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(54) **ANALYTICAL METHOD FOR FAB AND FAB' MOLECULES**

ANALYSEVERFAHREN FÜR FAB- UND FAB'-MOLEKÜLE

PROCÉDÉ ANALYTIQUE POUR DES MOLÉCULES FAB ET FAB'

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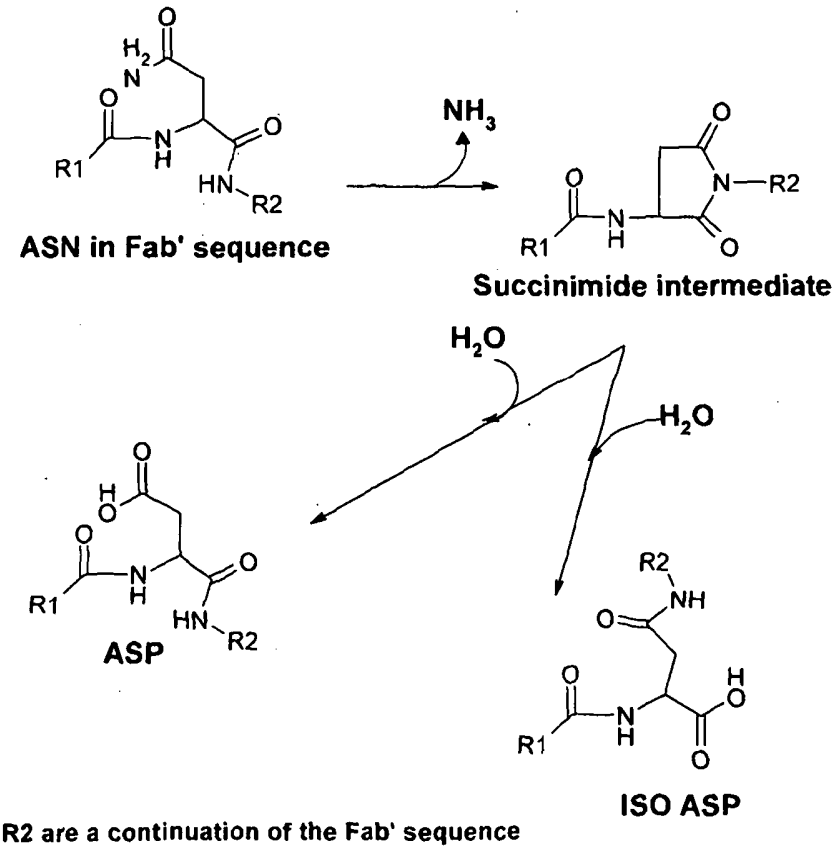
Description

[0001] The present invention relates to an improved assay method for measuring acidic species in PEGylated proteins. In particular, the present invention relates to an improved assay method for measuring acidic species for PEGylated Fab and PEGylated Fab' antibody formats.

[0002] PEGylated Fab and PEGylated Fab' antibody formats are useful in that they provide a circulating half-life *in vivo* similar to that of a whole antibody without the effector functions associated with a whole antibody. These formats have become useful in therapy and long term stability testing is required to support the regulatory approval process which licenses the sale of these therapeutic products. Furthermore, once approved for use by the general public manufactured product must be batch release tested before it is can be made available for sale.

[0003] The presence of acidic species in the formulations, for example after storage may be indicative of degradation (in particular deamidation) of the Fab or Fab'. Deamidation is classed by regulatory authorities as a degradation route and as such limits for the levels of deamidation are set for the product. These levels should not be exceeded during the shelf-life of the product. Whilst not wishing to be bound by theory it is thought that asparagine residues may be degraded via a succinimide intermediate by deamidation to generate acidic species, such as isoaspartic acid/aspartic acid, as set out in Scheme 1:



[0004] This deamidation of asparagine residues may result in a change in the proteins overall charge and may increase immunogenicity of the Fab or Fab'. Additionally, this deamidation may result in changes in the function/effcacy of the Fab or Fab', which may lead to unpredictable therapeutic effects/side effects or simply loss of activity. This can increase the adverse effects in patients after administration of the formulation. Thus degradation must be minimised and storage times and conditions must be limited to those when little or no degradation occurs. Therefore, it is important to be able to measure the deamidation in a given PEGylated Fab or Fab' formulation. The analysis may impact on the shelf-life and storage conditions given on the product labelling. It is also important to be able to monitor the levels of deamidation in the product because if predefined limits are exceeded then this may result in marketed products being withdrawn from sale or a block on the release for sale of certain batches of the products. In theory deamidation in the protein could be measured by quantifying the acidic species generated therein.

[0005] Zuang et al (2009. Anal Chem, 81, 1686-1692) disclose a method to measure deamination (i.e. measuring the formation of acidic species including isoAsp, aspartyl succinimide, ...) of an IgG antibody. The method comprises incu-

bating the antibody with trypsin at 37°C and separating and quantifying the digests via RP-HPLC and MS /MS. The trypsin digest generates peptide fragments

[0006] At the present time the total acidic species content of PEGylated Fab or PEGylated Fab' is measured using Cation Exchange (CEX)-HPLC.

[0007] The method measures an aggregate or total value of acidic species in the product. However, there are a number of routes by which the acidic species are generated and not all of those are associated with the deamidation (and hence degradation) of the protein. Thus the value obtained from the analysis is not a value for the actual amount of deamidation in the protein. In fact it includes acidic species generated by:

- degradation of the Fab' and
- hydrolysis of the linker joining the Fab' or Fab and the PEG molecule.

[0008] The hydrolysis of the PEG linker is thought to proceed through the succinimide ring as shown in Figure 4. This hydrolysis may in fact be the dominant effect and generate a large component of the aggregate value of the acidic species when analysed. The total acidic species content is represented diagrammatically in Figure 3, which shows acidic species generated in the protein by deamidation and acid species generated by hydrolysis of the linker. This can be represented diagrammatically as shown in Figure 5, which shows the relative proportions of acidic species generated by hydrolysis of the linker by deamidation and a combination thereof.

[0009] The aggregate value of acidic species in a molecule is not of primary interest. Instead what is of interest is the amount of deamidation in the protein. However, the analysis of the total acidic species content does not provide a value representative of the deamidation in the protein.

[0010] The amount of deamidation can be measured, for example employing ISOQUANT® aspartate detection kits. The deamidation is represented diagrammatically in Figure 2, which shows the deamidation in the protein only. However, this deamidation assay is not particularly robust (Alfaro et al., Anal. Chem, 2008, 80, 3882-3889).

[0011] To support the commercial manufacture of a therapeutic product, robust analytical techniques are required. The inventors believe that they have designed a robust and effective method for the analysis of the deamidation of PEGylated proteins, in particular PEGylated Fabs and Fab's.

[0012] The method of the present disclosure allows the direct measurement of acidic species in the Fab' molecule resulting from deamidation/degradation in the Fab' molecule and not in the PEG or the linker.

[0013] Thus there is provided a method of measuring acidic species generated by degradation of a Fab or Fab' component of a Fab'-PEG comprising the steps of:

a) cleaving the PEG and linker from the Fab'-PEG with an enzyme that cleaves the hinge portion of the Fab' and releases the peg and linker from the Fab',
b) separating the PEG and linker generated in step a) from the Fab', to provide a Fab or Fab' and
c) quantitatively analyzing acidic species associated with the cleaved Fab' and/or the cleaved PEG.

