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(54) **Title:** METHODS FOR PURIFYING TNFR:FC

(57) **Abstract:** The present invention relates to a method for purifying a TNFR:Fc fusion protein, comprising the steps of: (a) subjecting a solution comprising said TNFR:Fc to affinity chromatography; (b) subjecting the eluate of step (a) to anion exchange chromatography; and (c) subjecting the eluate or the flow-through of step (b) to hydrophobic interaction chromatography (HIC), and collecting the eluate to obtain purified TNFR:Fc. In a preferred embodiment, step (c) is carried out as multicolumn countercurrent solvent gradient purification (MCSGP). Further provided is a purified TNFR:Fc composition, obtained by the method disclosed herein.

Methods for purifying TNFR:Fc

5 The present invention is directed to methods for purifying TNFR:Fc, a fusion protein which is used in a variety of therapeutic applications. More specifically, the invention pertains to a method for purifying TNFR:Fc, as defined in the claims and to a purified TNFR:Fc composition, obtained by the method disclosed herein.

10 BACKGROUND OF THE INVENTION

Tumor Necrosis Factor alpha (TNF-alpha) is a member of a group of cytokines that stimulate the acute phase reaction, and thus is a cytokine involved in systemic inflammation. TNF-alpha is able to induce inflammation, induce apoptotic cell death, and to inhibit tumorigenesis and viral replication. Dysregulation of TNF-alpha production has been implicated in a variety of human diseases like autoimmune disease, ankylosing spondylitis, juvenile rheumatoid arthritis, psoriasis, psoriatic arthritis, rheumatoid arthritis, Wegener's disease (granulomatosis), Crohn's disease or inflammatory bowel disease, chronic obstructive pulmonary disease (COPD), Hepatitis C, endometriosis, asthma, cachexia, atopic dermatitis, Alzheimer as well as cancer.

Its receptor molecules include TNFR1 and TNFR2. TNF-R1 is expressed in most tissues and can be fully activated by both the membrane-bound and soluble trimeric forms of TNF, whereas TNF-R2 is found only in cells of the immune system and responds to the membrane-bound form of the TNF homotrimer. Upon contact with TNF-alpha, TNF receptors form trimers and thereby initiate intracellular cell signaling.

Accordingly, soluble TNFR molecules or fragments thereof, which are able to bind to TNF-alpha, can be used as a competitive inhibitor for TNF-alpha. The present disclosure relates to such soluble TNFR molecules fused to an Fc portion of a human immunoglobulin (TNFR:Fc), and more particularly to methods for purifying such TNFR:Fc molecules.

WO 94/06476 describes a hypothetical purification method for purifying a TNFR antagonist, wherein the method comprises a concentration step, followed by application of an affinity matrix, an anion exchange resin, and/or a cation exchange step, and a final reversed-phase high performance liquid chromatography (RP-HPLC) employing hydrophobic RP-HPLC media.

WO 03/059935 discloses a purification process for TNFR:Fc, wherein the TNFR:Fc is first purified by affinity chromatography on protein A, and leaching protein A is subsequently removed from the TNFR:Fc by hydroxyapatite chromatography.

WO 2005/075498 describes a method for purifying a protein using hydrophobic interaction chromatography as an intermediate step.

WO 2006/116886 discloses multicolumn purification processes in general and their optimizations.

WO 2008/025747 describes a method for purifying an Fc-fusion proteins, wherein the method comprises Protein A or Protein G affinity chromatography, cation exchange chromatography, anion exchange chromatography, and hydroxyapatite chromatography, in that order.

There is still a need in the art for new methods for purifying TNFR:Fc, in particular methods resulting in an improved yield, satisfactory purity, and at the same time at reduced costs.

SUMMARY OF THE INVENTION

The present invention relates to a method for purifying a TNFR:Fc fusion protein, comprising the steps of:

- (a) subjecting a solution comprising said TNFR:Fc to affinity chromatography;
- (b1) optionally subjecting the eluate of step (a) to anion exchange chromatography;
- (b2) subjecting the eluate of step (b1), or in absence of step (b1) the eluate of step (a) to multimodal anion exchange chromatography (MMC);
- (c) subjecting the flow-through of step (b2) to hydrophobic interaction chromatography (HIC), and collecting the eluate to obtain purified TNFR:Fc.

In an alternative embodiment, the present invention relates to a method for purifying a TNFR:Fc fusion protein, comprising the steps of:

- (a) subjecting a solution comprising said TNFR:Fc to affinity chromatography;
- (b2) subjecting the eluate of step (a) to multimodal anion exchange chromatography (MMC);
- (b1) subjecting the flow-through of step (b2), to anion exchange chromatography;
- (c) subjecting the eluate of step (b1) to hydrophobic interaction chromatography (HIC), and collecting the eluate to obtain purified TNFR:Fc.

In a preferred embodiment, step (c) is carried out as multicolumn countercurrent solvent gradient purification (MCSGP).

In another aspect, the present invention is directed to a purified TNFR:Fc composition, obtained by the method disclosed herein.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present disclosure provides a method for purifying a TNFR:Fc fusion protein, comprising the steps of:

- (a) subjecting a solution comprising said TNFR:Fc to affinity chromatography;
- (b1) optionally subjecting the eluate of step (a) to anion exchange chromatography;
- (b2) subjecting the eluate of step (b1), or in absence of step (b1) the eluate of step (a) to multimodal anion exchange chromatography (MMC);

- (c) subjecting the flow-through of step (b2) to hydrophobic interaction chromatography (HIC), and collecting the eluate to obtain purified TNFR:Fc.

In an alternative embodiment, the present invention provides a method for purifying a TNFR:Fc fusion protein, comprising the steps of:

- (a) subjecting a solution comprising said TNFR:Fc to affinity chromatography;
(b2) subjecting the eluate of step (a) to multimodal anion exchange chromatography (MMC);
(b1) subjecting the flow-through of step (b2), to anion exchange chromatography;
(c) subjecting the eluate of step (b1) to hydrophobic interaction chromatography (HIC), and collecting the eluate to obtain purified TNFR:Fc.

In the context of the present disclosure, the TNFR part of TNFR:Fc refers to any TNFR polypeptide having at least 90 %, preferably at least 91 %, such as at least 92 % or at least 93 %, more preferably at least 94 %, such as at least 95 %, or at least 96 %, even more preferably at least 97 %, such as at least 98 %, or at least 99 %, and most preferably 100 % identity over the full length of an amino acid sequence comprising at least 150-250, preferably at least 175-245 of TNFR1 or TNFR2, preferably TNFR2, more preferably 200-240, and most preferably 233-235 amino acids of the extracellular part of TNFR2, and still binding to TNF-alpha, as determined by ELISA or any other convenient assay. More preferably, said TNFR is capable of binding to TNF-alpha and Lymphotoxin alpha (LT-alpha), as determined by ELISA or any other convenient assay. Such assays are well-known to the skilled person.

The CDS and protein sequences of TNFR1 (TNF receptor type 1; CD120a; p55/60; for human: RefSeq (mRNA): NM_001065, RefSeq (protein): NP_001056 (SEQ ID NO:1)) and TNFR2 (TNF receptor type 2; CD120b; p75/80; for human: RefSeq (mRNA): NM_001066, RefSeq (protein): NP_001057 (SEQ ID NO:2)) are known in the art.

Generally, a polypeptide has "at least x % identity" over the full length of a defined length of amino acids with another polypeptide if the sequence in question is aligned with the best matching sequence of the amino acid sequence and the sequence identity between those two aligned sequences is at least x %. Such an alignment can be performed using for example publicly available computer homology programs such as the "BLAST" program, such as "blastp" provided at the NCBI homepage at <http://www.ncbi.nlm.nih.gov/blast/blast.cgi>, using the default settings provided therein. Further methods of calculating sequence identity percentages of sets of polypeptides are known in the art.

The Fc-region (fragment crystallisable region) refers to the tail region of an antibody, in the case of IgG composed of the second and third constant domain of the antibody's two heavy chains. In certain embodiments, the Fc polypeptide comprises the constant region of an IgG class heavy chain or a fragment and/or variant thereof and in other

embodiments the constant region of other immunoglobulin isotypes can be used to generate such TNFR:Fc fusions. For example, a TNFR:Fc polypeptide comprising the constant region of an IgM class heavy chain or a fragment and/or variant thereof could be used. Preferably, the Fc fragment is derived from IgG, more preferably from IgG1, even more preferably from human IgG1. The constant region of immunoglobulin heavy chains, with a specific example of a human IgG1 class heavy chain constant domain provided by SEQ ID NO: 3, comprises a CH1 domain (amino acids 1 through 98 of SEQ ID NO:3), a hinge region (amino acids 99 through 110 of SEQ ID NO:3), a CH2 domain (amino acids 111 through 223 of SEQ ID NO:3), and a CH3 domain (amino acids 224 through 330 of SEQ ID NO:3). As used herein, an Fc domain can contain one or all of the heavy chain CH1 domain, hinge region, CH2, and CH3 domains described above, or fragments or variants thereof. Certain embodiments of the invention include TNFR:Fc comprising all or a portion of the extracellular domain of TNFR1 (SEQ ID NO: 1) or TNFR2 (SEQ ID NO:2) fused to all or a portion of SEQ ID NO:3, optionally with a linker polypeptide between the TNFR portion and the Fc portion of the TNFR:Fc. For example, CH1, CH2 and the entire hinge region may be present in the molecule. In further embodiments, a heavy chain constant region comprising at least a portion of CH1 is the Fc portion of a TNFR:Fc. Certain embodiments can also include, for example, all of the hinge region or the C-terminal half of the hinge region to provide a disulfide bridge between heavy chains. If a dimeric TNFR:Fc is desired, it is important to include the portion of the hinge region implicated in disulfide bond formation between the heavy chains (for example, a portion of amino acids 99 through 110 of SEQ ID NO: 3 that includes amino acid 109 of SEQ ID NO: 3). In a preferred embodiment, the Fc portion consists of the full hinge region and the CH2 and CH3 domains. However, the TNFR:Fc can comprise portions of the CH3 domain that do not include the C-terminal lysine residue (amino acid 330 of SEQ ID NO: 3), as this residue has been observed to be removed in post-translational processing of IgG heavy chain polypeptides. Fc fusions and Fc fragments are well-known in the art.

Preferably, the TNFR:Fc is essentially identical / similar to etanercept, more preferably, the TNFR:Fc is etanercept. Etanercept is a dimer of two molecules of the extracellular portion of the p75 TNF-alpha receptor, each molecule consisting of a 235 amino acid TNFR2-derived polypeptide that is fused to a 232 amino acid Fc portion of human IgG1. The amino acid sequence of the monomeric component of etanercept is shown as SEQ ID NO:4. In the dimeric form of this molecule, two of these fusion polypeptides (or "monomers") are held together by three disulfide bonds that form between the immunoglobulin portions of the two monomers. The etanercept dimer therefore consists of 934 amino acids, and has an apparent molecular weight of approximately 150 kilodaltons. In North America, etanercept is marketed by Amgen under the trade name Enbrel®. Wyeth/Pfizer is the sole marketer of Enbrel® outside of North America excluding Japan where Takeda Pharmaceuticals markets the drug.

The term “essentially identical / similar to Etanercept” as used herein means that the amino acid sequence of the TNFR:Fc has at least 95% identity to the amino acid sequence shown in SEQ ID NO: 4, more preferably at least 96% identity, such as 97% identity, and most preferably 98% identity, such as 99% identity to the amino acid sequence shown in SEQ ID NO: 4. Alternatively or additionally, the TNFR:Fc may (only) differ from Etanercept by posttranslational modifications, e.g. by glycosylation. Suitable procedures for changing a glycosylation pattern, such as introducing or deleting a glycosylation site, and tests for determining a glycosylation pattern are well known to the skilled person.

The TNFR:Fc may be recombinantly produced, preferably by using a mammalian cell based expression system. Preferably, said mammalian cell-based expression system is at least one selected from the group consisting of Baby hamster kidney cell lines (e.g., BHK21); Chinese hamster ovary cell lines (e.g., CHO-K1, CHO-DG44, CHO-DXB, or CHO-dhfr-); Murine myeloma cell lines (e.g., SP2/0); Mouse myeloma cell lines (e.g., NS0); Human embryonic kidney cell lines (e.g., HEK-293); Human-retina-derived cell lines (e.g., PER-C6), and/or Amniocyte cell lines (e.g., CAP). Preferably, hamster cell based expression systems are being used. BHK21 (“Baby Hamster Kidney”) cells belong to a quasi-diploid established line of Syrian hamster cells, descended from a clone from an unusually rapidly growing primary culture of newborn hamster kidney tissue. Non limiting examples for BHK-21 cell lines which are commercially available and can be used in the context of the present invention are BHK-21 (C-13); BHK21-pcDNA3.1-HC; BHK570; Flp-In-BHK Cell Line; and/or BHK 21 (Clone 13) hamster cell line.

