2)-$.



- 24 -

25. The process of any one of claims 1 to 24, wherein $X = -N(R_1)(R_2)-$ and that there is/are 1-4 OR' groups, at a distance of 2 or 3 carbon atoms from the nitrogen atom in $-N(R_1)(R_2)-$ where R' is selected among the same groups as R_1 and R_2 .

5

26. The process of claim 24 or 25, wherein Y and/or A provide a hydroxy group at a distance of two or three atoms from the nitrogen atom in $-N(R_1)(R_2)-$.

27. The process of any one of claims 1 to 26, wherein in Y, R', R_1 and R_2 only one
10 atom selected among sulphur, oxygen and nitrogen binds to each carbon atom.

28. The process of any one of claims 1 to 27, wherein Z is selected among carboxylate, sulphonate, phosphonate, phosphate including charged protonated forms thereof.

15 29. The process of any one of claims 1 to 17, 27 or 28, wherein X is $-O-$ or $-S(R_1)-$.

30. The process of any one of claims 1 to 29, wherein Y is an aromatic group.

31. The process of claim 30 wherein Y is a benzene ring.

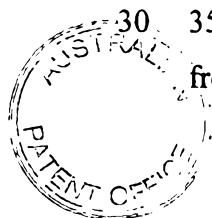
20

32. The process of any one of claims 1 to 31, wherein the ligand $-X-Y(-Z)_n$ is derived from hydroxybenzoic acid and/or sulphosalicylic acid.

33. The process of claim 32, wherein the ligand $-X-Y(-Z)_n$ is derived from para
25 hydroxybenzoic acid and/or para sulphosalicylic acid.

34. The process of claim 32 or 33, wherein the ligand $-X-Y(-Z)_n$ is derived via coupling of a ring bound OH group to the spacer.

30 35. The process of any one of claims 1 to 34, wherein the ligand $-X-Y(-Z)_n$ is derived from hydroquinone sulphonate.



- 25 -

36. The process of claim 35, wherein the ligand $-X-Y(-Z)_n$ is derived via coupling of a ring bound OH group to the spacer.

5 37. The process of claim 36, wherein $n = 2$, $Z_1 = -SO_3-$ and $Z_2 = -OH$ or $-O-$ and is coupled via a OH group which is meta or ortho to the $-SO_3-$.

38. The process of any one of claims 1 to 35, wherein Y is an aliphatic group selected among C_2-C_6 groups.

10

39. The process of claim 38, wherein Y is selected among C_2-C_4 groups.

40. The process of claim 38 or 39, wherein X is $-N(R_1)(R_2)-$, R_1 is a free electron pair of hydrogen, R_2 is tris(hydroxymethyl)methyl, Y is $-CH_2CH_2CH_2-$ or $-CH_2CHOHCH_2-$, Z is SO_3- and n is 1.

15

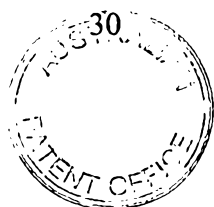
41. A cation exchanger suitable for use in a process as defined in any one of claims 1 to 40, wherein it is defined by formula (I) below



20 in which formula

- a) M is a hydrophilic matrix;
- b) A is a spacer, and
- c) $X-Y(-Z)_n$ is a cation exchanging ligand; wherein
- d) X is $-O-$, $-S(R_1)-$ or $-N(R_1)(R_2)-$, wherein R_1 and R_2 are selected among H, a free electron pair, $A-X-Y(-Z)_n$, $Y(-Z)_n$ and straight or branched aliphatic C_1-C_6 groups, which for C_{2-6} alkyl may have 1-4 of its hydrogen atoms replaced by OH groups in which a hydrogen may be replaced by a lower alkyl, with the proviso that only one of R_1 and R_2 can be a free electron pair;
- e) Y may be:
 - 1) a 5- or 6-membered cyclic structure containing double bonds, or

25



- 26 -

- 2) an alicyclic C₅-C₆ group or an aliphatic, straight or branched C₁-C₈ group with the possibility that the chain joining X with Z may be interrupted by one or two ether oxygen or thioether sulphur atoms and with the proviso that X is not -O- or -S-; and

5 f) Z is a cation-exchanging group.

42. The cation exchanger of claim 41, wherein X is -O-, -S(R₁)- or -N(R₁)(R₂)-, wherein R₁ and R₂ are selected among H, a free electron pair, A-X-Y(-Z)_n, Y(-Z)_n and straight or branched saturated aliphatic C₁-C₆ groups, which for C₂₋₆ alkyl may have 1-4 of
10 its hydrogen atoms replaced by OH groups in which a hydrogen may be replaced by a lower alkyl, with the proviso that only one of R₁ and R₂ can be a free electron pair.

43. The cation exchanger of claim 41 or claim 42 in which Y is a 5- or 6-membered aromatic cyclic structure.

44. The cation exchanger of claim 41 or claim 42 in which Y is an alicyclic C₅-C₆ group or a saturated aliphatic, straight or branched C₁-C₈ group with the possibility that the chain joining X with Z may be interrupted by one or two ether oxygen or thioether sulphur atoms and with the proviso that X is not -O- or -S-.