[0014] By removing the PEG and linker from the Fab' the amount of deamidation in the protein can be measured by quantifying the acidic species therein. The method is reproducible and robust and furthermore the cleavage of the PEG and linker does not interfere with or change the amount of deamidation in the protein. Thus the deamidation in the cleaved Fab' should be representative of the deamidation in the protein portion of the PEGylated Fab'.

[0015] In another embodiment the method also allows the extent of PEG linker hydrolysis to be determined by first determining the 'total' acidic species for the Fab'-PEG prior to cleavage in step (a) of the method and then subtracting the quantified acidic species associated with the Fab' component determined in step (c) of the method from the 'total' acidic species.

Brief Description of the Figures

[0016]

Figure 1 shows a diagrammatic representation of a PEGylated Fab'
Figure 2 shows of a diagrammatic representation deamidation occurring in a PEGylated Fab'
Figure 3 shows a diagrammatic representation of deamidation and hydrolysis of the linker both of which generate acidic species.
Figure 4 shows a diagrammatic representation of the chemical process of PEGylation of a Fab' and subsequent ring opening to generate an acidic species.
Figure 5 is a diagrammatic representation of the relative proportions of acidic species values which contribute to the total acidic species content.

Figure 6 is a diagrammatic representation of the species generated from a PEGylated Fab' after enzymatic digestion.
Figure 7 shows efficiency of a trypsin digest of a PEGylated Fab' over time.
Figure 8 is an CEX-HPLC analysis a the crude product resulting from trypsin digest of a PEGylated Fab'
Figure 9 is an iCEF of a PEGylated Fab' digested by trypsin
Figure 10 shows sequences 1 to 9
Figure 11. shows sequences 10 and 11

Brief Description of the Sequences

[0017] SEQ ID NO: 1 shows the amino acid sequence of CDRH1 of CDP870.
[0018] SEQ ID NO: 2 shows the amino acid sequence of CDRH2 of CDP870.
[0019] SEQ ID NO: 3 shows the amino acid sequence of CDRH3 of CDP870.
[0020] SEQ ID NO: 4 shows the amino acid sequence of CDRL1 of CDP870.
[0021] SEQ ID NO: 5 shows the amino acid sequence of CDRL2 of CDP870.
[0022] SEQ ID NO: 6 shows the amino acid sequence of CDRL3 of CDP870.
[0023] SEQ ID NO: 7 shows the nucleotide and predicted amino acid sequence of the light chain variable region CDP870.
[0024] SEQ ID NO: 8 shows the nucleotide and predicted amino acid sequence of the heavy chain variable region CDP870.
[0025] SEQ ID NO: 9 shows the amino acid sequence of a grafted anti-TNFα Fab CDP870 light chain.
[0026] SEQ ID NO: 10 shows the amino acid sequence of a grafted anti-TNFα Fab CDP870 heavy chain.

Detailed Description of the Invention

[0027] Acidic species as employed herein is intended to refer to a moiety, molecule, comprising a carboxylic acid i.e. comprising the group -C(O)OH.
[0028] In one embodiment the enzyme is a protease, for example trypsin or chymotrypsin, such as trypsin. When the enzyme employed is trypsin then the cleavage point is expected to be between the K and T in, for example the sequence SCDKTHTCAA (C-terminus Heavy Chain) of the Fab' fragment. Advantageously cleavage at that this point does not result in a change in the value of deamidation in the protein because the small portion of the hinge that is cleaved does not contain any asparagine residues.
[0029] Fab's naturally have a sequence in the hinge which is a suitable substrate for the enzyme. Fab molecules do not naturally have a substrate sequence for the enzyme but if desired an appropriate sequence can be engineered into an appropriate position allowing for removal of the PEG attached to the Fab by enzymatic digestion.
[0030] The enzymatic digestion may performed at a temperature in the range 20 to 40°C such as 25 to 38°C, in particularly it is optimally performed at 37°C.
[0031] In one embodiment when the starting entity is a PEGylated Fab' the enzyme cleaves the hinge portion of the Fab' and releases the PEG and linker from the Fab'.
[0032] The enzymatic digestion, for example tryptic digestion may be effected over a period of 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160 minutes or more.
[0033] The entities generated by digestion need not be separated because if a technique such as HPLC/clEF is employed for the quantification of acidic species the entities generated have different retention times and thus can be quantified individually without an additional separation step.
[0034] However, optionally the entities generated by step a) may be separated by known techniques, for example cation exchange chromatography, clEF, size exclusion chromatography and the like.
[0035] In one embodiment the acidic species associated with the Fab' and/or PEG is/are quantified.
[0036] In one embodiment the deamidation of the Fab or Fab' is measured.
[0037] In one embodiment acidic species in step c) are analysed employing HPLC analysis, for example employing an elution gradient. In one embodiment the HPLC analysis is CEX-HPLC analysis.
[0038] Alternatively, the amount of deamidation in the cleaved protein may be measured by capillary electrophoresis.
[0039] In one embodiment acidic species in step c) are analysed employing clEF analysis, suitable cartridges include iCE280 available from Convergent Bioscience.
[0040] In a preferred embodiment the antibody is an anti-TNF antibody, more preferably an anti-TNF Fab' CDP870, as described in WO01/094585.
[0041] In a one embodiment the antibody having specificity for human TNFα, comprises a heavy chain wherein the variable domain comprises a CDR having the sequence shown in SEQ ID NO:1 for CDRH1, the sequence shown in SEQ ID NO:2 for CDRH2 or the sequence shown in SEQ ID NO:3 for CDRH3.
[0042] In one embodiment the antibody comprises a light chain wherein the variable domain comprises a CDR having

the sequence shown in SEQ ID NO:4 for CDRL1, the sequence shown in SEQ ID NO:5 for CDRL2 or the sequence shown in SEQ ID NO:6 for CDRL3.

[0043] In one embodiment the antibody comprises a heavy chain wherein the variable domain comprises a CDR having the sequence shown in SEQ ID NO:1 for CDRH1, the sequence shown in SEQ ID NO:2 for CDRH2 or the sequence shown in SEQ ID NO:3 for CDRH3 and a light chain wherein the variable domain comprises a CDR having the sequence shown in SEQ ID NO:4 for CDRL1, the sequence shown in SEQ ID NO:5 for CDRL2 or the sequence shown in SEQ ID NO:6 for CDRL3.

[0044] In one embodiment the antibody comprises SEQ ID NO:1 for CDRH1, SEQ ID NO: 2 for CDRH2, SEQ ID NO:3 for CDRH3, SEQ ID NO:4 for CDRL1, SEQ ID NO:5 for CDRL2 and SEQ ID NO:6 for CDRL3.

[0045] The antibody is preferably a CDR-grafted antibody molecule and typically the variable domain comprises human acceptor framework regions and non-human donor CDRs.

[0046] Preferably, the antibody comprises the light chain variable domain CDP870 (SEQ ID NO:7) and the heavy chain variable domain CDP870 (SEQ ID NO:8).

[0047] According to the disclosure, the antibody can be a modified Fab fragment wherein the modification is the addition to the C-terminal end of its heavy chain one or more amino acids to allow the attachment of an effector or reporter molecule. Preferably, the additional amino acids form a modified hinge region containing one or two cysteine residue to which the effector or reporter molecule may be attached. Such a modified Fab fragment preferably has a heavy chain comprising or consisting of the sequence given as SEQ ID NO:10 and the light chain comprising or consisting of the sequence given as SEQ ID NO:9.

[0048] Preferences and/or embodiments may be combined as technically feasible.

[0049] The invention will now be described with reference to the following examples, which are merely illustrative and should not in any way be construed as limiting the scope of the present invention.