Chinese hamster ovary (CHO) cells are a cell line derived from the ovary of the Chinese hamster. They are often used in biological and medical research and are commercially utilized in the production of therapeutic proteins. They were introduced in the 1960s and were originally grown as a monolayer culture. Today, CHO cells are the most commonly used mammalian hosts for industrial production of recombinant protein therapeutics and are usually grown in suspension culture.

Non limiting examples for CHO cell lines which are commercially available and can be used in the context of the present invention are FreeStyle CHO-S cells; ER-CHO Cell Line; CHO 1-15 500 CHINESE HAM; CHO-DXB, CHO-dhfr-, CHO DP-12 clone#1934; CHO-CD36; CHO-ICAM-1; CHO-K1; Ovary; HuZP3-CHOlec3.2.8.1; xrs5; CHO-K1/BB2 Cells; CHO-K1/BB3 Cells; CHO-K1/EDG8/Gal α 15 Cells; CHO-K1/M5 Cells; CHO-K1/NK1 Cells; CHO-K1/NK3 Cells; CHO-K1/NMUR1 Cells; CHO-K1/NTSR1 Cells; CHO-K1/OX1 Cells; CHO-K1/PAC1/G α 15 Cells; CHO-K1/PTAFR Cells; CHO-K1/TRH1 Cells; CHO-K1/V1B Cells; 5HT1A Gal α -15-NFAT-BLA CHO-K1 Cell Line; AVPR2 CRE-BLA CHO-K1 Cell Line; CHO-S Cells SFM Adapted; DG44 Cells; Flp-In-CHO Cell Line; GeneSwitch-CHO Cell Line; NFAT-bla CHO-K1 Cell Line; T-REx-CHO Cell Line; GenoStat CHO K-1 Stable Cell Line; GenoStat CHO K-1 Stable Cell Line Kit; CHO-K1

Cell Line hamster, CHO-PEPT1 Cell line, CHO SSF3 and/or CHO-HPT1 Cell Line. In a particularly preferred embodiment, the hamster cell-based expression system is a CHO-dhfr- cell line.

The solution comprising the TNFR:Fc may be a cell culture material, such as a cell culture supernatant or a cell lysate. Preferably the solution is a cell culture supernatant, more preferably a cell free cell culture supernatant. In an even more preferred embodiment, the cell culture supernatant is serum-free supernatant, i.e. the supernatant is obtained from a cell culture in which the cells have been cultured under serum free conditions.

In a first step, the solution comprising the TNFR:Fc is subjected to affinity chromatography. The term "subjecting a solution comprising said TNFR:Fc to affinity chromatography" as used herein is intended to indicate that the affinity chromatography is specific for the TNFR:Fc, i.e. essentially only the TNFR:Fc is first bound to a resin via an interaction that is specific for the TNFR:Fc, then the resin is usually washed, whereafter the TNFR:Fc is eluted from the resin by applying suitable conditions. Affinity resins can be eluted by changing salt concentrations, pH, pl, charge and ionic strength in one or more steps or through a gradient to resolve the TNFR:Fc. The resin is typically a gel matrix, often of agarose, which has been modified in order to provide for specific interaction with TNFR:Fc.

For example, the affinity chromatography may be carried out on a resin modified with Protein A, Protein G, an antibody capable of binding the Fc-part of said TNFR:Fc, or an antibody directed against the TNFR-part of said TNFR:Fc. Preferably said resin is modified with Protein A or Protein G, and more preferably, said resin is modified with Protein A. Protein A is a protein originally found in the cell wall of *Staphylococcus aureus* which binds with high affinity to human IgG1 and IgG2 as well as mouse IgG2a and IgG2b. In addition, Protein A binds with moderate affinity to human IgA, IgE and IgM as well as to mouse IgG3 and IgG1. It does not react with human IgD or IgG3, or murine IgA, IgE and IgM. Alternatively, other Fc-binding bacterial proteins such as Protein G or Protein A/G may be used. Protein G has a binding affinity to human IgG1, IgG2 and IgG4, and to murine IgG2a and IgG2b that is comparable to Protein A. However, Protein G also binds to human IgG3 and rat immunoglobulins, and its binding affinity to murine IgG1 and IgG3 is increased as compared to Protein A. Protein G exhibits no apparent affinity to IgA, IgD, IgE, or IgM. Protein A/G is a recombinant fusion protein of both Protein A and Protein G. The binding of Protein A/G is less pH-dependent than Protein A, it binds to all subclasses of human and mouse IgG, binds to human IgA, IgE, IgM and (to a lesser extent) IgD, but does not bind mouse IgA or IgM. A particular suitable resin is MabSelect SuRe resin (GE Healthcare). Said resin has a mean particle size of 85 µm, and a loading capacity of 15-22 g/L resin. If the Fc-part of TNFR:Fc does not react with Protein A, Protein G or Protein A/G, one may use antibodies which are specific for said Fc-part or the TNFR-part.

Suitable antibodies will be apparent to those skilled in the art and are commercially available.

Binding of the TNFR:Fc to the affinity matrix or resin usually occurs at pH 6-8, preferably at pH 6.5-7.5, and more preferably at about pH 7.0. Hence, it may be necessary to adjust the pH of the solution prior to binding to the affinity resin. In a preferred embodiment, the resin having bound said TNFR:Fc is then washed with a suitable buffer having a pH at 6-8, preferably a pH at 6.5-7.5, and more preferably at a pH of about 7.0. For example, the buffer may be a phosphate buffer comprising 5-50 mM sodium phosphate. In a preferred embodiment, the washing buffer also comprises a salt such as sodium chloride, e.g. 20-200 mM sodium chloride.

The inventors have found that contamination with host cell proteins could be further reduced if the resin having bound said TNFR:Fc is subjected to another washing, which is preferably carried out after the previously described washing step. During this further washing, the affinity resin having bound said TNFR:Fc is washed with a second buffer having a slightly lower pH, such as a pH of 5-6.5, preferably a pH of 5.2-6.0. In one embodiment, said second buffer is also a phosphate buffer or a citrate buffer or an acetate buffer or a mixture of these buffers with a total molarity of 1-100 mM, preferably 5-50 mM. Preferably, this washing step can further comprise 0-750 mM sodium chloride, preferably 0-700 mM sodium chloride, more preferably 0-600 mM sodium chloride, and even more preferably 0-500 mM sodium chloride. The range of the sodium chloride given in this context of the second washing step during the affinity chromatography step should be understood as the washing may start at the highest concentration, and the concentration of sodium chloride is successively reduced, either stepwise or in form of a gradient. However, the skilled person will be aware that the final salt concentration does not need to go down to zero.

Elution of TNFR:Fc from the affinity matrix is preferably carried out by applying acidic conditions such as a pH ranging from 2.5 to 4.5, more preferably by applying a pH ranging from 3.0 to 3.5. In certain cases, it is desirable to apply a gradient starting from the higher pH towards the lower pH value. Elution may, for example, be carried out using a buffer comprising a buffer based on acetic acid, citric acid and/or phosphoric acid at concentrations of 1-100 mM, preferably 5-50 mM.

Additional parameters, such as flow rate, bed height of the column, etc. will have to be determined on a case by case basis using routine methods. However, to that end, it will be appreciated that affinity chromatographic procedures are well known in the art.

After the affinity chromatography, the TNFR:Fc is subjected to one or two steps of anion exchange chromatography (b), which allows separation and purification of molecules based on their charge. Preferably, the method comprises two steps of anion exchange chromatography.

The methods commonly used in the art apply a cation exchange step. In particular if a method contains two ion exchange chromatographic steps, it is general practice to apply at least one cation exchange chromatographic step. This is in contrast to the present method, which lacks any cation exchange chromatography step.

5 The anion exchange chromatography may be carried out in bind/elute mode or flow-through mode. If the TNFR:Fc is subjected to two steps of anion exchange chromatography, preferably one step is carried out in bind/elute mode, whereas the other step is carried out in flow-through mode. The two steps can be performed in any order. However, in certain instances, it can be preferred that the first anion exchange chromatography is carried out in bind/elute mode (e.g. as described in the following as
10 step (b1)) followed by a second anion exchange step carried out in flow-through mode (e.g. as described in the following as step (b2)).

The anion exchange chromatography may be carried out on any anion exchange resin suitable for use in the method disclosed herein. Depending on whether the charged group
15 of the resin is a weak or strong base, the anion exchange resin can be classified as being weak or strong. Wherein weak anion exchange resins can be positively charged below ~ pH 9, strong anion exchange resins remain positively charged throughout the pH range normally applied. The skilled person will know how to choose a suitable anion exchange resin. A particularly preferred anion exchange resin is Fractogel TMAE HiCap (M)
20 (commercially available from Merck).

The anion exchange chromatography may also use a multimodal chromatography (MMC) matrix. MMC, also called mixed mode chromatography, utilizes more than one form of interactions between the resin and the TNFR:Fc in order to achieve separation and purification. "Multimodal anion exchange chromatography (MMC)" is intended to mean a
25 chromatographic step using a (preferably strong) anion exchanger with multimodal functionality, the most pronounced of which are ionic interaction, hydrogen bonding and hydrophobic interaction. A suitable ligand for the resin is for example N-benzyl-N-methyl ethanol amine. Such resin is commercially available from GE Healthcare under the tradename Capto adhere. If the TNFR:Fc is subjected to two steps of anion exchange chromatography, the multimodal anion exchange chromatography is preferably applied in
30 the second step.

The method disclosed herein may thus comprise as the anion exchange chromatography step either step (b1) of subjecting the eluate of step (a) to anion exchange chromatography, OR step (b2) of subjecting the eluate of step (a) to multimodal anion
35 exchange chromatography (MMC). Preferably, the method disclosed herein comprises at least step (b2) of subjecting the eluate of step (a) to multimodal anion exchange chromatography (MMC).

More preferably, the method disclosed herein comprises in any order both step (b1) of subjecting the TNFR:Fc to classical anion exchange chromatography, AND step (b2) of

subjecting the TNFR:Fc to multimodal anion exchange chromatography (MMC). In a most preferred embodiment, the method comprises first step (b1) of subjecting the eluate of step (a) to anion exchange chromatography, followed by step (b2) of subjecting the TNFR:Fc resulting from step (b1) to multimodal anion exchange chromatography (MMC).

5 As noted above, in the latter case it may be preferred that the first anion exchange chromatography is carried out in bind/elute mode, whereas the second anion exchange step is carried out in flow-through mode. If the method only comprises multimodal anion exchange chromatography, it can be carried out in flow-through mode where the TNFR:Fc passes directly through the column while the contaminants are adsorbed.

10 The anion exchange chromatography step (b1) or (b2), or steps (b1) and (b2) allow for removal of leached Protein A, aggregates, host cell proteins, nucleic acids and viruses. Merely as an example, in the following a classical anion exchange chromatography step in bind/elute mode (b1) is described.

The TNFR:Fc is bound to the anion exchange resin at pH 7-8, preferably at pH 7.3-7.7, 15 more preferably at about pH 7.5. Accordingly, one will have to adjust the pH from the eluate from step (a), or dilute or dialyse the TNFR:Fc into an appropriate buffer.

Once the TNFR:Fc is bound to the anion exchange resin, said resin is washed with a buffer at pH 7-8, preferably at pH 7.3-7.7, more preferably at about pH 7.5. An appropriate washing buffer may be a phosphate buffer, e.g., a buffer comprising 1-50 mM sodium 20 phosphate, preferably 10-40 mM sodium phosphate.

Preferably, elution is carried out by changing the ionic strength while maintaining a similar pH. This can be done by increasing the salt concentration in the elution buffer. Hence, elution can be accomplished by using a buffer, such as a phosphate, citrate, or acetate buffer, or a mixture thereof, e.g. comprising 1-50 mM sodium phosphate, preferably 10-40 25 mM sodium phosphate, having a salt concentration that disturbs the ionic interaction between the TNFR:Fc and the anion exchange resin. For example, one may use 100-200 mM sodium chloride, preferably 110-190 mM sodium chloride, more preferably 120-180 mM sodium chloride. The range of the sodium chloride should be understood as the elution may start at the lowest concentration, and the concentration of sodium chloride is 30 successively increased, either stepwise or in form of a gradient.

Merely as an example, in the following a multimodal anion exchange chromatography step in flow-through mode (b2) is described.

