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(54) **Title:** IR ANALYSIS OF PROTEIN AGGREGATES

(57) **Abstract:** This invention relates to a method for infrared spectrophotometric quantitation of protein aggregates in samples containing biomolecules.

IR analysis of protein aggregates

Field of Invention

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This invention relates to a method for infrared spectrophotometric quantitation of protein aggregates in samples containing biomolecules.

Background

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Infrared (IR) spectroscopy is a commonly used analytical tool in research laboratories for analysis of samples. The IR region of the electromagnetic spectrum extends from the lower end of the visible region (wavenumber of approximately $14,300\text{ cm}^{-1}$) to the microwave region (near 20 cm^{-1}).

15

Usually, for a molecule to absorb IR, the vibrations or rotations within the molecule must cause a net change in the dipole moment of the molecule. The alternating electrical field of the radiation interacts with fluctuations in the dipole moment of the molecule and if the frequency of the radiation matches the vibrational frequency of the molecule, then the radiation is absorbed, thereby causing a change in the amplitude of the molecular vibration.

20

Purification of biotherapeutic antibodies produced in cell cultures is usually performed using a set of orthogonal purification techniques, typically starting with centrifugation and filtration to remove cellular debris and large particulates. This process is typically followed by protein A affinity chromatography for selective antibody capture, typically by low-pH elution, low-pH virus-inactivation, ion-exchange chromatography or hydrophobic interaction chromatography as orthogonal techniques. However, several of the aforementioned procedures may lead to antibody aggregate formation, particularly filtration-associated shear stress, stress due to agitation during upstream processing phase, the low-pH elution in affinity chromatography

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as well as low-pH virus inactivation step. Long-term storage, pH-shifts upon purification, freeze-thawing, lyophilization for antibody formulation and concentration-induced aggregate formation due to high antibody levels in the drug product are other possible sources of protein aggregation [Wang, W., Singh, S., Zeng, D. L., King, K. et al., Antibody structure, instability, and formulation. *J. Pharm. Sci.* 2007, 96, 1-26].

Aggregation levels during various phases of antibody purification vary between 0.5% and 60% depending on the intrinsic properties of the antibody molecule used and require monitoring as well as aggregate removal as aggregates are suspected to induce renal failure, increased immunogenicity and anaphylactic side reactions [Wang, W., Singh, S., Zeng, D. L., King, K. et al., Antibody structure, instability, and formulation. *J. Pharm. Sci.* 2007, 96, 1-26].

A common acceptance criterion for allowable aggregate levels in drug substances and products is to maintain levels below 5%.

The state of the art for aggregate analysis usually uses chromatographic methods such as size exclusion chromatography, and SDS-PAGE, capillary electrophoresis as well as light scattering [Brorson, K., Phillips, J., Defining Your Product Profile and Maintaining Control Over It, Part 4 Product-Related Impurities: Tackling Aggregates, Technical Bioprocess, BioProcess International November 2005]. These techniques, in particular SDS-PAGE, require a certain amount of time to obtain information about the aggregation content of a sample and therefore do not allow for fast data analysis and quick decision-making processes for the optimization of biotherapeutic processes.

Apart from the above given methods, it was realised that infrared spectroscopy can be used to analyze effects of storage and formulation on antibody structure [Matheus, S., Friess, W., Mahler, H.-C., FTIR and nDSC as Analytical Tools for High-Concentration Protein Formulations. *Pharm. Res.* 2006, 23, 1350-1363]

as well as for aggregate analysis [Joubert, M. K., Luo, Q., Nashed-Samuel, Y., Wypych, J. et al., Classification and Characterization of Therapeutic Antibody Aggregates. *J. Biol. Chem.* 2011, 286, 25118-25133]. However, these studies only used this technique for qualitative purposes only,
5 comparing specific wavenumber ranges.

Dry state IR protein quantification can be performed using developed methods and instruments (WO 2012/141831), there Amide I band area is used for general protein quantification. This method does not differentiate
10 between different protein types.

It would be desirable to find a simple and fast way to do quantitative analysis of protein aggregates in the presence of other proteins.