EXAMPLE

Tryptic Digestion Method

[0050] To an eppendorf, 1.0 mg Fab' PEG is added to 50mM NaOAc, 125mM NaCl pH 5.0 to 50μL total volume. 50μL 0.2M Na_2HPO_4 is added, followed by 40μL trypsin resuspension buffer (50mM Acetic Acid), the final pH should be in the region of pH 7.5. Vortex for 10 seconds.

[0051] The reaction is incubated at 37°C for 2 hours. Analyse by CEX HPLC or imaged capillary isoelectrophoresis.

Digest efficiency determined by HTRP HPLC

[0052] A tryptic digest using a Fab'PEG was set up using an Agilent 1100 series auto sampler incubated at 37°C, in order that the reaction could be injected directly onto HTRP HPLC assay. 9 injections of the Fab' Peg and Fab' control were carried out sequentially. Fab' PEG gave a digest profile shown in Figure 7. This demonstrates that within two hours, the Fab' PEG product content is reduced to ~20%.

Analysis

[0053] After digestion, samples were removed and diluted to 1mg/mL in sample dilution buffer (20mM Sodium Acetate pH 4.5). The Fab' PEG and cleaved Fab' can be identified along with their respective acidic species without pre-processing of the mixture obtained from the digestion step, as shown in Figure 8. The following as shown in figure 8 are eluted sequentially; undigested acidic Fab' PEG species, undigested Fab' PEG, acidic cleaved Fab' species and cleaved Fab'.

Suitable Chromatographic Conditions for the detection of cleaved Fab' Acidic species by CEX HPLC

[0054]

Solvent A	equilibration buffer 10mM 2-(N-morpholino)Ethane Sulfonic acid pH 6.2
Solvent B	elution buffer 10mM 2-(N-morpholino)Ethane Sulfonic acid, 50 mM sodium chloride pH 6.2
Column	Dionex Propac SCX-10 column
Flow Rate	0.5mL/min
Stop Time	75 min
Max Pressure	250 bar
Method Run Pressure (guide)	~60 bar

(continued)

Column Temperature	25°C
Injection Volume	100 μL
Autosampler Temperature	4 °C
Detection Wavelength 280nm	(16 bandwidth), 4nm slit width
Loading	100 μL at 1 mg/mL

Time	Solvent A (%)	Solvent B (%)	Flow Rate (mL/min)
0.0	100	0	0.5
2.0	100	0	0.5
62.0	40	60	0.5
62.5	0	100	0.5
63.5	0	100	0.5
64.0	100	0	0.5
75.0	100	0	0.5

[0055] Data Analysis may be carried out using HP Chemstation software where the peaks are integrated, see figure 8 for an example of chromatogram of Fab' PEG digest using the suitable chromatographic conditions described. The following are detected; undigested acidic Fab' PEG, undigested Fab' PEG, acidic cleaved Fab', cleaved Fab'.

Alternative Method of Analysis using Imaged Capillary Isoelectrophoresis

Sample Preparation for analysis using iCE280

[0056] A generic sample preparation of 200μL volume is given below:

- **Tryptic Digest** (desalted and free from any free ions at a concentration of 1mg/ml)-40ul (General rule: final concentration of the protein should be around 0.1-0.3mg/ml in final sample mixture).
- **1% Methyl Cellulose:** 70μL (Methyl cellulose concentration in the final the mixture should be 0.35%)
- **Carrier Ampholytes:** (3-10 Pharmalytes)- 8μL (Carrier ampholytes should have a concentration of 4% in the final sample mix).
- **pI markers:** 1μL each of two different pI markers are added whose pI values should lie on the either side of the protein and its related species.
- **HPLC grade water:** Add required amount of HPLC water to make up the volume to 200μL.

[0057] Mix the above sample by vortexing for 15-30 second to ensure proper mixing of different components. Spin the mixture at 16000g for 10 minutes to remove air bubbles and dust particles, which would interfere with the analysis.

Instrument settings and analysis employing iCE280 Technology

[0058] iCE 280 from Convergent biosciences (Isogen in Europe) is an imaged capillary isoelectrophoresis instrument, which is used to determine pIs of various protein samples and their related species.

[0059] High voltage is applied across the capillary using an anode and cathode, which are dipped, in small reservoirs containing catholyte (OH^-) and anolyte (H^+). Samples are prepared with carrier ampholytes and on application of high voltage the protein molecules migrate according to their respective pIs and finally focus at it. The anolyte may be prepared by adding calculated amount of phosphoric acid to give a final solution of 0.08 M phosphoric acid in 0.1% methyl cellulose. The catholyte may be prepared by adding 10.4 μl of 50% w/w NaOH solution to 2ml of 0.1% methyl cellulose. Catholyte must be freshly prepared and should not be reused. Generally 2ml is enough for a single fill of the reservoir.

[0060] Generally for all non-PEGylated samples, a focussing period of 5-6 minutes at 3000v should be enough, but since different proteins have different charge distribution, the focussing time would also vary accordingly. In that case the settings needs to be optimised by running two or three samples.

[0061] Data analysis may be carried out using EZChrom software. Profiles can be compared by overlaying them using 'Supercompare' and electrophoregrams can be integrated using 'Method development' options within the EZChrom software. See Figure 9 for an example of electrophoregram of Fab' PEG digest. The following are detected; low pI marker, acidic cleaved Fab' species, cleaved Fab' species, basic cleaved Fab' species, high pI marker.

Claims

1. A method of measuring acidic species generated by degradation of a Fab' component of a Fab'-PEG comprising the steps of:

   a) cleaving the PEG and linker from the Fab'-PEG with an enzyme that cleaves the hinge portion of the Fab' and releases the PEG and linker from the Fab',
   b) separating the PEG and linker generated in step a) from the Fab', to provide a Fab' and
   c) quantitatively analyzing acidic species associated with the cleaved Fab'.

2. A method according to claim 1, wherein the enzyme is trypsin or chymotrypsin.

3. A method according to claim 1 or 2 wherein the cleaving in step a) is performed at a temperature in the range 25 to 40°C, such as 37°C.

4. A method according to any one of claims 1 to 3, wherein the cleavage of step a) occurs between a lysine and a threonine, in particular in the C-terminal of the Fab' heavy chain.

5. A method according to claim 4, wherein the lysine and threonine are found in the sequence SCDKTHTCAA located at the C-terminal of a Fab'.

6. A method according to claim 1, wherein the separation is effected using cation exchange chromatography, cIEF, and/or size exclusion chromatography.

7. A method according to any one of claims 1 to 6, wherein the quantification of acidic species is performed employing cation exchange chromatography or imaged capillary isoelectrophoresis, such as CEX-HPLC.

8. A method according to any one of claims 1 to 7, wherein the Fab' comprises or consists of SEQ ID NO:10.

Patentansprüche

1. Verfahren zum Messen saurer Spezies, erzeugt durch Abbau einer Fab'-Komponente eines Fab'-PEG, umfassend die Schritte:

   a) Abspalten des PEG und Linkers von dem Fab'-PEG mit einem Enzym, das die Gelenkregion des Fab' abspaltet und das PEG und den Linker von dem Fab' löst,
   b) Trennen des in Schritt a) erzeugten PEG und Linkers von dem Fab', wodurch ein Fab' bereitgestellt wird, und
   c) quantitatives Analysieren saurer Spezies, die mit dem abgespalteten Fab' in Verbindung stehen.