For best results, conductivity of the TNFR:Fc containing solution, e.g. the eluate of step (a) or the eluate of step (b1), is adjusted prior to step (b2) to 20-60 mS/cm, preferably to 35 25-46 mS/cm; and to pH 5.5-6.5, preferably to pH 5.5-6.2. Loading and washing of said TNFR:Fc is also carried out in a buffer with a pH and a conductivity in the same ranges. The buffer may be a phosphate, citrate, or acetate buffer, or a mixture thereof, e.g. a buffer comprising 1-50 mM sodium phosphate, sodium citrate or sodium acetate, preferably 10-40 mM sodium phosphate, sodium citrate or sodium acetate; and 200-700

mM sodium chloride, preferably 250-600 mM sodium chloride. The concentration of the buffering salt and/or of the sodium chloride may be chosen as a gradient, or may be each a single concentration falling within the above ranges.

The fraction(s) obtained after the anion exchange chromatography step(s) comprising the TNFR:Fc is/are then subjected to a hydrophobic interaction chromatography (HIC). At high salt concentrations, nonpolar groups on the protein surface interact with the hydrophobic groups, e.g. octyl or phenyl groups, of the HIC resin. Particular useful HIC resins are the commercially available Phenyl Sepharose HP (GE Healthcare) and Toyopearl Phenyl 650, e.g. Toyopearl Phenyl 650 (M). Step (c) is particularly useful, since it allows separating undesired non-active or less active variants of TNFR:Fc (e.g. variants having false disulphide linkages). Since hydrophobic effects are augmented by increased ionic strength, the eluant is typically an aqueous buffer with decreasing salt concentrations, increasing concentrations of detergent (which disrupts hydrophobic interactions), and/or changes in pH. In a preferred embodiment, step (c) is carried out in a buffer having a pH ranging from 5.5-6.5, preferably pH 5.8-6.5, such as a pH of 6.0.

Further, prior to binding of the TNFR:Fc to the HIC resin, it may be necessary to adjust the fraction(s) comprising the TNFR:Fc obtained from step (b2), so that the conductivity of the solution is in the range of 50-100 mS/cm, preferably 70-85 mS/cm. This may be achieved, for example, by diluting the fraction(s) comprising the TNFR:Fc from step (b2) with a sodium citrate, sodium phosphate or sodium acetate buffer further comprising sodium sulphate at concentrations of or above 1 M sodium sulphate.

After loading of the TNFR:Fc, the HIC resin is washed with a suitable buffer. For example, the resin may be washed with a washing buffer comprising 50-150 mM sodium citrate, sodium phosphate or sodium acetate, preferably 50-100 mM sodium citrate or sodium phosphate; and sodium sulphate. In a preferred embodiment, the resin is washed with a washing buffer comprising 100 mM sodium phosphate and 0.6 M sodium sulphate; 50 mM sodium phosphate and 0.8 M sodium sulphate; 50 mM sodium phosphate and 0.95 M sodium sulphate; or 50 mM sodium citrate and 0.8 M sodium sulphate. The concentration of the buffer and/or of the sodium sulphate may be chosen as a gradient, or may be each a single concentration falling within the above ranges.

The TNFR:Fc is eluted in step (c) by applying a 0-100% gradient from said washing buffer to an elution buffer having a lower concentration of ions. For example, the elution buffer may be a citrate, phosphate or acetate buffer, preferably the same buffer system used in said washing buffer. More preferably, the elution buffer comprises 1-100 mM sodium citrate, sodium phosphate or sodium acetate, preferably 10-50 mM sodium citrate or sodium phosphate; and 0-100 mM sodium sulphate, and more preferably 0-10 mM sodium sulphate. Based on the actual data regarding yield and bioactivity for the eluted fractions obtained, the skilled person may select an optimal elution window, which represents the best compromise of yield, purity and bioactivity.

With the use of a HIC step, the degree of purity of the sample, as determined by size exclusion chromatography (SEC) can be increased to values above 90%, preferably above 92%, even more preferably above 95%. In particular, this HIC step (c) allows for the reduction of product-related impurities, such as degradation products (DPs) of TNFR:Fc, aggregation products (APs) of TNFR:Fc and other TNFR:Fc variants having reduced or no bioactivity. Such other TNFR:Fc variants might contain e.g. wrongly processed TNFR:Fc proteins or dimers, wrongly folded TNFR:Fc proteins or TNFR:Fc proteins or dimers with wrong intrachain and/or interchain disulphide bridging. It is understood by the skilled person that wrong disulphide bridging and wrong folding might be mutually dependent and/or synergistic.

In a particularly preferred embodiment of the method disclosed herein, step (c) is carried out as multicolumn countercurrent solvent gradient purification (MCSGP), preferably in a twin-column setup. The process principle of MCSGP for a twin-column setup is shown in Figure 1. The schematic chromatogram at the bottom of Figure 1 represents a batch chromatogram that has been divided into different sections (vertical dashed lines) according to the tasks that are carried out in the batch chromatography run (equilibration, feeding, elution, cleaning) and according to the elution order of the chromatogram: elution of weakly adsorbing impurities W, elution of the overlapping part W/P (W and the product P), elution of pure P, elution of the overlapping part of P/S (product P and S) and elution of the strongly adsorbing impurities S. In the MCSGP process these individual tasks correspond to unique flow paths in the system and are carried out analogously in pairs in the twin column setup. Thus the process tasks of the single column batch process and the MCSGP process are analogous and it is possible to derive the operating parameters for MCSGP from the batch operating parameters and the corresponding chromatogram. Briefly, the operating parameters are computed such that the elution volumes and the gradient start and end concentrations of the respective zones are the same in the batch and the MCSGP process.

A complete cycle of a twin-column MCSGP process comprises two "switches" with four pairs of tasks each (I1, B1, I2, B2) as illustrated in Figure 1. The phases in each switch are identical; the difference is only in the column position: In the first switch, column 1 is downstream of column 2 while in the second switch (not shown in Figure 1) column 2 is downstream of column 1. The four phases comprise the following tasks:

- Phase I1: The overlapping part W/P is eluted from the upstream column, and internally recycled into the downstream column (zones 5 and 1, respectively). In between the columns, the stream is diluted inline with solvent (indicated by the upward point arrow) to re-adsorb P (and overlapping W) in the downstream column. At the end of phase I1, pure product is ready for elution at the outlet of the upstream column (zone 5).

- Phase B1: Pure P is eluted and collected from the column in zone 5 (column 2 in Figure 1), keeping the overlapping part P/S and S in the column. At the same time, fresh feed is injected into the column in zone 2.
- Phase I2: The overlapping part P/S is eluted from the upstream column, and internally recycled into the downstream column (zones 7 and 3, respectively). In between the columns, the stream is diluted inline with solvent (indicated by the upward point arrow) to re-adsorb P in the downstream column. At the end of the step, all remaining P has been eluted from the upstream column and only S is left in the upstream column.
- Phase B2: The column in zone 8 (column 2 in Figure 1) is cleaned to remove S and re-equilibrated. At the same time, W is eluted from the other column in zone 4.

After having completed these tasks, the columns switch positions and in the next phase I1 (not shown in Figure 1), column 2 is in the downstream position (zone 1) and column 1 is in the upstream position (zone 5). At the beginning of this I1 phase, Column 2 is cleaned and re-equilibrated and ready for uptake of the W/P fraction from column 1. After having completed B1, I2, and B2 for the second time the columns are returning to their original positions and one cycle has been completed. At this point column 1 is clean and ready for uptake of W/P of the column 2 in phase I1 (as shown in Figure 1).

In contrast to the 3-column process, the twin-column process features an independent recycling of W/P and P/S which improves the flexibility in terms of the recycling phase durations at the cost of slightly increased solvent consumption. As in other countercurrent chromatographic processes, in practice the column movement is preferably simulated by connecting and disconnecting column inlets and outlets through valve switching and not by physical movement of the columns in MCSGP.

The settings of the boundaries for the recycling W/P and P/S fractions can be determined as described in Example 2 below. In a preferred embodiment in which step (c) is carried out as MCSGP, recycling fractions W/P are pooled from the point where the ascending UV_{280nm} value reaches 20% peak maximum to 90% peak maximum on the descending side of the elution peak, preferably from the point where the ascending UV_{280nm} value reaches 80% peak maximum to 95% peak maximum on the descending side of the elution peak;

product elution fractions P are pooled from the time point when the ascending UV_{280nm} value reaches 90% of the peak maximum until the descending UV_{280nm} value reaches 20% of the peak maximum, preferably from the time point when the descending UV_{280nm} value reaches 95 % of the peak maximum until the descending UV_{280nm} value reaches 30 % of the peak maximum;

recycling P/S fractions are pooled from the point where the descending UV_{280nm} value reaches 50% peak maximum to 10% peak maximum on the descending side of the elution

peak, preferably from the point where the descending UV_{280nm} value reaches 30% peak maximum to 20% peak maximum on the descending side of the elution peak.

However, it is noted that the above boundaries depend on the actual buffer system used and the relative concentrations of the product and the impurities.

5 For further information on the application of MCSGP, reference is made to WO 2006/116886, the disclosure of which is herein incorporated by reference.

This use of the MCSGP is to our knowledge the first demonstration of a continuous chromatography method for the purification of biological pharmaceutical substance on an industrial scale.

10 In further preferred embodiments, the method may further comprise a step (d), wherein the eluate of step (c) is subjected to nanofiltration, ultrafiltration and/or diafiltration, in order to separate any inactivated viruses or other contaminants from the purified solution and/or transfer the purified TNFR:Fc into a more suitable buffer in order to render the TNFR:Fc ready for further processing. For example, the purified TNFR:Fc may be
15 formulated into a pharmaceutical composition.

In another aspect, the present disclosure provides a purified TNFR:Fc composition, obtained by the methods as disclosed herein. Said purified TNFR:Fc composition is low in contaminants, such as host cell proteins, virus / DNA, endotoxins, and leached Protein A. In addition, it is low in non-desired variants of TNFR:Fc, such as wrongly processed or
20 folded variants, which are less or non-active. Specifically, the purification process as described herein may be used to reduce a product-related TNFR:Fc impurity wherein wrong intrachain disulphide bridging affects the TNF binding site, such as reducing a variant with a Cys₇₈-Cys₈₈ disulphide bridging when the TNFR:Fc is etanercept. In the latter, the method of the present disclosure is preferably combined with a method which is
25 capable of detecting the content of Cys₇₈-Cys₈₈ disulphide bridged TNFR:Fc in chromatographic fractions containing TNFR:Fc.

These advantages will be further apparent in detail from the following examples. These characteristics render the purified TNFR:Fc composition obtained by the method as disclosed herein particularly useful in medical applications, e.g. as disclosed in the
30 background section above.

The invention is further described by the following embodiments.

1. Method for purifying a TNFR:Fc fusion protein, comprising the steps of:

- (a) subjecting a solution comprising said TNFR:Fc to affinity chromatography;
- (b1) optionally subjecting the eluate of step (a) to anion exchange chromatography;
- 35 (b2) subjecting the eluate of step (b1), or in absence of step (b1) the eluate of step (a) to multimodal anion exchange chromatography (MMC);
- (c) subjecting the flow-through of step (b2) to hydrophobic interaction chromatography (HIC), and collecting the eluate to obtain purified TNFR:Fc.

2. Method for purifying a TNFR:Fc fusion protein, comprising the steps of:

- (a) subjecting a solution comprising said TNFR:Fc to affinity chromatography;
- (b2) subjecting the eluate of step (a) to multimodal anion exchange chromatography (MMC);
- (b1) subjecting the flow-through of step (b2) to anion exchange chromatography;
- (c) subjecting the eluate of step (b1) to hydrophobic interaction chromatography (HIC), and collecting the eluate to obtain purified TNFR:Fc.