15 It has been found that protein aggregates can be analysed quantitatively by IR spectrometry if more than one infrared band area and/or band height within the mid infrared spectrum is used for the analysis. Additionally, it has been found that if analysis is performed on a dried sample, water molecule absorption, otherwise masking and interfering with relevant protein
20 absorption bands, is avoided. The interference by other substances on quantification results is reduced, as quantification relies on several infrared bands, preferably present in different regions of the mid infrared range.

Summary of the Invention

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The present invention is thus directed to a method for quantitation of protein aggregates in a sample, the method comprising the steps of:

- (a) providing a support
- (b) applying the sample to the support
- 30 (c) optionally drying the sample on the support
- (c) exposing the sample on the support to an infrared beam comprising a wavelength in the spectral range of $4000\text{--}400\text{ cm}^{-1}$ or any portion of the

spectral range of 4000-400 cm^{-1} , thereby to obtain an infrared absorption spectrum; wherein two or more absorption band areas and/or band heights in the infrared absorption spectrum correlate with protein aggregate quantity within the sample.

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The present invention provides IR based methods for quantitation of protein aggregates, especially antibody aggregate content in a sample comprising biomolecules, in particular antibodies.

10 Quantitation requires less time to perform, no additional buffer and eluent consumption as well as less sample volume than most methods known in the art. Further, this particular invention does not require any special sample preparation steps.

15 In one method for quantitation of aggregate content in a sample containing one or more biomolecules, in particular for quantitating protein aggregation forms, in particular antibody aggregation forms, in a given sample according to the present invention, the method comprises the steps of:

(a) providing a support, also called sample holder, preferably comprising a
20 porous membrane which comprises a hydrophilic region surrounded by a hydrophobic region for sample containment;

(b) contacting the hydrophilic region of the membrane with the sample;

(c) optionally drying the sample on the membrane;

(d) exposing the sample on the membrane to an infrared beam comprising
25 a wavelength in the spectral range of 4000-400 cm^{-1} , or any portion of the spectral range, thereby to obtain an infrared absorption spectrum; where two or more absorption peak areas in the infrared absorption spectrum correlate with the quantity of protein aggregation forms, in particular antibody aggregation forms and protein aggregate amount, in particular
30 antibody aggregate amount in a sample.

Surprisingly, it has been found, that protein aggregates, in particular antibody aggregates can be quantified using two or more, preferably between 3 and 12, more preferable between 3 and 7, wavenumber ranges within the infrared spectrum. Preferably these ranges are associated to certain secondary structure elements (e.g. α -helix, β -sheet, etc.), which may undergo changes during aggregation and thus enable selective antibody aggregation, or are associated to differences in the hydrogen-bonding pattern between aggregated and non-aggregated protein, also allowing quantification of protein aggregates.

10

Suitable wavenumber ranges encompass either the total or part of the ranges between 2000-800 cm^{-1} , preferably the total or part of the ranges between 1700-1340 cm^{-1} , more preferably the total or part of the ranges 1695-1601 cm^{-1} , 1579-1480 cm^{-1} , 1478- 1423 cm^{-1} and/or 1360-1355 cm^{-1} .

15

Preferably the two or more absorption band areas and/or heights for protein aggregate quantification are selected from one or more of the following ranges: 1695-1601 cm^{-1} , 1579-1480 cm^{-1} , 1478- 1423 cm^{-1} and/or 1360-1355 cm^{-1} .

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In some embodiments according to the claimed methods, the one or more protein aggregates are selected from aggregates formed by antibodies, in particular mono- or polyclonal antibodies, host cell proteins and/or different antibody forms.

25

In various embodiments, each of the two or more absorption bands in the spectrum correlate with the quantity of aggregated protein forms, e.g. aggregation forms and aggregate amount of an antibody in the sample.

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In further embodiments, the protein aggregation forms may show differences in their absorption bands, depending on whether these

aggregates are of non-covalent or covalent origin. Consequently, the here described methods allow differentiation of these different aggregate forms.

5 In some embodiments according to the present invention, one or more calibration curves are pre-loaded onto the instrument used, thereby obviating the need to generate a calibration curve each time a user wishes to quantitate protein aggregation forms and aggregate amount, in particular antibody aggregation forms and aggregate amount in a sample.