2. Verfahren nach Anspruch 1, wobei das Enzym Trypsin oder Chymotrypsin ist.

3. Verfahren nach Anspruch 1 oder 2, wobei das Abspalten in Schritt a) bei einer Temperatur im Bereich von 25 bis 40 °C, wie 37 °C, durchgeführt wird.

4. Verfahren nach einem der Ansprüche 1 bis 3, wobei das Abspalten aus Schritt a) zwischen einem Lysin und einem Threonin auftritt, insbesondere im C-Terminus der schweren Kette des Fab'.

5. Verfahren nach Anspruch 4, wobei das Lysin und Threonin in der Sequenz SCDKTHTCAA, die sich am C-Terminus eines Fab' befindet, zu finden ist.

6. Verfahren nach Anspruch 1, wobei die Trennung unter Verwendung von Kationenaustauschchromatographie, cIEF

und/oder Größenausschlusschromatographie bewirkt wird.

7. Verfahren nach einem der Ansprüche 1 bis 6, wobei die Quantifizierung saurer Spezies unter Einsatz von Kationenaustauschchromatographie oder bildgebender Kapillar-Isoelektrophorese wie CEX-HPLC durchgeführt wird.

8. Verfahren nach einem der Ansprüche 1 bis 7, wobei das Fab' SEQ ID NR.: 10 umfasst oder daraus besteht.

Revendications

1. Procédé de mesure d'espèces acides se formant par dégradation d'un constituant Fab' d'un Fab'-PEG, comprenant les stades de :

   a) clivage du PEG et du segment de liaison du Fab'-PEG par un enzyme qui clive la partie charnière du Fab' et libère le PEG et le segment de liaison du Fab',
   b) séparation du PEG et du segment de liaison formés au stade a) du Fab' pour obtenir un Fab' et
   c) analyse quantitative des espèces acides associées au Fab' clivé.

2. Procédé suivant la revendication 1, dans lequel l'enzyme est la trypsine ou la chymotrypsine.

3. Procédé suivant la revendication 1 ou 2, dans lequel le clivage au stade a) est effectué à une température allant de 25 à 40°C, telle qu'à 37°C.

4. Procédé suivant l'une quelconque des revendications 1 à 3, dans lequel le clivage du stade a) se produit entre une lysine et une thréonine, en particulier à la terminaison C de la chaîne lourde de Fab'.

5. Procédé suivant la revendication 4, dans lequel la lysine et la thréonine se trouvent dans la séquence SCDKTHTCAA placée à la terminaison C d'un Fab'.

6. Procédé suivant la revendication 4, dans lequel on effectue la séparation en utilisant une chromatographie par échange de cation, cIEF et/ou une chromatographie par exclusion de dimension.

7. Procédé suivant l'une quelconque des revendications 1 à 6, dans lequel on effectue la quantification des espèces acides en employant une chromatographie par échange de cation ou une isoélectrophorèse capillaire imagée, telle que CEX-HPLC.

8. Procédé suivant l'une quelconque des revendications 1 à 7, dans lequel le Fab' comprend l'identification de séquence N° 10 ou consiste en l'identification de séquence N° 10.

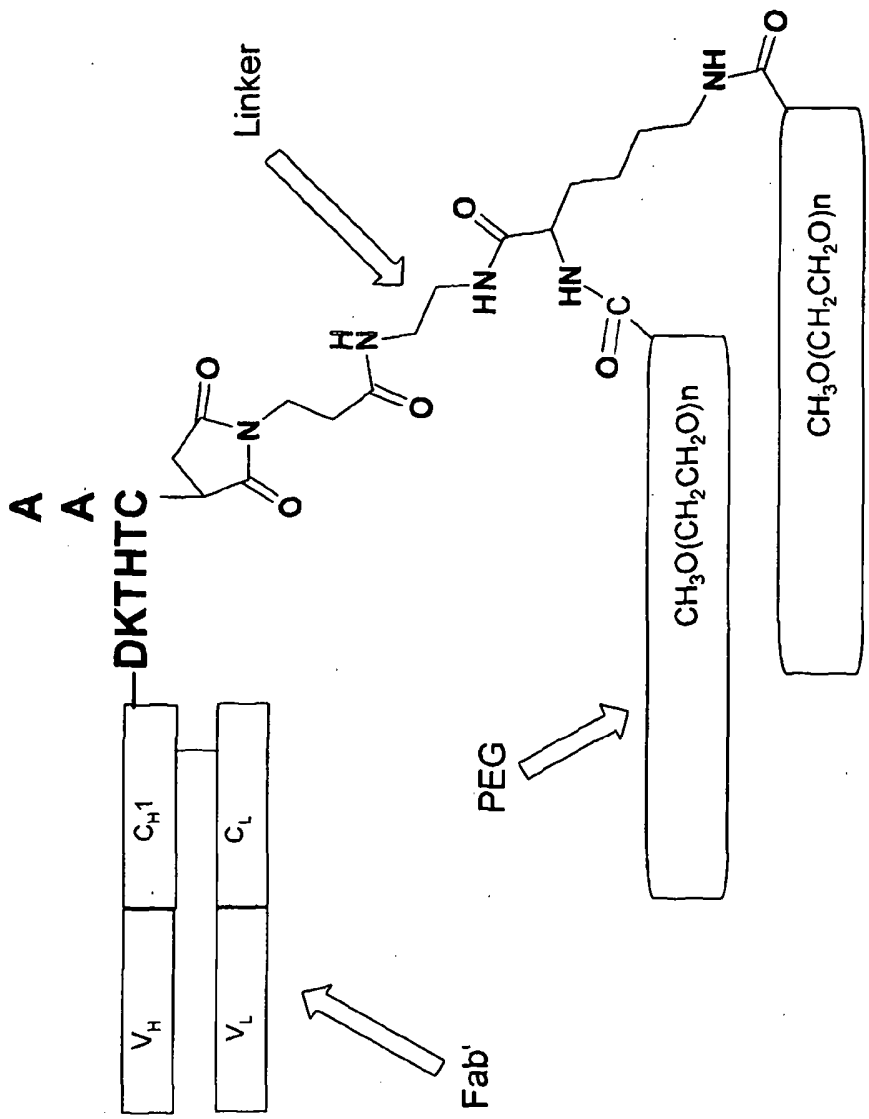


Figure 1 Schematic diagram of a PEGylated Fab' format.