3. The method of embodiment 1 or 2, wherein step (c) is carried out as multicolumn countercurrent solvent gradient purification (MCSGP).
4. The method of any one of embodiments 1 to 3, wherein the purification process does not use any cation exchange chromatography step.
5. The method of any one of embodiments 1 to 4, wherein in step (a) is carried out on a resin modified with Protein A, Protein G, an antibody capable of binding the Fc-part of said TNFR:Fc, or an antibody directed against the TNFR-part of said TNFR:Fc; preferably wherein said resin is modified with Protein A or Protein G, and more preferably, wherein said resin is modified with Protein A.
6. The method of any one of embodiments 1 to 5, wherein said TNFR:Fc is bound in step (a) to the affinity resin at pH 6-8, preferably at pH 6.5-7.5, more preferably at about pH 7.0.
7. The method of any one of embodiments 1 to 6, wherein in step (a) the resin having bound said TNFR:Fc is washed with a buffer at pH 6-8, preferably at pH 6.5-7.5, more preferably at about pH 7.0.
8. The method of embodiment 7, wherein said resin is washed with a phosphate buffer comprising 5-50 mM sodium phosphate, preferably in the presence of a salt, more preferably in the presence of 20-200 mM sodium chloride.
9. The method of embodiment 7 or 8, wherein in step (a) the resin is subsequently washed with a second buffer which is a phosphate buffer, a citrate buffer, an acetate buffer or a mixture of any of these buffers at pH 5-6.5, preferably pH 5.2-6.0.
10. The method of embodiment 9, wherein said second buffer comprises 1-100 mM, preferably 5-50 mM of the buffering agent, and 0-750 mM sodium chloride, preferably 0-700 mM sodium chloride, more preferably 0-600 mM sodium chloride, and even more preferably 0-500 mM sodium chloride.
11. The method of any one of embodiments 1 to 10, wherein said TNFR:Fc is eluted in step (a) at a pH ranging from 2.5 to 4.5, preferably at a pH ranging from 3.0 to 3.5.
12. The method of embodiment 11, wherein said TNFR:Fc is eluted in step (a) using a buffer based on acetic acid, citric acid and/or phosphoric acid at concentrations of 1-100 mM, preferably 5-50 mM.
13. The method of any one of embodiments 1-12, wherein said TNFR:Fc is bound in step (b1) to the anion exchange resin at pH 7-8, preferably at pH 7.3-7.7, more preferably at about pH 7.5.

14. The method of any one of embodiments 1-13, wherein in step (b1) the resin having bound said TNFR:Fc is washed with a buffer at pH 7-8, preferably at pH 7.3-7.7, more preferably at about pH 7.5.
- 5 15. The method of embodiment 14, wherein said buffer comprises 1-50 mM phosphate, citrate or acetate, preferably 10-40 mM sodium phosphate.
16. The method of any one of embodiments 1-15, wherein in step (b1) said TNFR:Fc is eluted with a buffer at pH 7-8, preferably at pH 7.3-7.7, more preferably at about pH 7.5.
- 10 17. The method of embodiment 16, wherein said buffer comprises 1-50 mM phosphate, citrate, acetate, or mixtures thereof, preferably 10-40 mM sodium phosphate, and wherein said buffer further comprises 100-200 mM sodium chloride, preferably 110-190 mM sodium chloride, more preferably 120-180 mM sodium chloride.
- 15 18. The method of any one of embodiments 1-17, wherein the eluate of step (a) or step (b1) is adjusted prior to step (b2) in conductivity to 20-60 mS/cm, preferably to 25-46 mS/cm, and to pH 5.5-6.5, preferably to pH 5.5-6.2.
19. The method of any one of embodiments 1-18, wherein in step (b2) loading and washing of said TNFR:Fc is carried out in a buffer having a pH ranging from 5.5-6.5, preferably pH 5.5-6.2.
- 20 20. The method of embodiment 19, wherein said buffer comprises 1-50 mM phosphate, citrate, acetate, or mixtures thereof, preferably 10-40 mM sodium phosphate, sodium citrate or sodium acetate, and 200-700 mM sodium chloride, preferably 250-600 mM sodium chloride.
21. The method of any one of embodiments 1-20, wherein step (c) is carried out in a buffer having a pH ranging from 5.5-6.5, preferably pH 5.8-6.5, more preferably having a pH of 6.0.
- 25 22. The method of embodiment 21, wherein the TNFR:Fc containing fraction to be applied to step (c) is diluted to a conductivity in the range of 50-100 mS/cm, preferably 70-85 mS/cm.
- 30 23. The method of embodiment 22, wherein the TNFR:Fc containing fraction to be applied to step (c) is diluted with a buffer comprising sodium citrate, sodium phosphate and/or sodium acetate; and sodium sulphate at a concentration of 1 M or higher.
24. The method of embodiment 20 or 23, wherein the resin having bound said TNFR:Fc is washed with a washing buffer comprising sodium sulphate and 50-150 mM sodium citrate, sodium phosphate or sodium acetate, preferably 50-100 mM sodium citrate or sodium phosphate.
- 35 25. The method of embodiment 24, wherein said TNFR:Fc is eluted in step (c) applying a 0-100% gradient from said washing buffer to an elution buffer;

wherein the elution buffer comprises 1-100 mM sodium citrate, sodium phosphate or sodium acetate, preferably 10-50 mM sodium citrate or sodium phosphate;
and 0-100 mM sodium sulphate, preferably 0-10 mM sodium sulphate.

26. The method of any one of embodiments 3-25, wherein step (c) is carried out as MCSGP, and wherein in step (c) elution fractions are pooled from the time point when the descending UV_{280nm} value reaches 95 % of the peak maximum until the descending UV_{280nm} value reaches 30 % of the peak maximum.
27. The method of embodiment 26, wherein the recycling fractions W/P and P/S are pooled from the time point when the ascending UV_{280nm} value reaches 80 % of the peak maximum until the descending UV_{280nm} value reaches 95 % of the peak maximum and from the time point when the descending UV_{280nm} value reaches 30 % of the peak maximum until the descending UV_{280nm} value reaches 20 % of the peak maximum
28. The method of any one of embodiments 1-27, wherein the method further comprises step (d), subjecting the eluate of step (c) to nanofiltration, ultrafiltration and/or diafiltration.
29. The method of any one of embodiments 1-28, wherein the method further comprises formulating said purified TNFR:Fc into a pharmaceutical composition.
30. The method of any one of embodiments 1-29, wherein the TNFR:Fc is etanercept.
31. A purified TNFR:Fc composition, obtained by the method according to any one of embodiments 1-30.

In the following, the present invention is further illustrated by the following figures and examples, which are not intended to limit the scope of the present invention. All references cited herein are explicitly incorporated by reference.

DESCRIPTION OF THE FIGURES

Figure 1: Schematic illustration of the twin-column MCSGP process principle (1st switch). The dashed vertical lines separate the different MCSGP process tasks corresponding to the zones of the schematic batch chromatogram shown in the lower part of the figure. Phases I1, B1, I2, B2 are carried out sequentially.

Figure 2: Schematic of MCSGP experimental optimization. The grey rectangle represents the product elution window. The dashed vertical lines indicate the initial position of the product elution window. The sloped dashed line stands for the linear gradient.

Figure 3: 24h-robustness run BK. The light grey signal (cond) indicates the conductivity measured in front of column 2. The medium grey line (UV3 col 2) refers to the A_{280} signal recorded at the outlet of column 2. The dark grey line (UV4 col 1) refers to the A_{280} signal recorded at the outlet of column 1. The UV signals have different heights due to individual

behavior of the UV detectors. The decreasing A_{280} signals starting at ca. 1500 min indicate the beginning of the shutdown of the process.

Figure 4: 24h-robustness run BP. The light grey signal (cond) indicates the conductivity measured in front of column 2. The medium grey line (UV col 2) refers to the A_{280} signal recorded at the outlet of column 2. The dark grey line (UV col 1) refers to the A_{280} signal recorded at the outlet of column 1. The UV signals have different heights due to individual behavior of the UV detectors. The decreasing A_{280} signals starting at ca. 1400 min indicate the beginning of the shutdown of the process.

Figure 5: 24h-robustness run BK. Purity determined by SEC (size exclusion chromatography) and yield determined by analytical protein A chromatography are shown.

Figure 6: 24h-robustness run BP. Purity determined by SEC and yield determined by analytical protein A chromatography are shown.

Figure 7: 24h-robustness run BK. Overlay of the chromatograms of column 1 of the last three cycles before shutdown. The long vertical lines indicate the borders of the feeding interval and the product collection interval, respectively. Note that the feeding interval of column 1 corresponds to the product collection interval of column 2 and vice versa. The sharp peak in the feeding interval of column 1 indicates product losses due to non-binding.

Figure 8: 24h-robustness run BP. Overlay of the chromatograms of column 1 of the last three cycles before shutdown. The long vertical lines indicate the borders of the feeding interval and the product collection interval, respectively. Note that the feeding interval of column 1 corresponds to the product collection interval of column 2 and vice versa. The sharp peak in the feeding interval of column 1 indicates product losses due to non-binding.

DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1 (human TNF receptor type 1; CD120a; p55/60; RefSeq (protein): NP_001056)

MGLSTVPDLL	LPLVLLELLV	GIYPSGVIGL	VPHLGDREKR	DSVCPQGKYI	HPQNNsicCT	60
KCHKGTyLYN	DCPGPGQTD	CRECESGSFT	ASENHLRHCL	SCSKCRKEMG	QVEISSCTVD	120
RDTVCGCRKN	QYRHYWSEN	FQCFNCSLCL	NGTVHLSCQE	KQNTVCTCHA	GFFLRENECV	180
SCSNCKKSLE	CTKLCLPQIE	NVKGTEDSGT	TVLLPLVIF	GLCLLSLLFI	GLMYRYQRWK	240
SKLYSIVCGK	STPEKEGELE	GTTTKPLAPN	PSFSPTPGFT	PTLGFSPVPS	STFTSSSTYT	300
PGDCPNFAAP	RREVAPPYQG	ADPILATALA	SDPIPNPLQK	WEDSAHKPQS	LDTDDPATLY	360
AVVENVPPLR	WKEFVRRRLGL	SDHEIDRLEL	QNGRCLREAQ	YSMLATWRRR	TPRREATLEL	420
LGRVLRDMDL	LGCLIEDIEEA	LCGPAALPPA	PSLLR			455

WO 2016/009049

PCT/EP2015/066427

SEQ ID NO: 2 (human TNF receptor type 2; CD120b; p75/80; RefSeq (protein): NP_001057)

	MAPVAVWAAL	AVGLELWAAA	HALPAQVAFT	PYAPEPGSTC	RLREYYDQTA	QMCCSKCSPG	60
	QHAKVFCTKT	SDTVCDSCED	STYTQLWNWV	PECLSCGSRC	SSDQVETQAC	TREQNRICTC	120
5	RPGWYCALSK	QEGCRLCAPL	RKCRPGFGVA	RPGETSDVV	CKPCAPGTFS	NTTSSTDICR	180
	PHQICNVVAI	PGNASMDAVC	TSTSPTRSMA	PGAVHLPQPV	STRSQHTQPT	PEPSTAPSTS	240
	FLLPMGPSPP	AEGSTGDFAL	PVGLIVGVTA	LGLLIIGVVN	CVIMTQVKKK	PLCLQREAKV	300
	PHLPADKARG	TQGPEQQHLL	ITAPSSSSSS	LESSASALDR	RAPTRNQPQA	PGVEASGAGE	360
	ARASTGSSDS	SPGGHGTQVN	VTCIVNVCSS	SDHSSQCSSQ	ASSTMGDTDS	SPSESPKDEQ	420
10	VPFSKEECAAF	RSQLETPETL	LGSTEEKPLP	LGVPDAGMKP	S		461

SEQ ID NO: 3 (human IgG1 class heavy chain constant domain)

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser
15 Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu
Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His
Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys
Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
20 Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu
Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg
Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
25 Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln
Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
Leu Ser Leu Ser Pro Gly Lys

30 SEQ ID NO: 4 (Etanercept)

	LPAQVAFTPY	APEPGSTCRL	REYYDQTAQM	CCSKCSPGQH	AKVFCTKTS	TVCDSCEDST	60
	YTQLWNWVPE	CLSCGSRCSS	DQVETQACTR	EQNRICTCRP	GWYCALSKQE	GCRLCAPLRK	120
	CRPGFGVARP	GTETSDVCK	PCAPGTFSNT	TSSTDICRPH	QICNVVAIPG	NASMDAVCTS	180
	TSPTRSMAPG	AVHLPQPVST	RSQHTQPTPE	PSTAPSTSFL	LPMGPSPPAE	GSTGDEPKSC	240
35	DKTHTCPPCP	APELLGGPSV	FLFPPKPKDT	LMISRTPEVT	CVVVDVSHED	PEVKFNWYVD	300
	GVEVHNAKTK	PREEQYNSTY	RVVSVLTVLH	QDWLNGKEYK	CKVSNKALPA	PIEKTISKAK	360
	GQPREPQVYT	LPPSREEMTK	NQVSLTCLVK	GFYPSDIAVE	WESNGQPENN	YKTTTPVLDS	420
	DGSFFLYSKL	TVDKSRWQQG	NVFSCSVMHE	ALHNHYTQKS	LSLSPGK		467

EXAMPLES

All used substances were Ph. Eur. Grade or of comparable quality. The buffers were prepared with purified and de-ionized water.