10 In some embodiments according to the present invention, these calibration curves may be based on multivariate calibration, using several suitable wavenumber ranges for quantification, whereby these wavenumber ranges may be located at different areas within the mid infrared spectrum.

15 In some embodiments according to the present invention, the spectra may be treated by mathematical steps first, before using wavenumber ranges and bands for establishing a calibration curve. Exemplary mathematical treatment steps include but are not limited to first derivative, second derivative, higher order derivative, normalization of spectra, vector
20 normalization, minima-maxima normalization.

The here described methods according to the present invention can be used for quantitating protein aggregates in a very small sample volume. In various embodiments according to the claimed methods, the sample
25 volume ranges from 0.1 to 20 μl . In a particular embodiment, the sample volume is about 2 to 5 μl or less, or less than 1 μl .

In some embodiments, the sample comprises a biological fluid such as, for example, a pharmaceutical sample, cell culture fluid, drug product, drug
30 substance, blood, plasma, serum and urine. In other embodiments, the sample is an environmental sample or a food sample. In yet other embodiments, the sample is a fuel sample.

In some embodiments, the sample comprises one or more antibodies, which may be contained in cell culture fluid, drugs substance, drug product. In some embodiments, samples for protein aggregation quantification
5 comprise the whole production process of biotherapeutic proteins, comprising samples taken from a bioreactor, from centrifugation, filtration, chromatography steps, virus inactivation, final polishing phase.

10 In some embodiments, the sample may comprise one or more, also different, aggregation forms and aggregate amounts, composed of protein aggregates and in particular, of antibody aggregates, derived from one protein or from two or more different proteins.

15 In some embodiments, two or more different aggregates, that means two or more different aggregate forms of one protein and/or two or more aggregates of different proteins can be analyzed in the sample with the method of the present invention. For this, for each aggregate form two or more specific absorption band areas and/or heights are selected. The selected absorption band areas can then be individually analyzed so that
20 two or more aggregate forms can be analyzed in parallel in one measurement.

25 In one embodiment, for example, two or more absorption band areas and/or band heights correlate with the amount of non-covalent protein aggregates and two or more different absorption band areas and/or heights correlate with the amount of covalent protein aggregates.

In some embodiments, the sample comprises cell lysate or tissue lysate. In various embodiments, the sample is a crude sample.
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In some embodiments, the porous support is a membrane, e.g. an ultrafiltration or microporous membrane.

- 5 In various embodiments according to the claimed methods, the support or sample holder comprises a porous support, e.g. a membrane as described in WO/2012/141831 which comprises an area within which the sample volume is contained on the membrane. The area for sample containment may comprise a hydrophilic region within a hydrophobic region, e.g. as described in WO/2012/141831, where the sample volume is contained within the hydrophilic region.
- 10 It is important that the sample applied to the support is contained within the diameter of the IR beam diameter, preferably within a diameter smaller than the IR beam diameter. This ensures that the entire sample is visible to IR beam and that accurate protein aggregate quantitation is achieved. A suitable support may e.g. contain, but is not limited to a hydrophilic region
- 15 ranging from 2.0 mm through 9.2 mm.

Brief Description of the Drawings

- 20 Figure 1 is an exemplary calibration curve generated for quantification of protein aggregate amount, particularly antibody aggregate amount compared to overall antibody amount. A sample containing monoclonal antibody at a concentration total concentration of 4 mg/ml was mixed with sample of the same antibody, which was exposed to repeated pH-stress inducing covalent antibody aggregate forms. Calibration was then
- 25 generated using prepared samples of different aggregate amounts by mixing aggregated and non-aggregated antibody at different ratios but maintaining same overall antibody concentration.

- 30 Figure 2 is a caption of an infrared absorption spectrum, depicting the second derivative of a representative experiment to quantitate antibody aggregation amount in cell culture fluid solution. For simplicity only small amount of wavenumber range is shown. It should be understood that also additional as well as other than here shown wavenumber ranges may be

used for aggregate quantification with potential wavenumber ranges for aggregate quantification being between 4000- 400cm⁻¹. Within the here chosen wavenumber range between 1520- 1480 cm⁻¹, a clear absorption pattern is visible, showing band intensity correlating with aggregate amount.