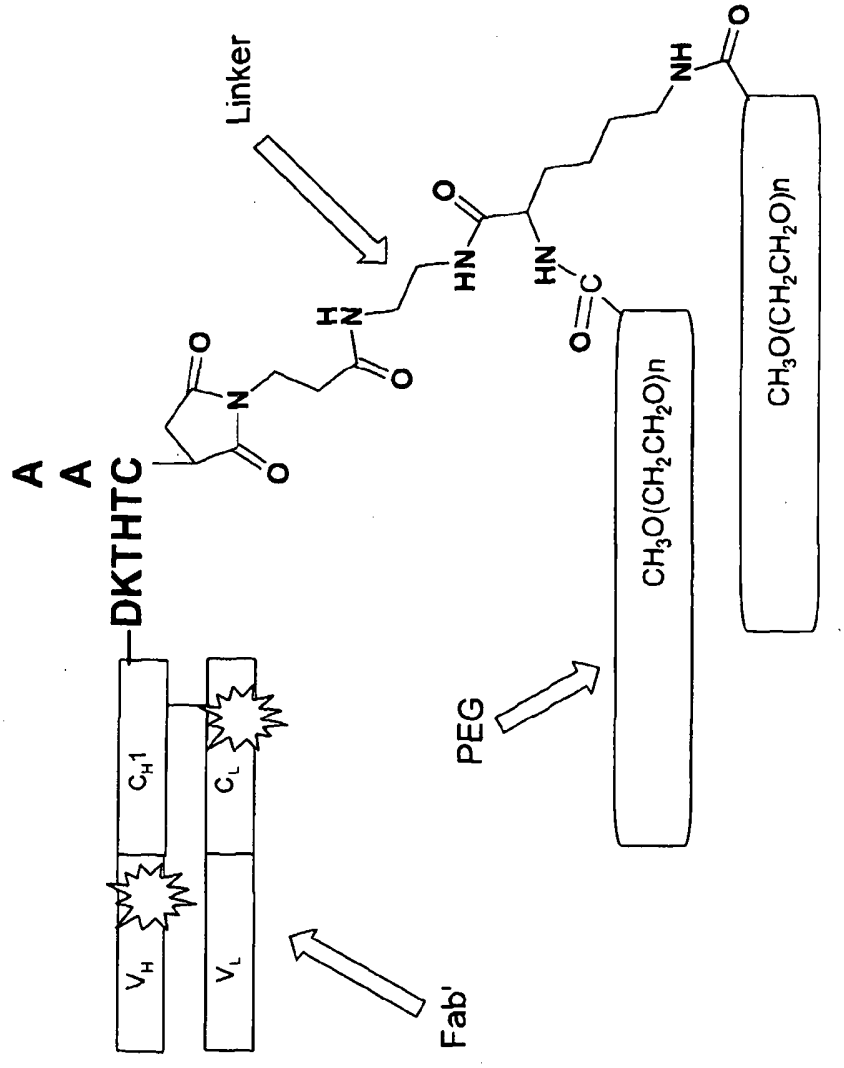


Figure 2 Schematic diagram of a PEGylated Fab' format with deamidation in the Fab' fragment

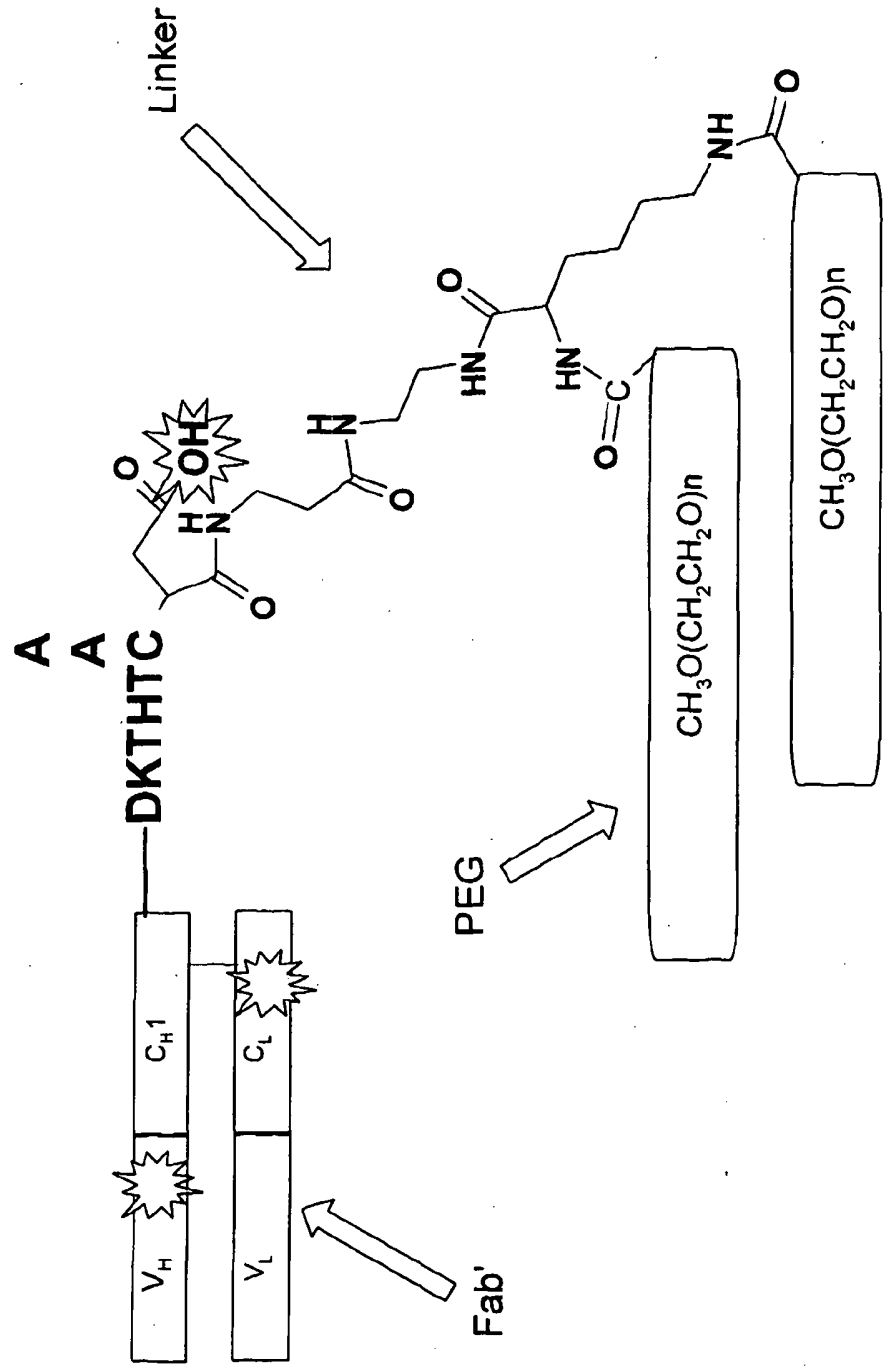


Figure 3 Schematic diagram of a PEGylated Fab' format with deamidation in the Fab' fragment and hydrolysis of the linker to generate acidic species

Figure 4 Schematic representation of the hydrolysis of Fab' and PEG maleimide followed by ring opening of the PEG linker succinimide ring