Example 1: Purification of TNFR:Fc

5 Affinity Chromatography

The purification process starts from cell free culture supernatants. The material was 0.2 μ m filtered. Utilizing the Fc part of the fusion protein, TNFR:Fc was captured by affinity chromatography on Protein A resin. The Protein A interaction with the Fc part is very specific. Therefore, the capture chromatography very efficiently separates host cell
10 proteins (HCPs), DNA and virus from the product.

Process temperature was 21 °C. The cell culture supernatant was loaded onto MabSelect SuRe resin (GE Healthcare), equilibrated with sodium phosphate buffer of pH 7.0 further comprising 150 mM sodium chloride. Then, the column was washed with the same buffer until UV₂₈₀ returns to signal close to baseline (about 2 to 6 column volumes).

15 To increase the HCP removal capacity of the capture step, an additional wash step was introduced. This wash buffer contained 20 mM acetic acid, equilibrated to pH 5.5 with sodium hydroxide. It was followed by product elution with an acidic buffer having a pH of ~3.2. The eluate was processed to the next purification step.

Anion Exchange Chromatography

20 The intermediate resulting from the affinity chromatography step was adjusted with sodium hydroxide to pH 7.0 and loaded onto a Fractogel TMAE HiCap (M) resin (Merck). Subsequently, the column was rinsed with sodium phosphate buffer and finally the product was eluted in a gradient up to 250 mM NaCl in sodium phosphate buffer. The eluate was processed to the next purification step.

25 Multimodal Anion Exchange Chromatography

In a next step, multimodal anion exchange (MM) chromatography combining the principles of anion exchange chromatography with those of *inter alia* hydrophobic interaction chromatography was carried out in flow-through mode.

The eluate from the anion exchange chromatography step was adjusted in conductivity
30 using 4 M sodium chloride to about 25 mS/cm, and the pH was adjusted to pH 5.5 using a phosphoric acid solution of pH $\leq$ 2. Then the intermediate was equilibrated with 20 mM sodium phosphate, 250 mM sodium chloride pH 5.6, loaded onto Capto adhere resin (GE

Healthcare) and the column was then washed with 20 mM sodium phosphate, 250 mM sodium chloride pH 5.6. The flow-through comprising the product was collected and pooled.

Hydrophobic Interaction chromatography

5 Hydrophobic interaction chromatography (HIC) was used for separating TNFR:Fc degradation products (DPs), aggregation products (APs), and other product related impurities having no or reduced TNFR:Fc bioactivity. It further serves for separating of HCPs and virus. Therefore, HIC is particularly suitable as a final polishing step.

The flow-through from the MM chromatography was diluted first with 20 mM sodium phosphate buffer, pH 7.5, then with 100 mM sodium phosphate buffer pH 6.5 comprising 1.4 M sodium sulfate, so that the conductivity of the solution was above 80 mS/cm. Then, the solution was loaded onto Toyopearl Phenyl 650 (M) and equilibrated with sodium phosphate buffer pH 6.5 comprising sodium sulfate. The column is then rinsed with the same buffer. Finally, the column is eluted using a 0-100% gradient from the equilibration buffer to elution buffer (25 mM sodium phosphate pH 6.5).

The purity of the product was determined using size exclusion chromatography (SEC), and by determining the amount of host cell proteins (HCP), Protein A, and endotoxin. Further, the step yield and total yield was calculated for each purification step. The following Table 1 shows data obtained with the above described method for at least three runs.

Table 1

	Purity (%) acc. to SEC	Step yield (%)	Total yield (%)	HCP (ppm)	Protein A (ppm)
Affinity chrom. (Protein A)	89.2		set as 100.0	2398	2.4
Anion exchange chrom.	89.1	93.7	93.7	730	< LOQ
MM chrom.	89.9	92.9	87.0	453	< LOQ
HIC chrom.	96.2	35.9	31.2	138	< LOQ

In a separate experiment, the HIC step was performed on Phenyl Sepharose HP (GE Healthcare) resin instead with equivalent purification results.

Example 2: Purification of TNFR:Fc

Affinity Chromatography

The purification process starts from cell free culture supernatants. The material was 0.2 μ m filtered. Utilizing the Fc part of the fusion protein, TNFR:Fc was captured by affinity chromatography on Protein A resin. The Protein A interaction with the Fc part is very specific. Therefore, the capture chromatography very efficiently separates host cell proteins (HCPs), DNA and virus from the product.

Process temperature was 21 °C. The cell culture supernatant was loaded onto MabSelect SuRe resin (GE Healthcare), equilibrated with sodium phosphate buffer of pH 7.0 further comprising 150 mM sodium chloride. Then, the column was washed with the same buffer until UV₂₈₀ returns to signal close to baseline (about 2 to 6 column volumes).

To increase the HCP removal capacity of the capture step, an additional wash step was introduced. This wash buffer contained sodium acetate and 0-500 mM sodium chloride. It was followed by product elution with an acidic buffer having a pH of ~3.2. The eluate was processed to the next purification step.

Anion Exchange Chromatography

The intermediate resulting from the affinity chromatography step was adjusted to pH 7.5 and loaded onto a Fractogel TMAE HiCap (M) resin (Merck). Subsequently, the column was rinsed with sodium phosphate buffer and finally the product was eluted with sodium phosphate buffer containing 150 mM sodium chloride. The eluate was processed to the next purification step.

Multimodal Anion Exchange Chromatography

The eluate from the anion exchange chromatography step was adjusted in conductivity to above 40 mS/cm using 4 M sodium chloride, and the pH was adjusted to pH 6.0 using a phosphoric acid solution of pH $\leq$ 2. Then the intermediate was equilibrated with 20 mM sodium phosphate, 450 mM sodium chloride pH 6.0, loaded onto Capto adhere resin (GE Healthcare), and the column was then washed with 20 mM sodium phosphate, 450 mM sodium chloride pH 6.0. The flow-through comprising the product was collected and pooled.

Hydrophobic Interaction chromatography

Hydrophobic interaction chromatography (HIC) was used for separating TNFR:Fc degradation products (DPs), aggregation products (APs), and other product related

impurities having no or reduced TNFR:Fc bioactivity. It further serves for separating of HCPs and virus. Therefore, HIC is particularly suitable as a final polishing step.

The flow-through from the MM chromatography was diluted with sodium citrate buffer pH 6.0 comprising 1.4 M sodium sulfate. The conductivity of the solution was about 80 mS/cm. Then, the solution was loaded onto Toyopearl Phenyl 650 (M) and equilibrated with sodium citrate buffer pH 6.0 comprising sodium sulfate. The column is then rinsed with the same buffer. Finally, the column is eluted using a 0-100% gradient from the equilibration buffer to elution buffer (25 mM sodium citrate pH 6.0).

The purity of the product was determined using size exclusion chromatography (SEC), and by determining the amount of host cell proteins (HCP), Protein A, and endotoxin. Further, the step yield and total yield was calculated for each purification step. The following Table 1 shows data obtained with the above described method for at least three runs.

Table 2

	Purity (%) acc. to SEC	Step yield (%)	Total yield (%)	HCP (ppm)	Protein A (ppm)
Affinity chrom. (Protein A)	82.1		set as 100.0	1500	1.7
Anion exchange chrom.	82.8	87.9	87.9	560	0.6
MM chrom.	83.4	91.3	80.3	229	0.2
HIC chrom.	96.0	47.2	37.9	112.7	< LOQ

The overall process yield of the above methods could be further improved. However, as can be seen in the above tables, while HIC chromatography is important for increasing the degree of purity to more than 95%, it is also the step, wherein most of the product is lost.

Example 3: Purification of TNFR:Fc using HIC chromatography in continuous mode (MCSGP)

TNFR:Fc was purified as described above, except that HIC chromatography was used in continuous mode (MCSGP).

Size exclusion analytics were carried out using a combination of a guard column TSKgel 3000SWXL, 6 x 40 mm, in sequence with two TSKgel G3000SWXL 7.8x300 mm columns.

The running buffer (150 mM potassium phosphate pH 6.5) was applied at a flow rate of 0.4 ml/min. The injection volume was typically 2 or 3 µl, and detection was done at 210 nm. Performance of the columns was ensured prior to, during and after the measurements by injecting reference and molecular weight standards.

- 5 For fine-tuning of the MCSGP operating parameters, an elution profile for a run conducted in batch mode was made. The results are shown in Table 2 below.

Table 2. Fractionation at % Absorption (UV_{280nm}) of elution peak maximum

	SEC purity (%)	Bioactivity (%)
-90 %	86.9	86
-95 %	92.6	87
100 %	93.6	82
90 %	96.3	85
80 %	98.7	94
60 %	98.3	94
55 %	97.9	83
50 %	97.3	79
45 %	96.9	< 67
Load	93.2	70
Eluate (100%-55%)	97.8	90

- 10 In Figure 2 it is shown schematically how fine-tuning of the operating parameters is carried out in MCSGP based on the MCSGP runs BK, BN and BO. The chromatograms in Figure 2 represent schematically chromatograms of the MCSGP process that are identical to the batch design chromatogram.

- 15 The starting point for optimization of the MCSGP procedure was run BK that featured a suboptimal yield of 55% (purity 95.1 %) and losses in the flow-through stream, indicating that too much P has accumulated within the process. One action would be the reduction of the feed flow rate, but this would lead to a lowering of the productivity.

- 20 As a measure to improve the yield, new MCSGP operating parameters were determined as follows: The product collection was started at a lower gradient concentration (Run BN) and also the final gradient concentration was changed proportionally. Thus, the P elution window is shifted to the front. The product elution flow rate and the elution time were not changed, so the product elution window size remained the same. Note the analogy to

batch chromatography: The shift corresponds to the pooling of a certain number of earlier samples instead of pooling late samples (e.g. pooling fractions 6, 7, 8 instead of fractions 7, 8, 9). The MCSGP run BN was performed and showed a yield of 65 % (purity 96.6 %). Due to the shifting of the elution window to the front, the yield is improved. However, still
5 not enough P is eluted from the columns, so a large fraction of P is recycled and P starts to break through during the loading phase.

To improve the yield further, new MCSGP parameters were determined as follows: In order to elute more material from the column, the elution window was widened towards the front. As in the previous case this is done by shifting the P collection start towards a
10 lower gradient concentration, but in contrast to the earlier case, the product collection stop point remained the same (Run BO). Thus, the product elution volume was increased. In batch chromatography, this action corresponds to taking additional early fractions into the product pool (e.g. pooling fractions 5, 6, 7, 8 instead of only 6, 7, 8). The evaluation of the experimental verification of run BO showed that yields of 78 % were obtained (purity 95.6
15 %) with the new operating parameters.

Note that during the entire parameter optimization process, the gradient slope and duration were not changed. For MCSGP design it is sufficient to use a batch gradient chromatogram as starting point that shows satisfactory purity in a few fractions. The product elution window can then be shifted, widened or narrowed as described above.
20 Due to the internal recycling of the impure side fractions, the yield is kept high (provided that the columns are not operated at their dynamic capacity limits and no breakthrough occurs).

In order to demonstrate process robustness, a number of MCSGP experiments were operated for 24 hrs. In order to shut down the MCSGP process, the process was
25 continued loading buffer instead of feed.

Figure 3 shows the conductivity signal, measured before the second column of the MCSGP process, the A_{280} signals recorded at the outlets of columns 1 and 2, respectively. The A_{280} signals have different heights due to individual differences of the UV detectors. The startup cycle is included in the figures. Shutdown was initiated after 12 cycles
30 (includes the startup cycle) at ca. 1500 min. Figure 4 shows the same signals for the robustness run BP. Shutdown was initiated after 11 cycles (includes the startup cycle) at ca. 1400 min. Both figures show that the signal heights of each cycle were similar and that there were no undesired perturbations.

Figure 5 and Figure 6 show yield and purity for runs BK and BP, respectively. Yield and
35 purity were determined for the product collected during each complete cycle using offline analytics (Protein A chromatography and SEC). Within a sequence of fine-tuning steps as

outlined above, the yield could be improved from 55-60% (run BK) to 75-80% (run BP) without compromising the purity (Run BK, Run BN, Run BO, Run BP), as also shown in the below Table 3.

Table 3

Cycle	Run BK Yield (%)	Run BK Purity (%)	Run BP Yield (%)	Run BP Purity (%)
1	15.9	95.3	20.6	96.6
2	61.1	96.2	80.4	97.7
3	60.1	96.8	77.5	97.7
4	59.4	96.4	77.0	96.8
5	60.4	96.7	77.1	97.0
6	57.9	96.2	76.3	97.1
7	57.7	96.9	74.9	97.1
8	57.5	96.3	75.4	97.2
9	57.3	96.2	75.2	96.9
10	58.4	96.5	85.8	96.8
11	57.2	96.3	75.7	96.9
12	54.8	95.6		

The yield increase can be understood more clearly if the chromatograms of runs BK (Figure 7) and BP (Figure 8) are compared more closely.