5 Figure 3 depicts the second derivative of an infrared absorption spectrum depicting the results of a representative experiment to quantitate aggregation forms of an antibody in cell culture fluid solution after employing an affinity chromatography step. The X-axis represents the wavenumber in cm⁻¹ and the Y-axis represents the absorbance units. The
10 spectrum demonstrates that the spectral intensity decreases at different wavenumber regions as the concentration of antibody aggregates in the sample decreases from 14.8 % (w/w) to 1 % (w/w) compared to the overall antibody amount.

Figure 4 depicts the second derivative of an infrared absorption spectrum
15 depicting the results of a representative experiment to distinguish and selectively quantitate different aggregation forms in a sample, comprising antibody. Antibody solution with non-covalent aggregate forms was used directly after affinity chromatography elution and spiked to antibody solution, containing no aggregates as well as antibody solution containing
20 covalent aggregate forms.

Selective spectral window areas were used to distinguish different aggregate form types, either of covalent or non-covalent form type, using single samples containing both aggregates in same sample. If more than 50% of aggregates compared to total aggregate amount (w/w) were from
25 non-covalent aggregate type, spectrum was colored in black. If more than 50% of aggregates compared to total aggregate amount (w/w) were covalent aggregate type, spectrum was colored in grey.

The X-axis depicts the wavenumber in cm⁻¹ and the Y-axis depicts the absorbance units. The spectrum demonstrates that different aggregate
30 types can clearly be distinguished using the here described methods, as they show spectral differences.

Figure 5 is a comparison of antibody aggregate quantification using size exclusion chromatography, the standard method known and used in the art for aggregate quantification and aggregate quantification using the here described invention.

- 5 Figure 6 depicts the calibration of the here described method, to be used for antibody aggregate quantification comprising samples of two different types of cell culture fluid and post protein A affinity purification samples. Figure 7 depicts the calibration of the here described method for quantification of antibody aggregates, using different samples comprising
- 10 different monoclonal antibodies each. A total of three different antibodies were used, all in cell culture fluid.

Detailed Description of the Invention

- 15 The present invention provides improved methods for differentiation and selective quantitation of protein aggregates, preferably antibody aggregates, in a sample, using infrared spectroscopy (IR). In order that the present disclosure may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout
- 20 the detailed description.

I. Definitions

- The term "quantify," "quantitation," "quantitate," "measure" or
- 25 "measurement," as used interchangeably herein, refers to the determination of amount or concentration of an analyte in a sample, using the methods according to the claimed invention. The term "analyte," as used herein, refers to any molecule of interest that is desirable to quantitate using the methods described herein. In various embodiments, an analyte is a protein
- 30 aggregate.

The term "multivariate" as used in this invention refers to correlation of the concentration of an analyte to more than one reference data point. Within

the context of this invention, multivariate refers to using several suitable wavenumber ranges, bands, for quantification of a specific type of analyte, in this case, protein aggregates. These suitable wavenumber ranges may be located at different positions within the mid infrared spectrum.

5 The term "biomolecule" as used herein, refers to any biological material, preferably to proteins.

The term "protein" as used herein refers to large biological molecules consisting of one or more chains of amino acids, which are bonded together via peptide or amide bonds, forming polypeptide chains making up the
10 protein. The term protein as used herein comprises different types of proteins, e.g. biotherapeutic proteins, host cell proteins, impurity proteins, any protein of interest and particularly antibodies.

The term "antibody" refers to a protein which has the ability to specifically bind to an antigen. Typically, antibodies are having a basic four-polypeptide
15 chain structure consisting of two heavy and two light chains, said chains being stabilized, for example, by interchain disulfide bonds. Antibodies may be monoclonal or polyclonal and may exist in monomeric or polymeric form, for example, IgM antibodies which exist in pentameric form and/or IgA antibodies which exist in monomeric, dimeric or multimeric form. Antibodies
20 may also include multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they retain, or are modified to comprise, a ligand-specific binding domain. The term "fragment" refers to a part or portion of an antibody or antibody chain comprising fewer amino acid residues than an intact or complete antibody or antibody chain. Fragments
25 can be obtained via chemical or enzymatic treatment of an intact or complete antibody or antibody chain. Fragments can also be obtained by recombinant means. When produced recombinantly, fragments may be expressed alone or as part of a larger protein called a fusion protein.