45. The cation exchanger of any one of claims 41 to 44, wherein X of formula (I) is -N(R₁)(R₂)-.

46. The cation exchanger of any one of claims 41 to 45, wherein X = -N(R₁)(R₂)- and that there is 1-4 OR' groups, at a distance of 2 or 3 carbon atoms from the nitrogen atom in -N(R₁)(R₂)- where R' is selected among the same groups as R₁ and R₂.

47. The cation exchanger of claim 46, wherein there is 1-3 OR' groups.

48. The cation exchanger of any one of claims 41 to 47, wherein Y and/or A provide a hydroxy group at a distance of two or three atoms from the nitrogen atom in -N(R₁)(R₂)-.



- 27 -

49. The cation exchanger of any one of claims 41 to 48, wherein in Y, R', R₁ and R₂ only one atom selected among sulphur, oxygen and nitrogen binds to each carbon atom.

5 50. The cation exchanger of claim 41 substantially as hereinbefore described with reference to the Examples.

51. The use of the process of any one of claims 1 to 40 for the separation of positively charged biomolecules from a solution.

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52. The use of the process of any one of claims 1 to 40 for the separation of positively charged amphoteric biomolecules from a solution.

53. The use of the process of any one of claims 1 to 40 for the separation of positively charged proteins from a solution.

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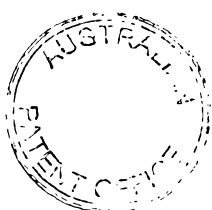
54. The process of claim 25 wherein there is/are 1-3 OR' groups.

DATED this 28th day of January, 2003

20 **Amersham Biosciences AB**

by DAVIES COLLISON CAVE
Patent Attorneys for the Applicant

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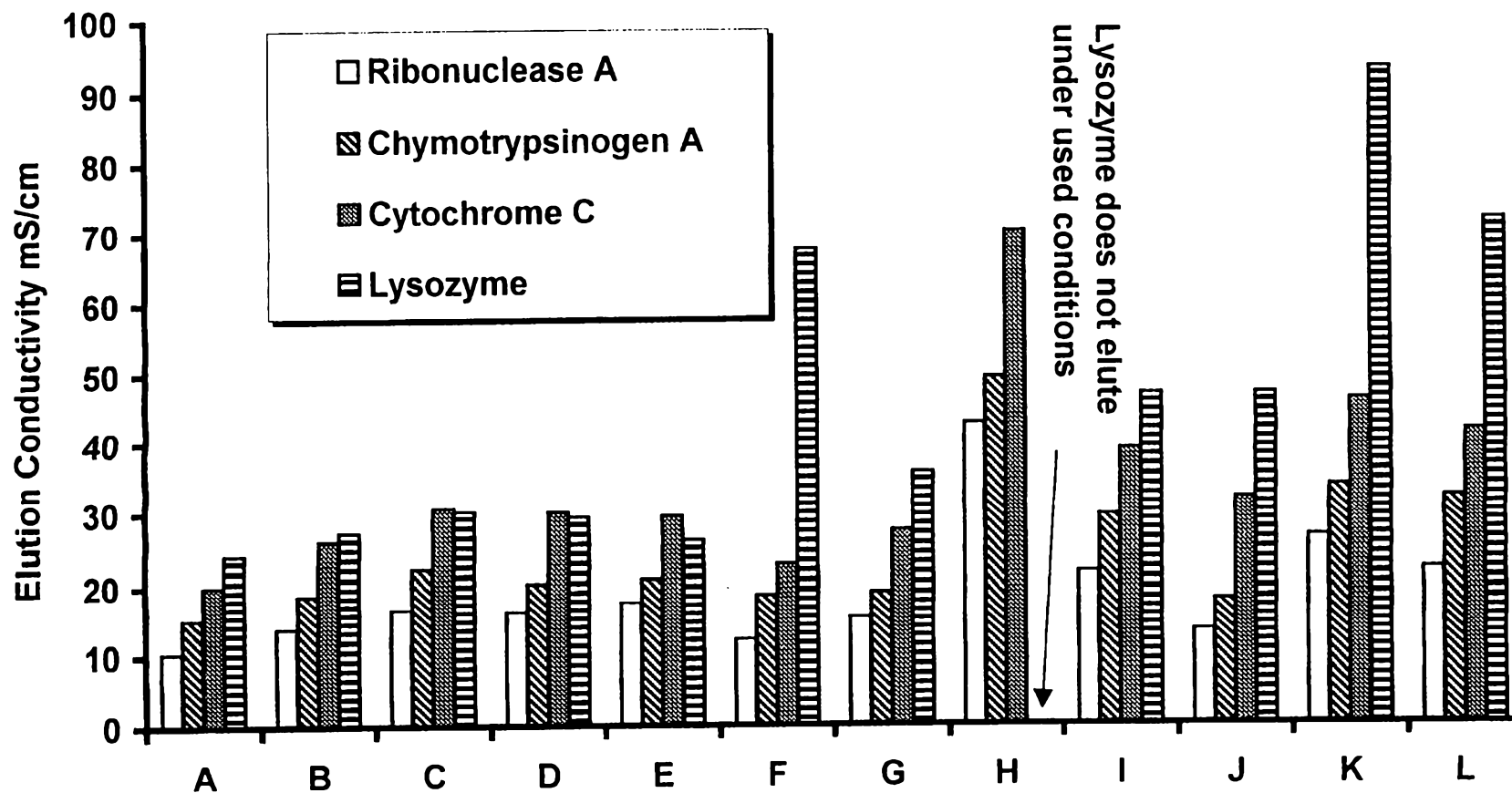