Each figure shows an overlay of the chromatograms of column 1 of the last three cycles before initiation of shutdown. The chromatograms match excellently which indicates that cyclic steady state has been reached. Now, when comparing Figure 7 and Figure 8 it becomes obvious that in the case of run BK the elution window (indicated by the long vertical lines within the main peak in the chromatogram) is located more towards the peak tail than the elution window in the case of run BP. This means on the one hand, that more product is eluted within the window in run BP (particularly when considering that also the product elution flow rate was increased from 0.18 mL/min (BK) to 0.25 mL/min (BP). On the other hand, if more product is eluted from the system, less product is internally recycled, leaving more capacity for freshly fed material. Consequently, the amount of product lost in run BP during the loading step (loading step indicated by long vertical lines in the left part of the chromatogram, Figure 8) is much less than in run BK (Figure 7). Offline analyses confirmed that ca. 30-35% of the product was lost during the feed step of run BK (note the sharp peak in the feeding interval, Figure 7) and only ca. 8% of the

product was lost during the feed step of run BP (note the much smaller peak in the feeding interval in Figure 8).

The results are further summarized in the following Table 4.

Table 4

	Batch	MCSGP (BP)
SEC Purity (%)	96.0	96.0
Bioactivity (%)	94	97
Yield (%)	47	76
Buffer consumption (L/g)	~ 4.0	2.5
Final product concentration (g/L)	1.0	4.0

5

Hence, a yield improvement by about 30 percentage points or about 50% as compared to the regular batch procedure could be achieved, moreover, up to 75% reduced buffer consumption and 20% higher product concentration in the final product. Due to the reduced buffer consumption, it is furthermore feasible to use smaller and/or less process equipment, thereby also achieving a better plant utilization.

10

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Claims

- 5 1. Method for purifying a TNFR:Fc fusion protein, comprising the steps of:
- (a) subjecting a solution comprising said TNFR:Fc to affinity chromatography;
 - (b1) optionally subjecting the eluate of step (a) to anion exchange chromatography;
 - (b2) subjecting the eluate of step (b1), or in absence of step (b1) the eluate of step
 - 10 (a) to multimodal anion exchange chromatography (MMC);
 - (c) subjecting the flow-through of step (b2) to hydrophobic interaction chromatography (HIC), and collecting the eluate to obtain purified TNFR:Fc.
2. Method for purifying a TNFR:Fc fusion protein, comprising the steps of:
- (a) subjecting a solution comprising said TNFR:Fc to affinity chromatography;
 - 15 (b2) subjecting the eluate of step (a) to multimodal anion exchange chromatography (MMC);
 - (b1) subjecting the flow-through of step (b2) to anion exchange chromatography;
 - (c) subjecting the eluate of step (b1) to hydrophobic interaction chromatography (HIC), and collecting the eluate to obtain purified TNFR:Fc.
- 20 3. The method of embodiment 1 or 2, wherein step (c) is carried out as multicolumn countercurrent solvent gradient purification (MCSGP).
4. The method of any one of embodiments 1 to 3, wherein the purification process does
- 25 not use any cation exchange chromatography step.
5. The method of any one of embodiments 1 to 4, wherein in step (a) is carried out on a resin modified with Protein A, Protein G, an antibody capable of binding the Fc-part of said TNFR:Fc, or an antibody directed against the TNFR-part of said TNFR:Fc;
- 30 preferably wherein said resin is modified with Protein A or Protein G, and more preferably, wherein said resin is modified with Protein A.
6. The method of any one of embodiments 1 to 5, wherein said TNFR:Fc is bound in step
- 35 (a) to the affinity resin at pH 6-8, preferably at pH 6.5-7.5, more preferably at about pH 7.0.

7. The method of any one of embodiments 1 to 6, wherein in step (a) the resin having bound said TNFR:Fc is washed with a buffer at pH 6-8, preferably at pH 6.5-7.5, more preferably at about pH 7.0.
- 5 8. The method of embodiment 7, wherein said resin is washed with a phosphate buffer comprising 5-50 mM sodium phosphate, preferably in the presence of a salt, more preferably in the presence of 20-200 mM sodium chloride.
9. The method of embodiment 7 or 8, wherein in step (a) the resin is subsequently
10 washed with a second buffer which is a phosphate buffer, a citrate buffer, an acetate buffer or a mixture of any of these buffers at pH 5-6.5, preferably pH 5.2-6.0.
10. The method of embodiment 9, wherein said second buffer comprises 1-100 mM, preferably 5-50 mM of the buffering agent, and
15 0-750 mM sodium chloride, preferably 0-700 mM sodium chloride, more preferably 0-600 mM sodium chloride, and even more preferably 0-500 mM sodium chloride.
11. The method of any one of embodiments 1 to 10, wherein said TNFR:Fc is eluted in step (a) at a pH ranging from 2.5 to 4.5, preferably at a pH ranging from 3.0 to 3.5.
20
12. The method of embodiment 11, wherein said TNFR:Fc is eluted in step (a) using a buffer based on acetic acid, citric acid and/or phosphoric acid at concentrations of 1-100 mM, preferably 5-50 mM.
- 25 13. The method of any one of embodiments 1-12, wherein said TNFR:Fc is bound in step (b1) to the anion exchange resin at pH 7-8, preferably at pH 7.3-7.7, more preferably at about pH 7.5.
14. The method of any one of embodiments 1-13, wherein in step (b1) the resin having
30 bound said TNFR:Fc is washed with a buffer at pH 7-8, preferably at pH 7.3-7.7, more preferably at about pH 7.5.
15. The method of embodiment 14, wherein said buffer comprises 1-50 mM phosphate, citrate or acetate, preferably 10-40 mM sodium phosphate.
- 35 16. The method of any one of embodiments 1-15, wherein in step (b1) said TNFR:Fc is eluted with a buffer at pH 7-8, preferably at pH 7.3-7.7, more preferably at about pH 7.5.

17. The method of embodiment 16, wherein said buffer comprises 1-50 mM phosphate, citrate, acetate, or mixtures thereof, preferably 10-40 mM sodium phosphate, and wherein said buffer further comprises 100-200 mM sodium chloride, preferably 110-190 mM sodium chloride, more preferably 120-180 mM sodium chloride.
18. The method of any one of embodiments 1-17, wherein the eluate of step (a) or step (b1) is adjusted prior to step (b2) in conductivity to 20-60 mS/cm, preferably to 25-46 mS/cm, and to pH 5.5-6.5, preferably to pH 5.5-6.2.
19. The method of any one of embodiments 1-18, wherein in step (b2) loading and washing of said TNFR:Fc is carried out in a buffer having a pH ranging from 5.5-6.5, preferably pH 5.5-6.2.
20. The method of embodiment 19, wherein said buffer comprises 1-50 mM phosphate, citrate, acetate, or mixtures thereof, preferably 10-40 mM sodium phosphate, sodium citrate or sodium acetate, and 200-700 mM sodium chloride, preferably 250-600 mM sodium chloride.
21. The method of any one of embodiments 1-20, wherein step (c) is carried out in a buffer having a pH ranging from 5.5-6.5, preferably pH 5.8-6.5, more preferably having a pH of 6.0.
22. The method of embodiment 21, wherein the TNFR:Fc containing fraction to be applied to step (c) is diluted to a conductivity in the range of 50-100 mS/cm, preferably 70-85 mS/cm.
23. The method of embodiment 22, wherein the TNFR:Fc containing fraction to be applied to step (c) is diluted with a buffer comprising sodium citrate, sodium phosphate and/or sodium acetate; and sodium sulphate at a concentration of 1 M or higher.
24. The method of embodiment 20 or 23, wherein the resin having bound said TNFR:Fc is washed with a washing buffer comprising sodium sulphate and 50-150 mM sodium citrate, sodium phosphate or sodium acetate, preferably 50-100 mM sodium citrate or sodium phosphate.

25. The method of embodiment 24, wherein said TNFR:Fc is eluted in step (c) applying a 0-100% gradient from said washing buffer to an elution buffer;
wherein the elution buffer comprises 1-100 mM sodium citrate, sodium phosphate or sodium acetate, preferably 10-50 mM sodium citrate or sodium phosphate;
5 and 0-100 mM sodium sulphate, preferably 0-10 mM sodium sulphate.
26. The method of any one of embodiments 3-25, wherein step (c) is carried out as MCSGP, and wherein in step (c) elution fractions are pooled from the time point when the descending UV_{280nm} value reaches 95 % of the peak maximum until the descending
10 UV_{280nm} value reaches 30 % of the peak maximum.
27. The method of embodiment 26, wherein the recycling fractions W/P and P/S are pooled from the time point when the ascending UV_{280nm} value reaches 80 % of the peak maximum until the descending UV_{280nm} value reaches 95 % of the peak maximum
15 and from the time point when the descending UV_{280nm} value reaches 30 % of the peak maximum until the descending UV_{280nm} value reaches 20 % of the peak maximum
28. The method of any one of embodiments 1-27, wherein the method further comprises step (d), subjecting the eluate of step (c) to nanofiltration, ultrafiltration and/or
20 diafiltration.
29. The method of any one of embodiments 1-28, wherein the method further comprises formulating said purified TNFR:Fc into a pharmaceutical composition.
- 25 30. The method of any one of embodiments 1-29, wherein the TNFR:Fc is etanercept.
31. A purified TNFR:Fc composition, obtained by the method according to any one of embodiments 1-30.