Exemplary fragments include Fab, Fab', F(ab')₂, Fc and/or Fv fragments.
30 Exemplary fusion proteins include Fc fusion proteins. According to the present invention fusion proteins are also encompassed by the term "antibody". As discussed above, in some embodiments, an antibody is an

Fc region containing protein, e.g., an immunoglobulin. In some embodiments, an Fc region containing protein is a recombinant protein which includes the Fc region of an immunoglobulin fused to another polypeptide or a fragment thereof. Exemplary polypeptides include, e.g.,

5 renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α -1 -antitrypsin; insulin α -chain; insulin β -chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIc, factor

10 IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor - α and - β ; enkephalinase; RANTES (regulated on activation normally T- cell expressed and secreted); human macrophage inflammatory protein (MIP-

15 1- α); a serum albumin such as human serum albumin; Muellierian-inhibiting substance; relaxin α -chain; relaxin β -chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as β -lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA) (e.g.,

20 CTLA-4); inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone- derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT- 6), or a nerve growth factor such as NGF- β .; platelet-derived growth factor (PDGF);

25 fibroblast growth factor such as α FGF and β FGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF- β , including TGF- β 1, TGF- β 2, TGF- β 3, TGF- β 4, or TGF- β 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins (IGFBPs); CD proteins such as CD3, CD4,

30 CD8, CD 19 CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon- α , - β , and - γ ; colony stimulating factors (CSFs), e.g., M-CSF,

GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments and/or variants of any of the above-listed polypeptides. In addition, an antibody according to the present invention is any protein or polypeptide, fragment or variant thereof, that binds specifically to any of the above-listed polypeptides. "Antibody" and "antibodies" may be used interchangeably in the here described invention and method.

The term "aggregate" or "protein aggregate" as used herein, refers to any protein that is at least aggregated, e.g. accumulated and clumped together with at least one other protein. Protein aggregates include antibody aggregates like antibody dimers, multimers, quadramers, low and high molecular weight aggregates, multiple aggregation forms, denatured proteins, denatured antibodies.

The term "sample," as used herein, refers to any medium, which includes an analyte (e.g., a protein aggregate) to be quantitated using the methods according to the present invention. A sample may include, but is not limited to, e.g., a food substance (e.g., poultry, fresh meat, milk, yogurt, dairy products, bakery products, beverages, beer, lemonade, juices, cheeses, vegetables, fruit, fish etc.), cell culture fluid, e.g. cell culture fluid from a bioreactor, from various phases during production and purification of biopharmaceutical proteins, drug solutions, drug substance, drug formulations in general, a water or sewage body (e.g., pond, lake, river, ocean, sewage canals, drinking tap water etc.), a clinical specimen (e.g., blood, plasma, serum, sputum, tissue, urine, saliva, sample/fluid from respiratory tract etc.), soil, and cosmetics and pharmaceuticals (e.g., lotions, creams, ointments, solutions, medicines, eye and ear drops etc.). In a particular embodiment, a sample comprises a cell or tissue lysate. In

various embodiments of the claimed invention, a sample may constitute a crude sample, i.e., it does not require any preparation or treatment prior to use in the claimed methods.

5 The term "support" as used herein is any suitable support allowing application of a sample and measurement of that sample when exposing the support to an infrared beam. A suitable support must maintain the sample within the beam of the infrared laser, e.g. by being composed of a hydrophilic part to contain the sample and a hydrophobic surrounding, preventing the applied sample from flowing from the spot of application.

10 Furthermore, a suitable support must be permeable to infrared light, at least for the relevant wavenumber ranges used in the method herein for differentiation and selective quantification of protein aggregates. Such a suitable support is e.g. a membrane, as described in WO/2012/141831, but may also be an IR permeable chip. An example of a suitable hydrophilic

15 membrane is a hydrophilic PTFE membrane.