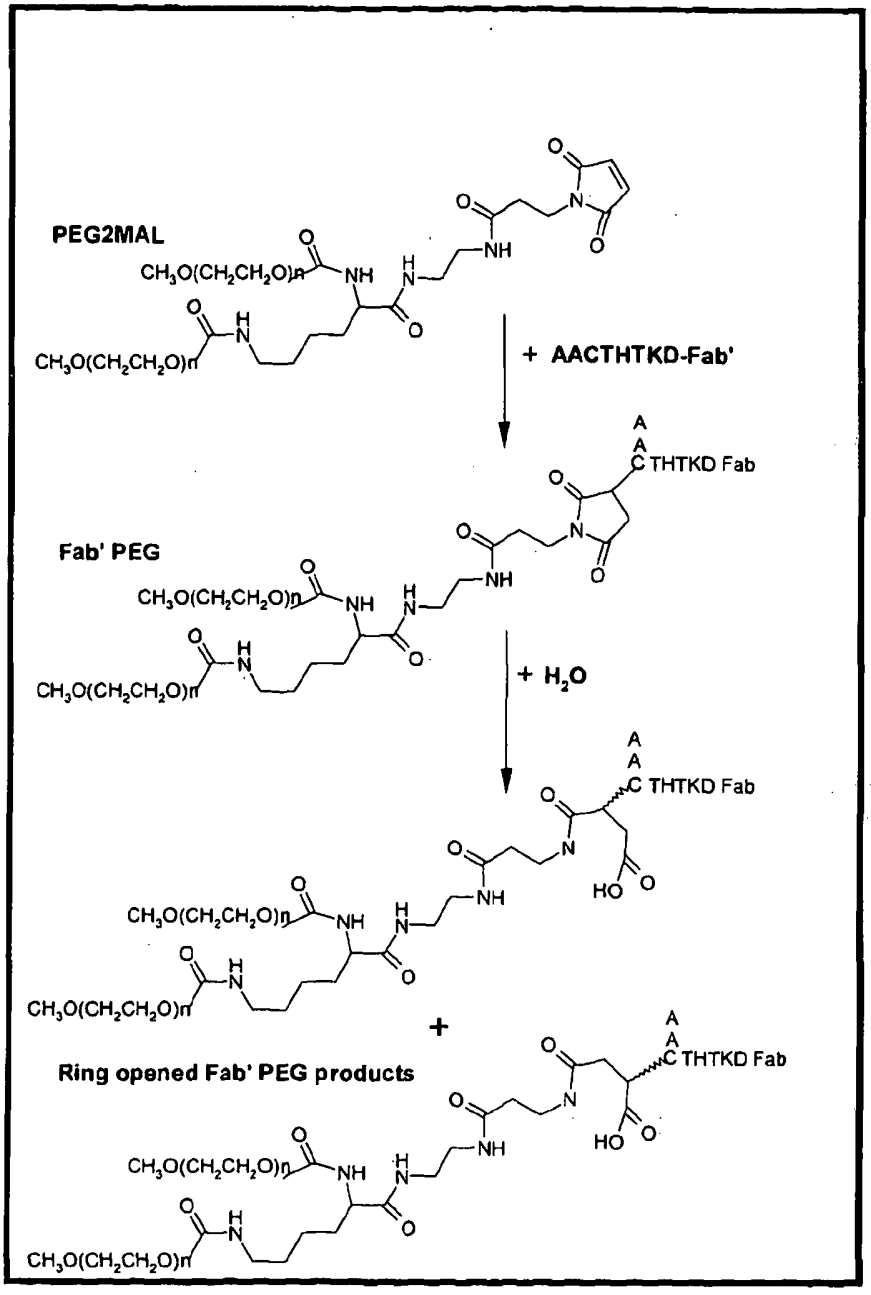
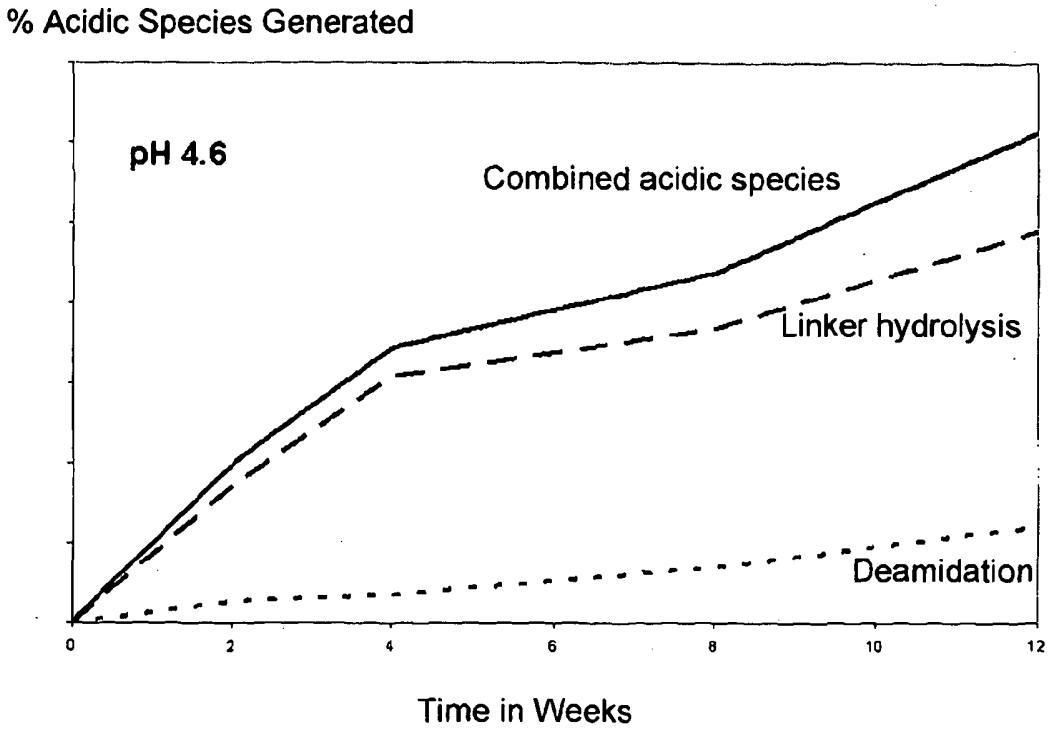


Figure 5

Analysis of combined acidic species, linker hydrolysis generated acidic species and acidic species generated through deamidation



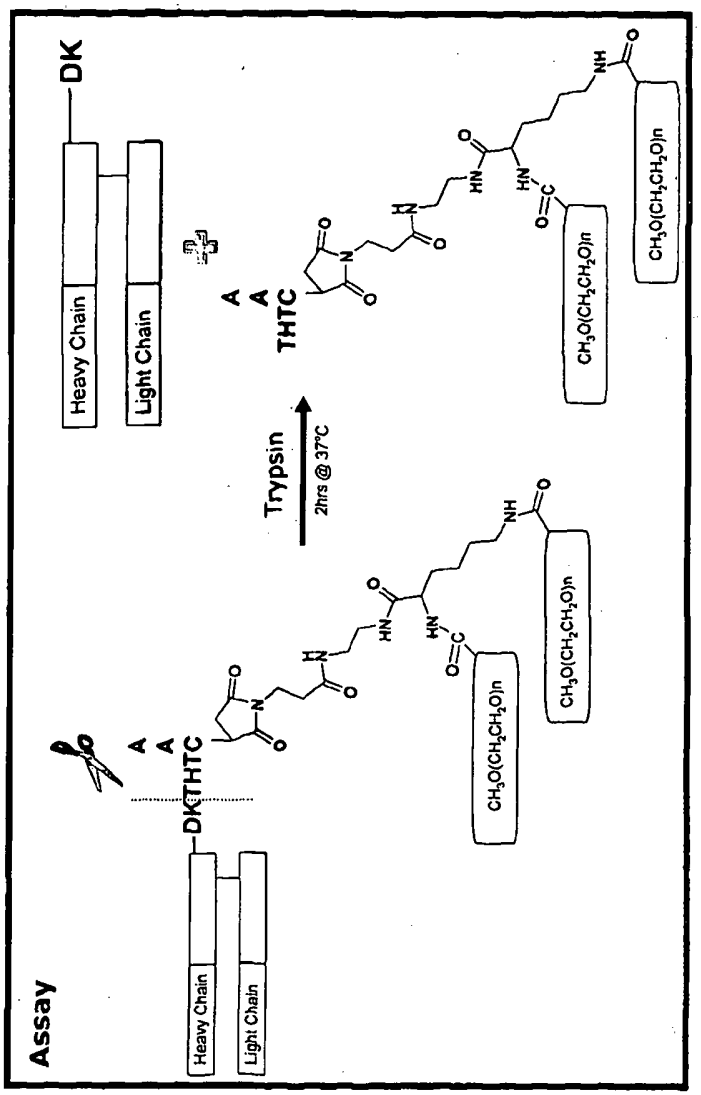

Assay
Heavy Chain
Light Chain
DK
A
A
THTC
DK
Trypsin
2hrs @ 37°C
Heavy Chain
Light Chain
A
A
THTC
CH3O(CH2CH2O)n
CH3O(CH2CH2O)n
CH3O(CH2CH2O)n
CH3O(CH2CH2O)n