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Fig. 1

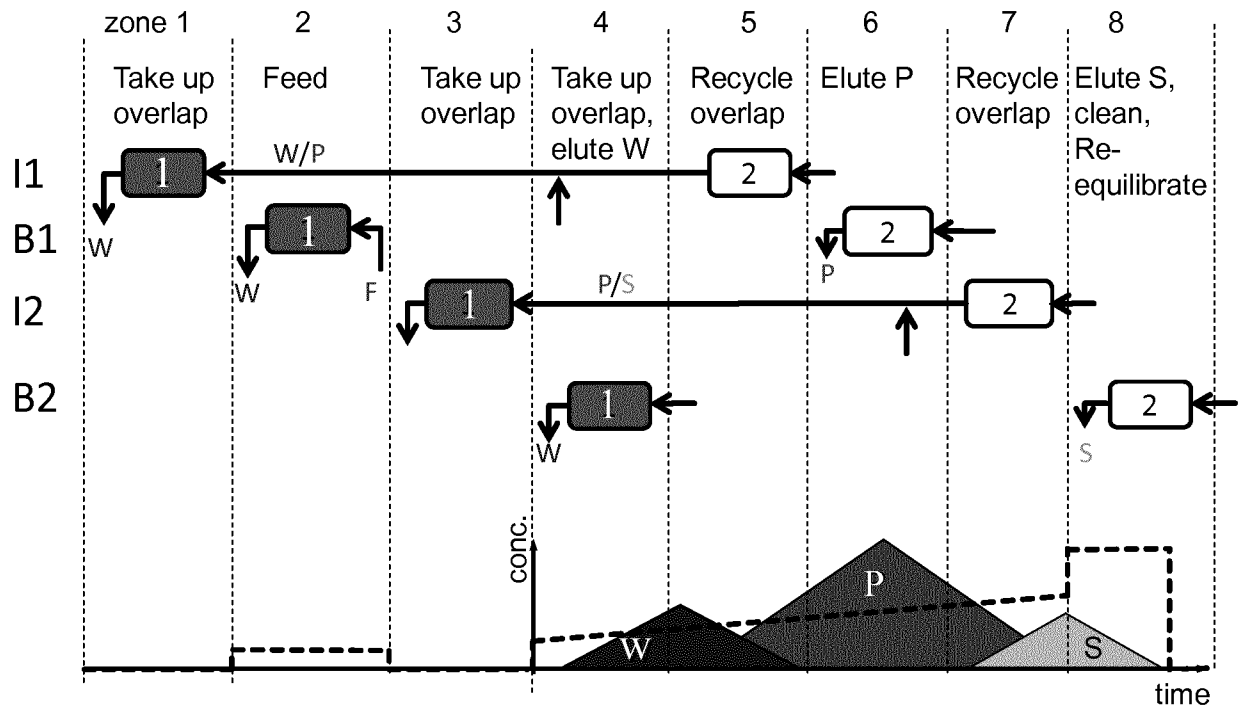


Fig. 2

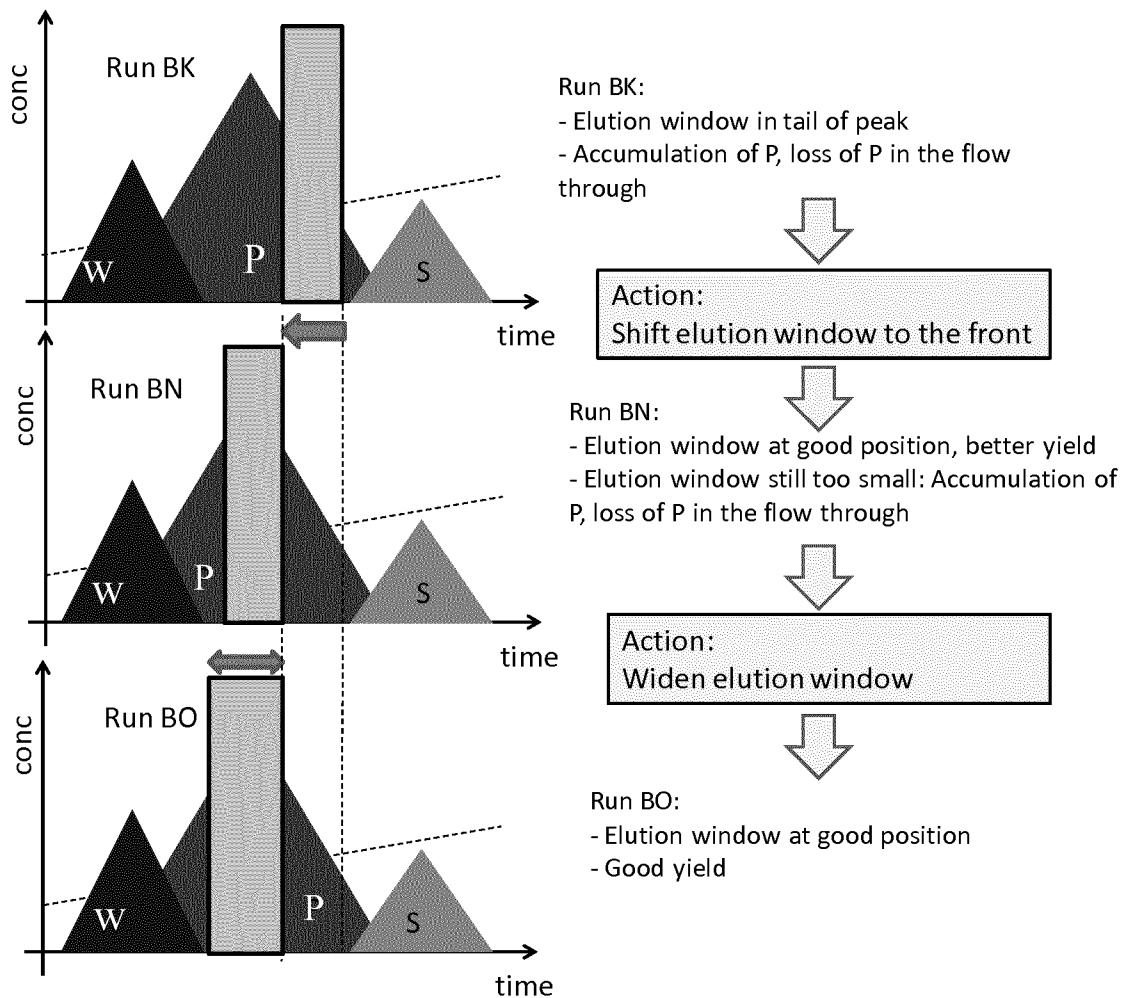


Fig. 3

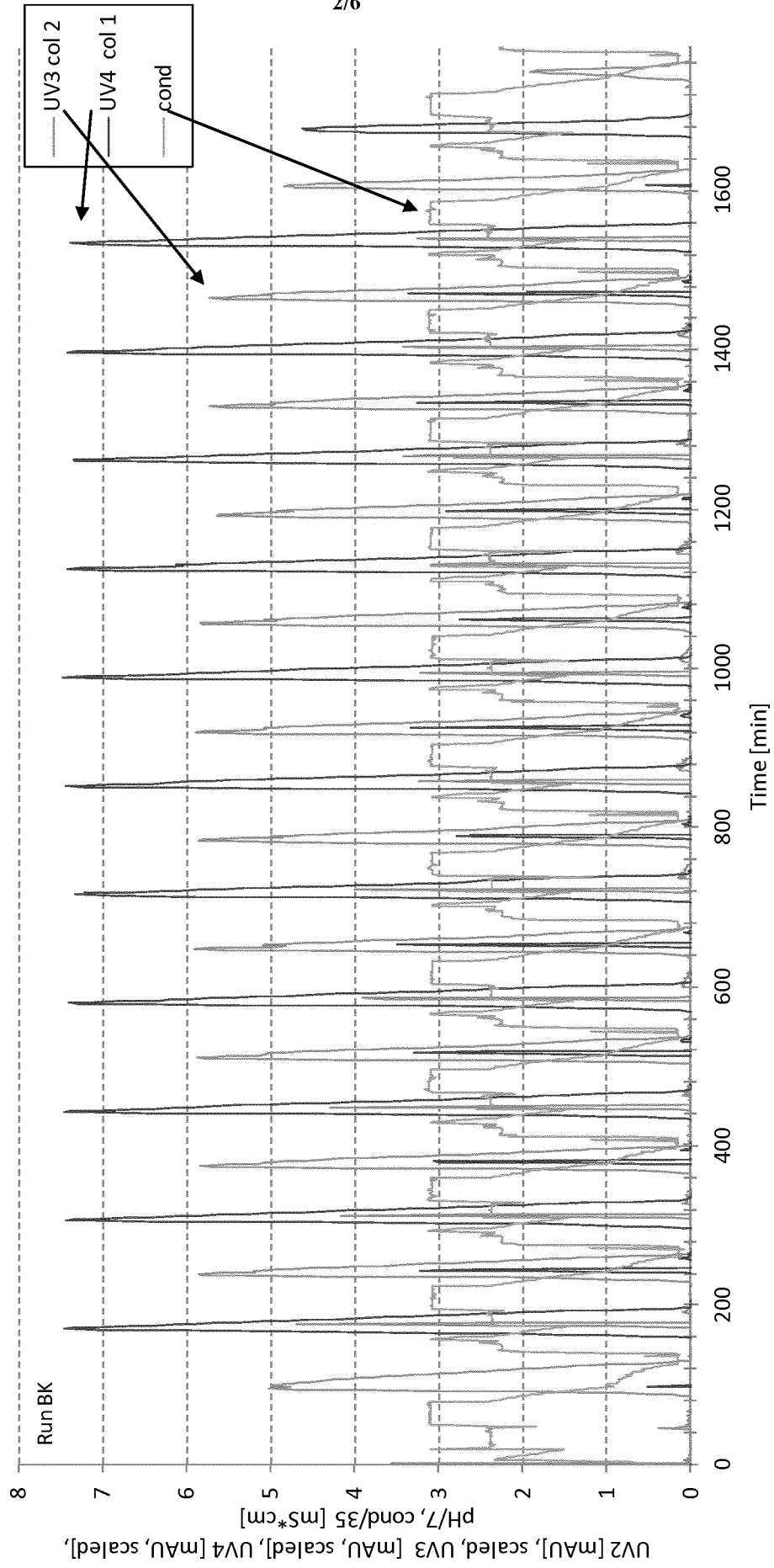


Fig. 4

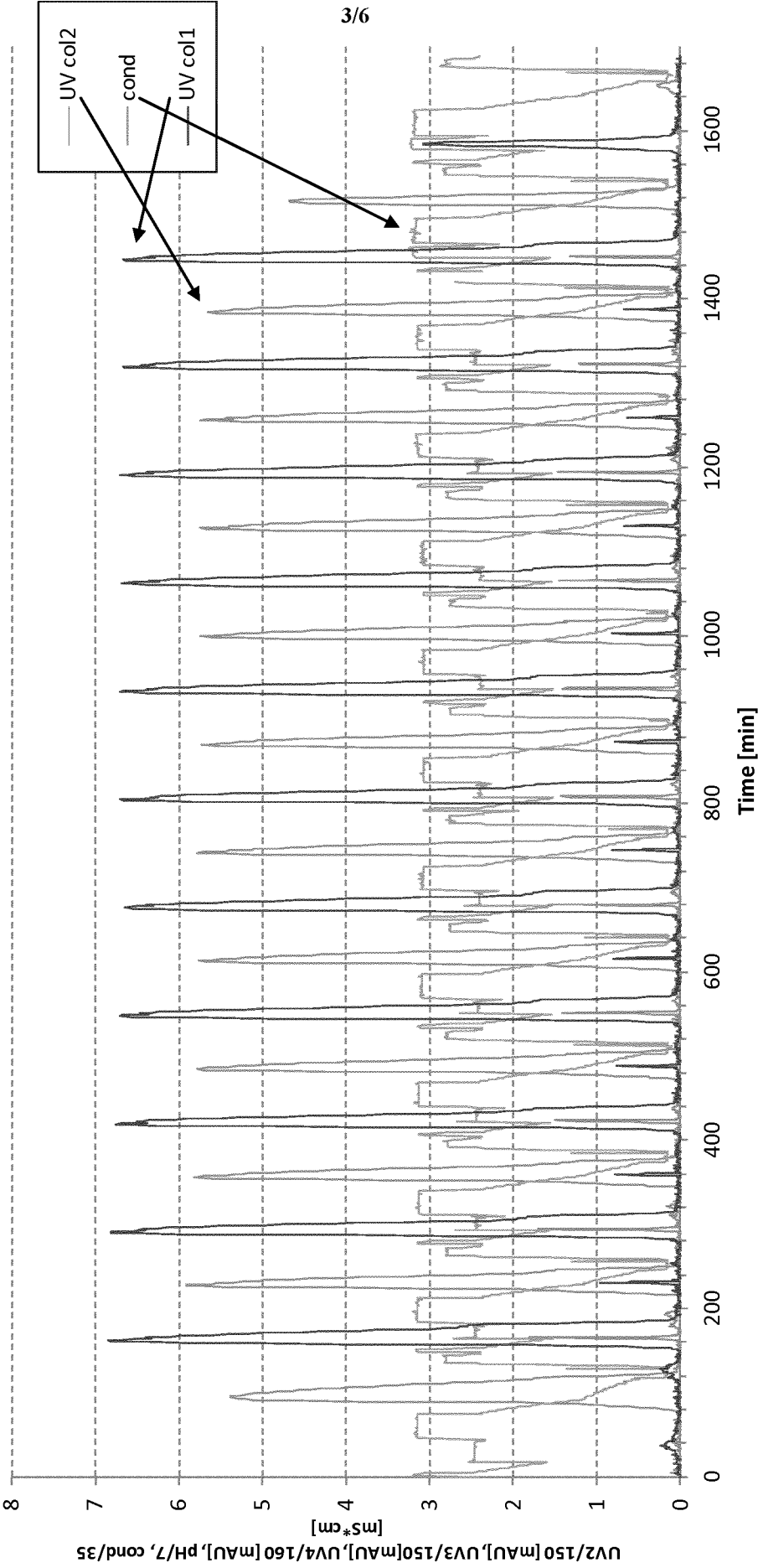
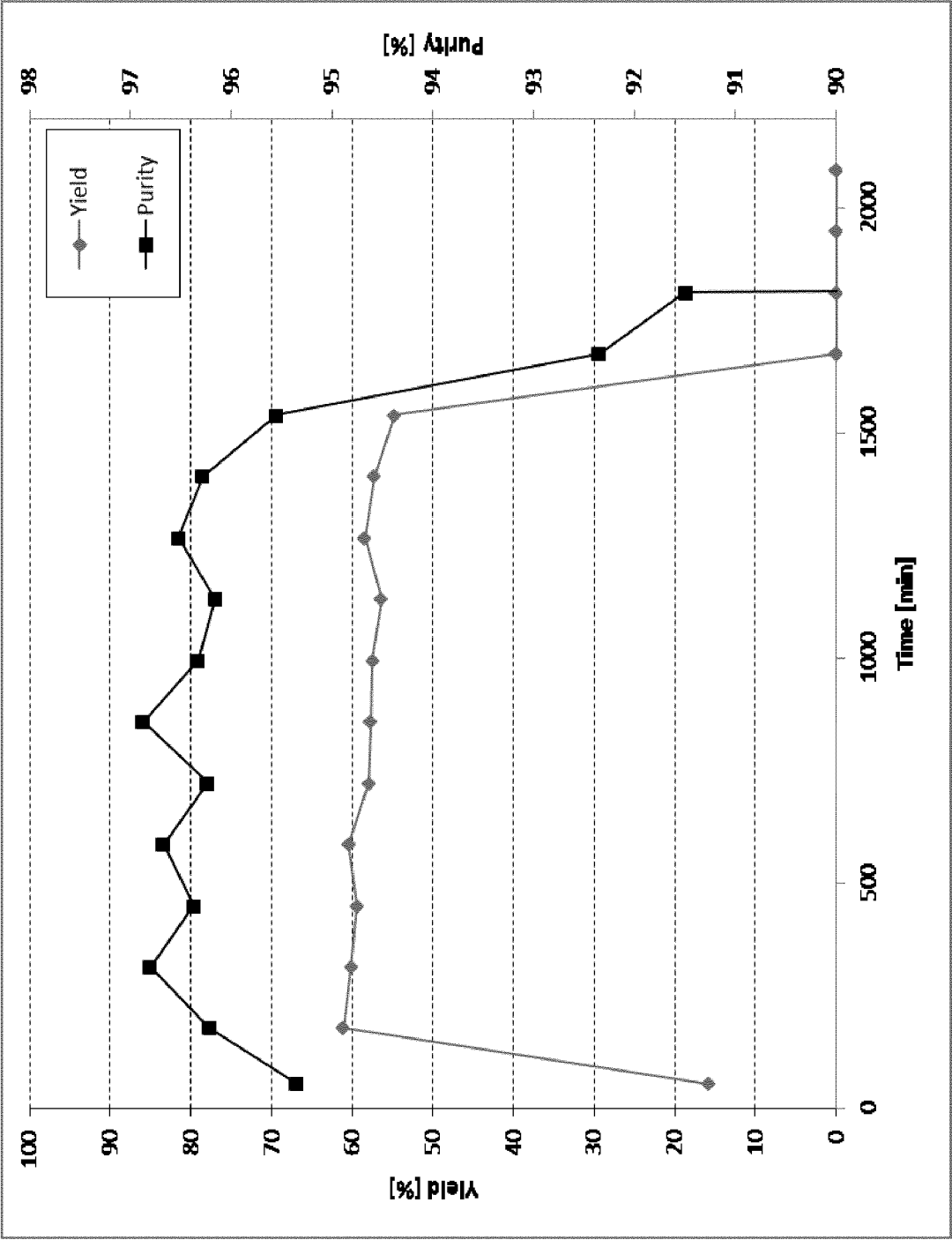