In some embodiments, a small volume of a sample (e.g., 0.1-20 μ l) is pipetted or spotted onto a support, suitable for being used for infrared based quantification purposes. The support may be composed of a

20 membrane, composed of e.g. a hydrophilic region on the support contained in a sample holder (e.g., in the form of a card) and subsequently optionally dried followed by exposing the sample to IR based spectroscopy. The sample on the support may be dried using any suitable method. For example, the sample may be air dried or dried using a convection heater or

25 a conventional oven or even a microwave oven. Other suitable drying methods are the use of compressed air/nitrogen or any other inert gas. It is contemplated that in some embodiments, a drying mechanism is incorporated into the IR system. Often it is desirable that there are no traces of water present in a sample prior to quantitation of protein

30 aggregates as water may present a hindrance to obtaining accurate quantitation. If the presence of water presents a hindrance to obtaining accurate results, the sample is preferably dried prior to IR measurement.

Infrared absorbance to detect the presence of water may be used to assure correct drying of the support prior to quantitation. According to the present invention "drying" thus means that water is removed from the sample on the support at least until the amount of water left in the sample does not present a hindrance to obtaining accurate quantification any more.

The methods according to the claimed invention enable the use of a very small sample volume to achieve accurate quantitation of protein aggregates in the sample. The sample volume is about 0.05 μ l, 0.1 μ l or 0.2 μ l or 0.3 μ l or 0.4 μ l or 0.5 μ l or 0.6 μ l or 0.7 μ l or 0.8 μ l or 0.9 μ l or 1.0 μ l or 1.5 μ l or 2 μ l or 2.5 μ l or 3 μ l or 3.5 μ l or 4 μ l or 4.5 μ l or 5 μ l or 5.5 μ l or 6 μ l or 6.5 μ l or 7 μ l or 7.5 μ l or 8 μ l or 8.5 μ l or 9 μ l or 9.5 μ l or 10 μ l or 10.5 μ l or 11 μ l or 11.5 μ l or 12 μ l or 12.5 μ l or 13 μ l or 13.5 μ l or 14 μ l or 14.5 μ l or 15 μ l or 15.5 μ l or 16 μ l or 16.5 μ l or 17 μ l or 17.5 μ l or 18 μ l or 18.5 μ l or 19 μ l or 19.5 μ l or 20 μ l or greater than 20 μ l. Although, the devices and methods described herein facilitate the use of very small volumes for protein aggregate quantitation in order to increase lower limit of detection and quantitation using the methods described herein, multiple aliquots of a sample can be applied to the support, with drying of the sample in between the aliquots. For examples, in case of samples containing very low levels of protein aggregates, which may be difficult to detect or undetectable using the methods described herein, multiple aliquots of a certain sample (e.g., 10-20 μ l) can be spotted on the support, with drying of the sample in between the different aliquots. Therefore, although, only a small volume of 10-20 μ l is spotted onto the support in each application, a total sample volume of 50 μ l to 100 μ l or more could be applied in total over the multiple applications of the sample volume, with drying of each sample volume in between the applications.

In some embodiments, a sample volume of 100 μ l or more is applied to the support by applying 20 μ l of sample to the support and drying the support.

Once that sample is completely dry, additional 20 μ l of sample can be applied to the support and the support can be dried again. This process can be repeated multiple times to spot the requisite sample volume on the

support. The advantage of this technique is that it increases the limit of the analyte detection/quantitation.

As used herein, the term "lyse," "lysis" or "lysing" refers to any means by which a cell or tissue can be broken open, e.g., by compromising the cell membrane and possibly causing the contents of the cell to be liberated.

Exemplary methods which may be used for lysing a cell or tissue include, but are not limited to, enzymatic methods, ultrasound, mechanical action (e.g., shear and impaction) and chemical methods.

The term "wavelength," generally refers to the distance between one peak or crest of a wave and the next peak or crest. It is equal to the speed of the wave divided by its frequency, and to the speed of a wave times its period.

Wavelength is a characteristic of both traveling waves and standing waves, as well as other spatial wave patterns. Wavelength is commonly designated by the Greek letter lambda (λ). Assuming that a sinusoidal wave is moving at a fixed wave speed, wavelength is inversely proportional to the frequency of the wave. Therefore, waves with higher frequencies have shorter wavelengths, and waves with lower frequencies have longer wavelengths.