Figure 6

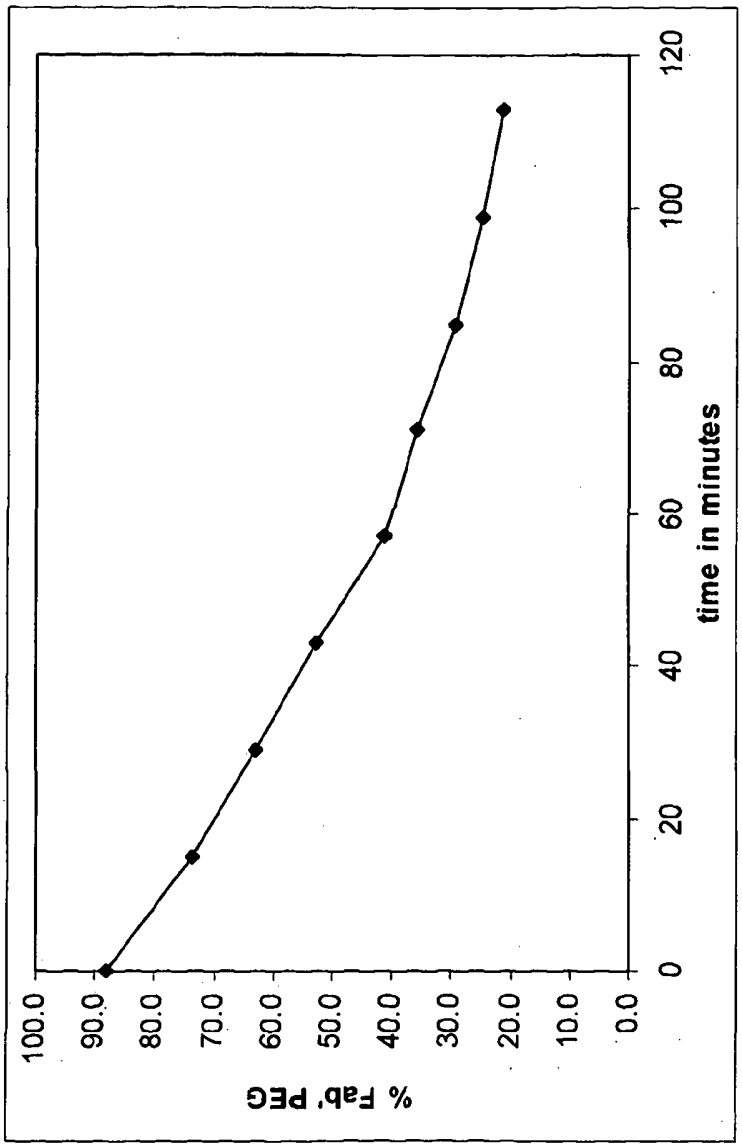


Figure 7 Efficiency of Tryptic Digest Conditions followed by HTRP HPLC

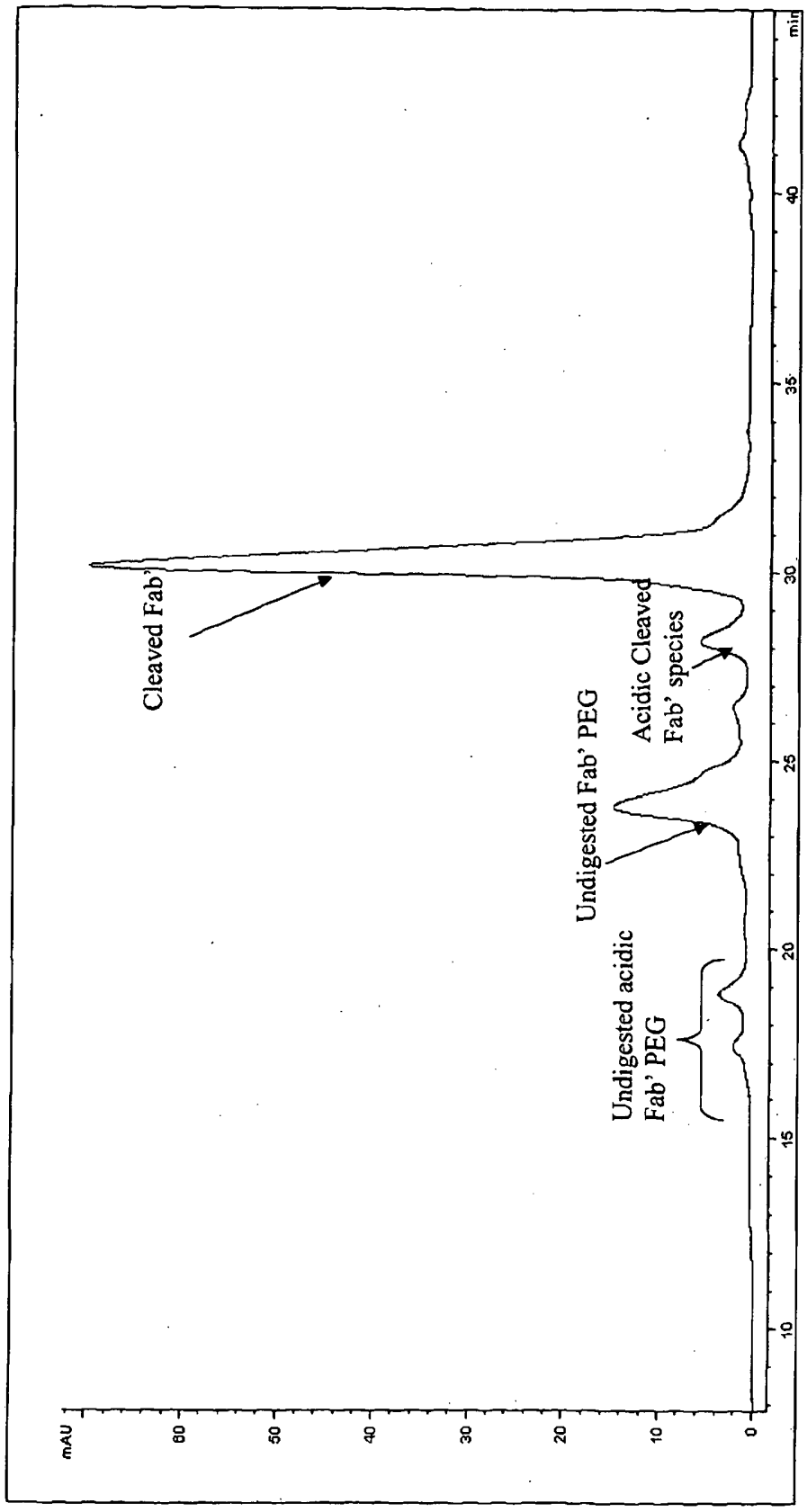


Figure 8 Fab' PEG CEX HPLC digest profile

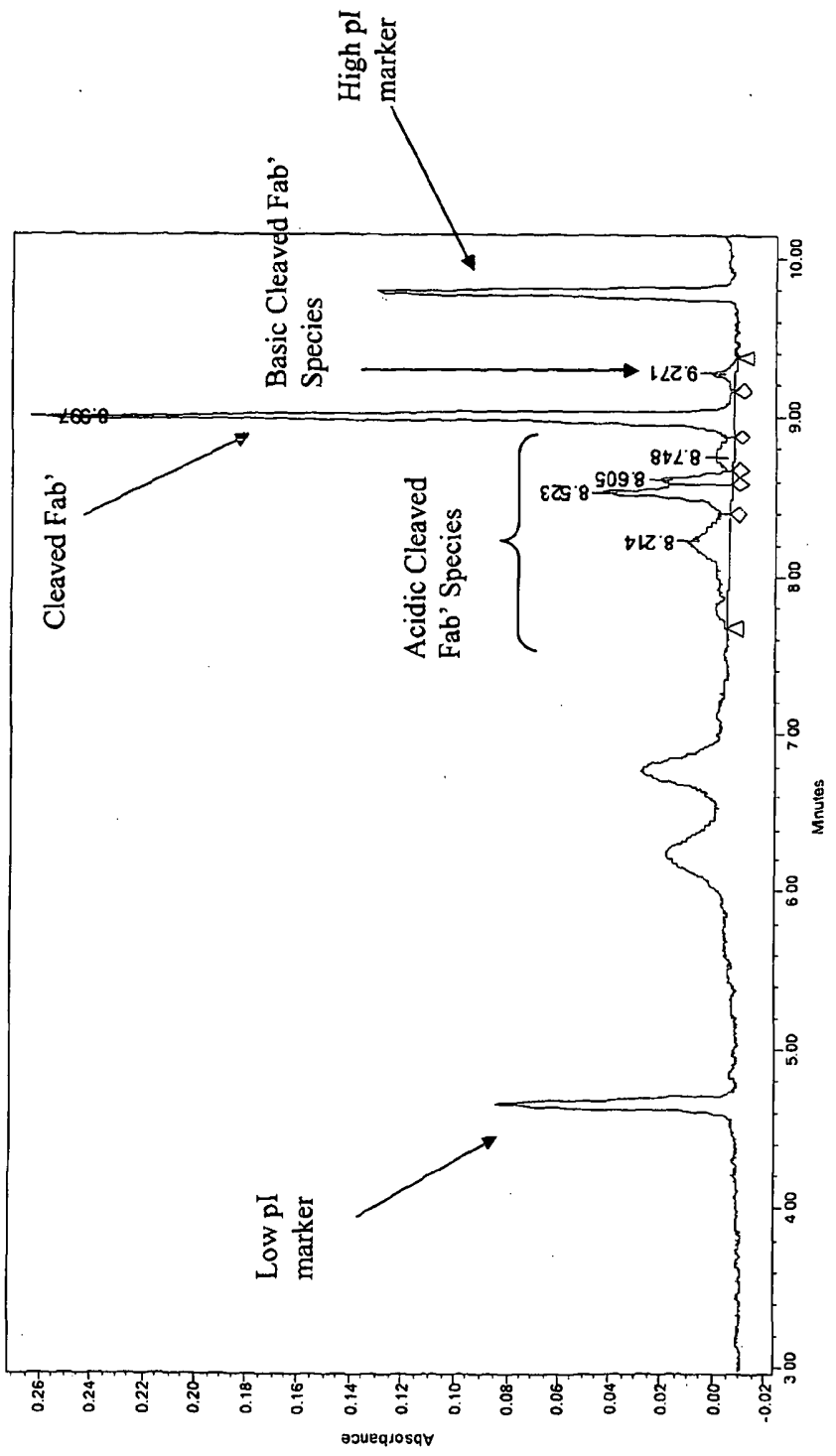


Figure 9 cIEF profile of tryptic digest of Fab' PEG

Figure 10

SEQ ID NO: 1 shows the amino acid sequence of CDRH1 of CDP870.
Asp Tyr Gly Met Asn

SEQ ID NO: 2 shows the amino acid sequence of CDRH2 of CDP870
Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Ala Asp Ser Val Lys Gly

SEQ ID NO: 3 shows the amino acid sequence of CDRH3 of CDP870.
Gly Tyr Arg Ser Tyr Ala Met Asp Tyr

SEQ ID NO: 4 shows the amino acid sequence of CDRL1 of CDP870.
Lys Ala Ser Gln Asn Val Gly Thr Asn Val Ala

SEQ ID NO: 5 shows the amino acid sequence of CDRL2 of CDP870.
Ser Ala Ser Phe Leu Tyr Ser

SEQ ID NO: 6 shows the amino acid sequence of CDRL3 of CDP870.
Gln Gln Tyr Asn Ile Tyr Pro Leu Thr

SEQ ID NO: 7 shows the amino acid sequence of the light chain variable region CDP870
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Ala Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Tyr Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ile Tyr Pro Leu Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys

SEQ ID NO:8 shows the amino acid sequence of the heavy chain variable region CDP870.
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Val Phe Thr Asp Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Met Gly Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Gly Tyr Arg Ser Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser

Figure 11

SEQ ID NO: 9 shows the amino acid sequence of a grafted anti-TNFα Fab CDP870 light chain.
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Ala Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Tyr Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ile Tyr Pro Leu Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys

SEQ ID NO: 10 shows the amino acid sequence of a grafted anti-TNFα Fab CDP870 heavy chain.