Fig. 5



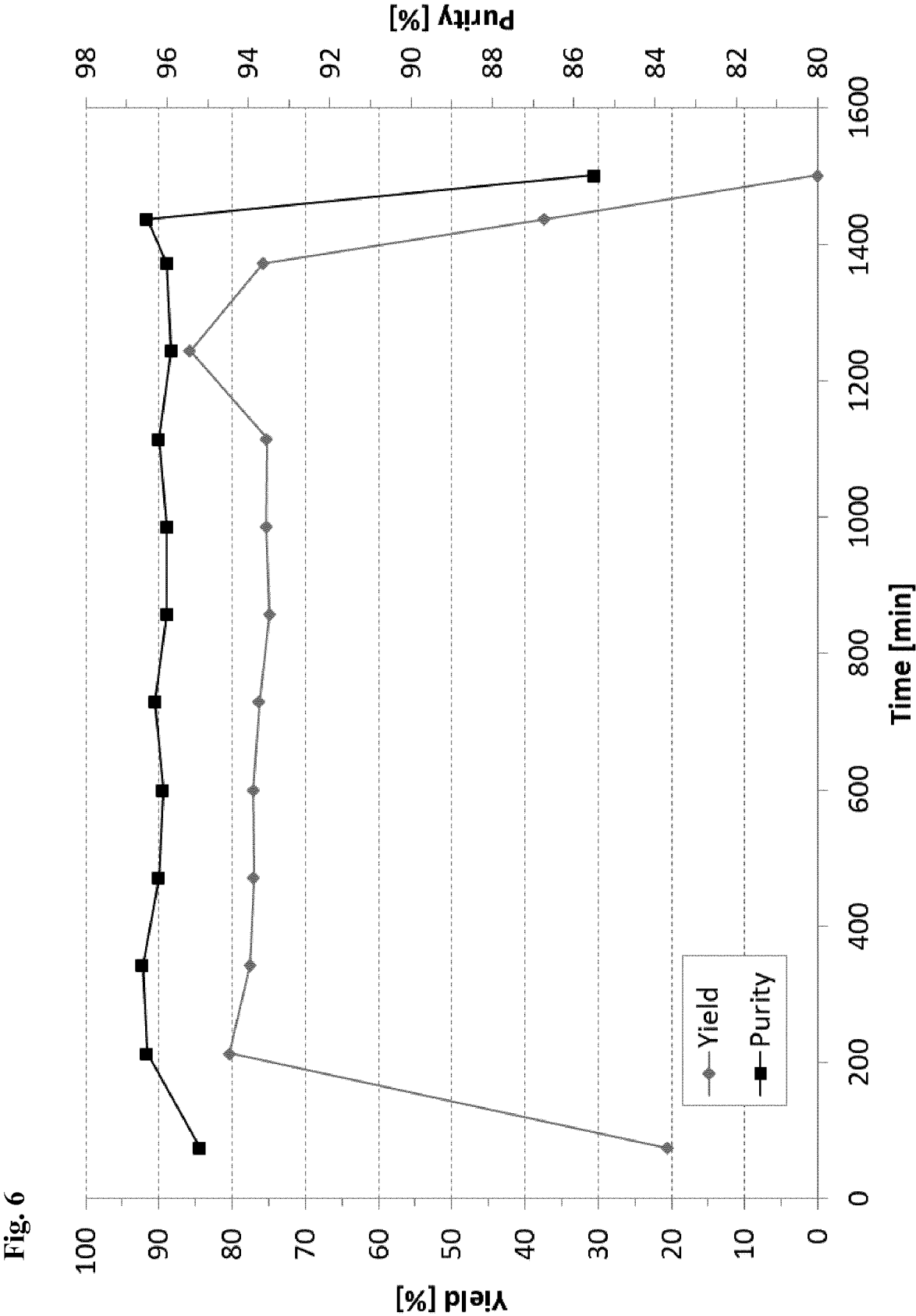


Fig. 7

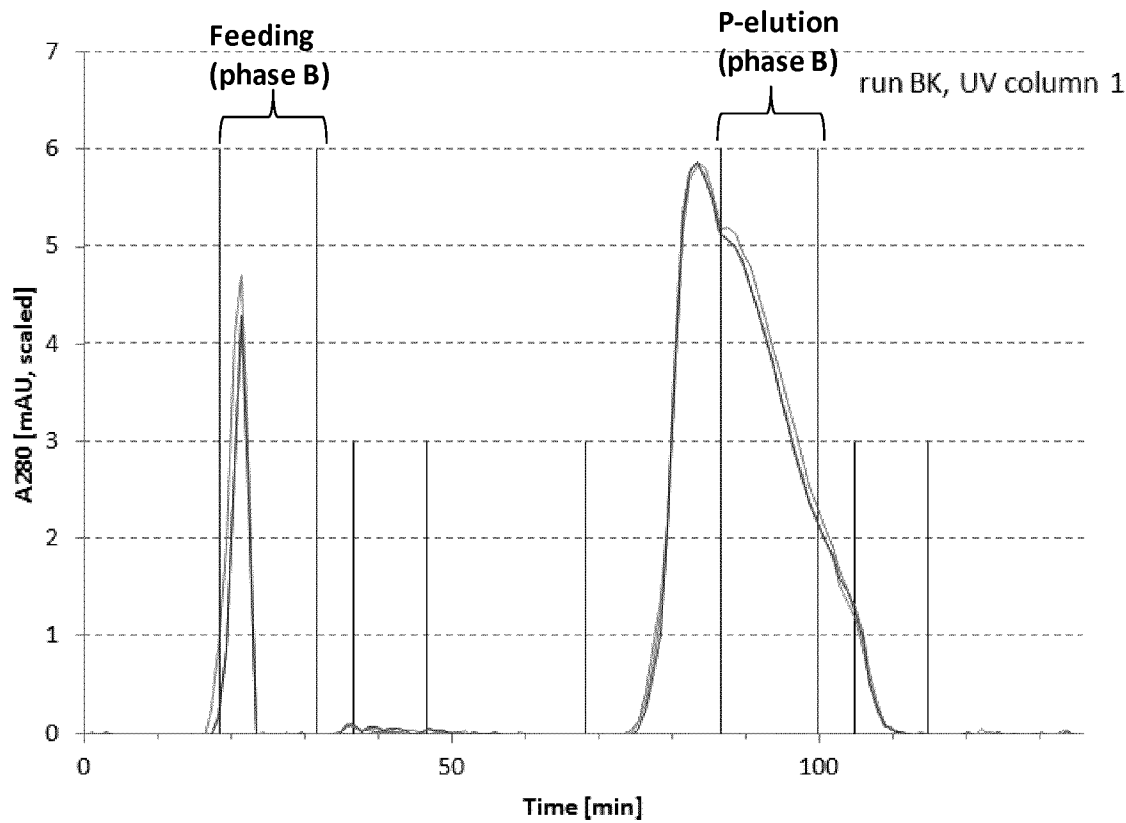
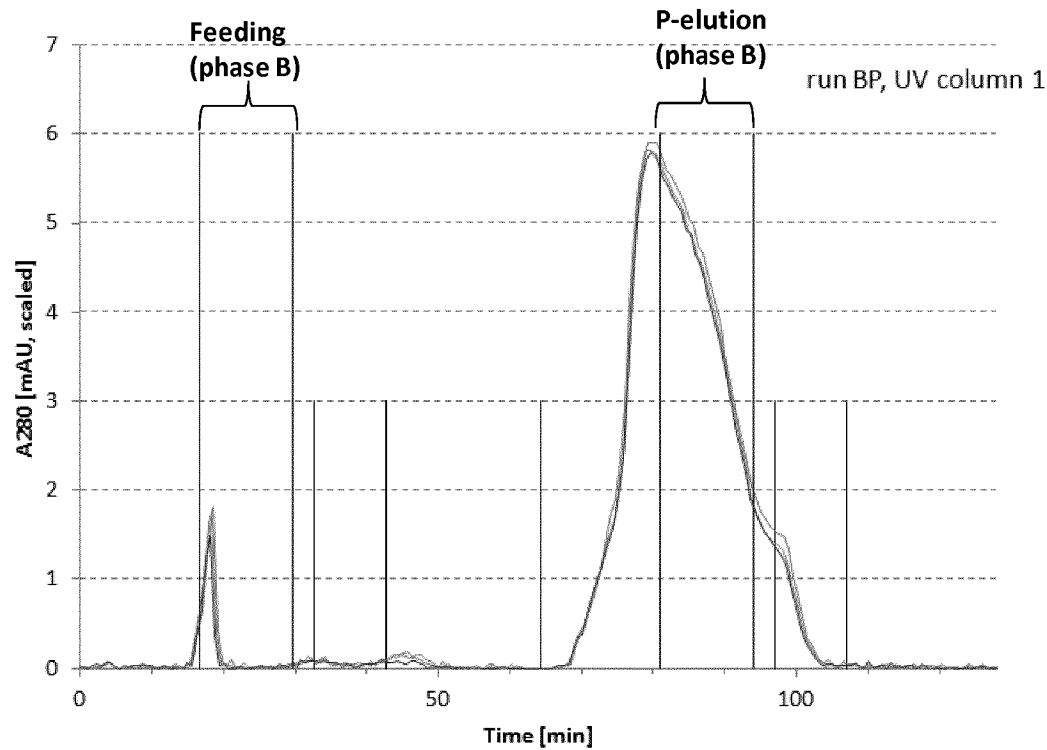


Fig. 8



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/066427

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K1/36 C07K14/705
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/078627 A1 (MERCK SHARP & DOHME [US]; KASHI RAMESH S [US]; PATEL SHONA P [US]; MIT) 22 May 2014 (2014-05-22) example 1	1-31
X	WO 2014/102814 A1 (INTAS BIOPHARMACEUTICALS LTD [IN]) 3 July 2014 (2014-07-03) page 7, line 16 - line 30; claims; figures; examples 1,2	1-31
X	WO 2012/176158 A1 (REDDYS LAB LTD DR [IN]; KULKARNI SAMIR [IN]; DEVAKATE RAVIKANT [IN]; G) 27 December 2012 (2012-12-27) claim 8	1-31



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2015/066427

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