The term "wavenumber," is a property of a wave proportional to the reciprocal of its wavelength. It is generally measured in units of cm^{-1} and can be defined by the number of wavelengths per unit distance, i.e., $1/\lambda$, where λ is the wavelength.

The term "absorption band" as used herein, refers to one or more parts of an infrared absorption spectrum observed following the exposure of a sample to IR spectroscopy, as described herein. Once an infrared absorption spectrum is obtained using the IR based methods described herein, either the area under one or more bands in the spectrum is calculated by drawing a baseline across the band and measuring the area enclosed in the band. Alternatively, the band intensity can be determined measuring the height from baseline to top position of a band.

Description:

The present invention for the first time offers a simple and fast method for quantitative analysis of protein aggregates.

5 It is based on the finding that two and more band areas and/or band heights correlate with the concentration or quantity of protein aggregates, in particular antibody aggregates. It is further based on the finding that protein aggregates persist when the sample is dried on the support so that very sensitive measurements can be made without water hindering the
10 measurement.

In some embodiments, the concentration of aggregates in a sample is measured as follows. As a first step, a calibration curve is generated using a calibrant, which includes protein standards of known aggregate amount
15 and concentration, and the calibration curve is pre-loaded onto an IR spectrometer instrument. The same calibration curve can subsequently be used each time that the instrument is used for quantitating aggregates in a sample. Further, it is not necessarily required that the calibrant be present in the sample being analyzed, or in other words, it could be derived from a
20 completely different source than the sample. The calibration samples are applied to a suitable support, the support containing the sample is optionally dried and the absorption spectrum is measured using an IR instrument, e.g., the Bruker IR instrument. Without wishing to be bound by theory, it is contemplated that any suitable IR instrument may be used in
25 the methods according to the present invention.

In some cases, it may be beneficial to perform a mathematical pre-treatment of spectra before using them for calibration and/or analysis of unknown samples. Exemplary mathematical pre-treatment steps include but are not limited to first derivative, second derivative, higher order
30 derivative, normalization of spectra, vector normalization, minima maxima normalization.

Most modern IR absorption instruments use Fourier-transform techniques with a Michelson interferometer. In some embodiments according to the claimed methods, in order to obtain an IR absorption spectrum, one mirror of the interferometer moves to generate interference in the radiation
5 reaching the detector. Since all wavelengths are passing through the interferometer, the interferogram is a complex pattern. The absorption spectrum as a function of wavenumber (cm^{-1}) is obtained from the Fourier transform of the interferogram, which is a function of mirror movement (cm). This design does not have the reference cell of a dispersive instrument, so
10 a reference spectrum is recorded and stored in memory to subtract from the sample spectrum.

Other exemplary IR absorption instruments include dispersive IR absorption instruments and single wavelength IR instruments. Dispersive IR spectrometers use diffraction grating in a monochromator in order to
15 disperse the different wavelengths of light. In general, dispersive IR spectrometers have been replaced with FTIR instruments. Single wavelength IR instruments may be used for monitoring a single IR wavelength to measure the kinetics of a fast reaction.

In some embodiments according to the claimed invention, an FTIR
20 instrument is used for the quantitation of protein aggregates. Area under the curve which encompasses suitable bands, also multiple band areas, e.g. using a multivariate approach, for protein aggregate quantification ($4000 - 400 \text{ cm}^{-1}$) is calculated by the software inbuilt into the instrument. In some cases, it may be beneficial to perform a mathematical pre-treatment
25 of spectra before using them for calibration and/or analysis of unknown samples. Exemplary mathematical pre-treatment steps include but are not limited to first derivative, second derivative, higher order derivative, normalization of spectra, vector normalization, minima maxima normalization.

30 For example, the Bruker IR spectrometer includes the Bruker Opus software. A calibration curve is then set up which plots area under the band vs. aggregate concentration of the protein aggregate standard. Using the

calibration curve generated by the standard of known aggregate concentration, the concentration of an aggregate in a sample is subsequently measured.