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Val Phe Thr Asp Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Met Gly Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Gly Tyr Arg Ser Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser 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 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 Ala Ala

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- WO 01094585 A **[0040]**

Non-patent literature cited in the description

- **ZUANG et al.** *Anal Chem,* 2009, vol. 81, 1686-1692 **[0005]**
- **ALFARO et al.** *Anal. Chem,* 2008, vol. 80, 3882-3889 **[0010]**

ANALITIKAI ELJÁRÁS FAB ÉS FAB' MOLEKULÁKHOZ

Szabadalmi igénypontok

1. Eljárás Fab'-PEG Fab'-komponensének bomlásakor keletkező savas speciesek mérésére, amely magában foglalja a következő lépéseket:

a) a PEG és a kötőcsoport hasítása az Fab'-PEG-ről olyan enzimmel, amely lehasítja az Fab' csuklórészét és felszabadítja a PEG-et és a kötőcsoportot az Fab'-ról,

b) az a) lépésben képzett PEG és kötőcsoport elválasztása a Fab'-tól, ezáltal Fab' biztosítása, és

c) a lehasított Fab'-vel kapcsolódó savas speciesek mennyiségi analízise.

2. Az 1. igénypont szerinti eljárás, ahol az enzim tripszin vagy kemotripszin.

3. Az 1. vagy 2. igénypont szerinti eljárás, ahol a hasítás az a) lépésben 25-40°C-os hőmérsékleten, például 37°C-on megy végbe.

4. Az 1-3. igénypontok bármelyike szerinti eljárás, ahol a hasítás az a) lépésben egy lizin és egy treonin között megy végbe, különösen az Fab'-nehézlánc C-terminálisán.

5. A 4. igénypont szerinti eljárás, ahol a lizin és a treonin az Fab' C-terminálisán elhelyezkedő SCDKTHTCAA szekvenciában található.

6. Az 1. igénypont szerinti eljárás, ahol az elválasztás kationcserélő kromatográfiával, cIEF-fel (kapilláris izoelektromos fókuszálás) és/vagy méretkizárásos kromatográfiával valósul meg.

7. Az 1-6. igénypontok bármelyike szerinti eljárás, ahol a savas speciesek mennyiségi analízise kationcserélő kromatográfia vagy képalkotó kapilláris elektroforézis, például CEX-HPLC alkalmazásával történik.

8. Az 1-7. igénypontok bármelyike szerinti eljárás, ahol az Fab' tartalmaz SEQ ID NO:10 szerinti szekvenciát vagy SEQ ID NO:10 szerinti szekvenciából áll.