Figure 1. Comparison of Cation Ligands at pH 8,0

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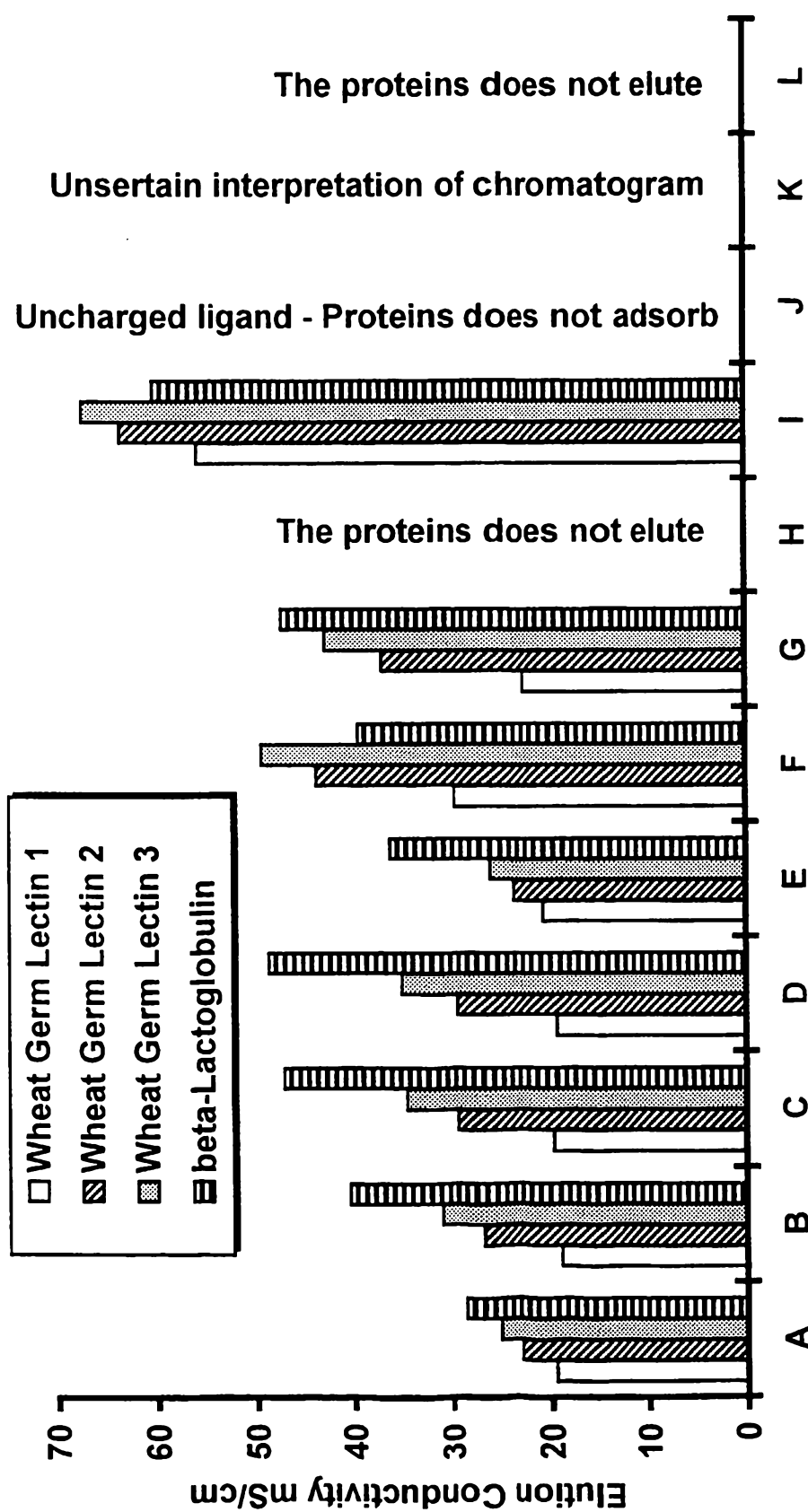


Figure 2. Comparison of Cation Ligands at pH 4,0