5 In general, a calibration curve refers to a graphical display of the functional relationship between the expected value of the observed signal to the analyte amount or concentration. Typically, standards encompassing a range of known concentrations of a calibrant are used to generate a calibration curve. The spectrum obtained with the sample is then compared with the standards to obtain the concentration of the desired analyte.

10

Some of the methods described herein further include a step of monitoring/detecting absorption by water in the sample. Water absorbs at ~3400 and 1600 cm^{-1} , thereby overlapping with the spectrum obtained with protein samples. In some embodiments according to the present invention, an IR instrument is used which includes an in-built software to monitor the change in spectral intensity associated with absorption by water, which decreases as the amount of water in the sample reduces. Accordingly, by monitoring the spectral intensity associated with water, a user can determine if the sample needs to be further dried. Typically, the spectral intensity associated with absorption by water is measured a few times during the drying process, until the same spectral intensity is observed for 2 or 3 or more consecutive reads, thereby confirming that the sample is dry for the actual quantitation.

15

20

25 In a preferred embodiment, the support that is provided in the method of the present invention is a porous membrane, e.g., an ultrafiltration membrane or a microporous membrane. In general, an ultrafiltration membrane is understood to have a pore size smaller than 0.1 μm .

Preferably, the support comprises a hydrophilic region surrounded by a hydrophobic region, as described in WO/2012/141831

30

5 In one embodiment, the support that is provided in the method of the present invention is within a device. The device can be any holder in which the support can be fixed, e.g. by mechanical forces or with a glue. The device can be made e.g. of plastic. It can be in the form of a flat card onto which the support is fixed. It is also possible that more than one support is within the device or that the device has more than one opening which provides access to the support so that more than one sample application area is present on the device and more than one sample can be analyzed with said device.

10

Sample holder cards for use in infrared spectrophotometric analysis have been previously described in the art which include a microporous membrane as support, e.g., those discussed in U.S. Patent Nos. 5,470,757 and 5,764,355, incorporated by reference herein in their entirety. Other suitable devices and supports, e.g. sample holder cards and membranes are described in WO/2012/141831, whereby these supports are cost effective, easy to manufacture and disposable.

15

The methods of quantitating protein aggregates, preferably antibody aggregates in a sample described herein obviate the requirement for sample preparation steps and eluent or buffer preparation. This is an improvement to methods known and used in the art, such as chromatography, SDS-PAGE, capillary electrophoresis.

20

25 This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application and of corresponding EP application 13001626.4 filed March 28, 2013, as well as the Figures, are incorporated herein by reference.

30

Examples

Example 1: Generation of a standard curve for protein aggregate quantitation

5 In methods described herein, a user generates a calibration or standard curve once and can use that for future analyses. In one experiment, a calibration curve was generated as follows. Solutions of various concentrations of antibody aggregates were prepared and analyzed using FTIR based detection methods as described herein. Before establishing the

10 calibration curve, spectra were mathematically transformed to second derivative to increase apparent resolution. Several suitable wavenumber ranges, showing a correlation of band intensity with antibody aggregate concentration, were selected for calibration on a multivariate approach. These wavenumber ranges encompassed 1694-1690 cm^{-1} , 1665-1654 cm^{-1} , 1624-1616 cm^{-1} , 1580-1567 cm^{-1} , 1560-1555 cm^{-1} , 1546-1527 cm^{-1} , 1502-1496 cm^{-1} , 1475-1440 cm^{-1} , 1360-1346 cm^{-1} , 1317-1306 cm^{-1} and 914-903 cm^{-1} . The graph in Figure 1 shows the predicted protein aggregate quantification as determined from multiple band areas (Y axis) versus concentration or amount of protein aggregates as determined from a

15 reference method (X axis). The linear range of this calibration is used for calculation of protein aggregate concentration in an unknown sample when the peak area is known.

20

25 Example 2: General Protocol for quantitation of antibody aggregates in a sample

In a representative experiment, a sample containing a defined amount of protein aggregates is prepared, using e.g. aggregated antibody in a defined mixture with non-aggregated antibody.

Approximately 0.2 - 10 μl of a blank solution (e.g., deionized water, drug substance, drug product, post protein A sample, cell culture fluid) is applied

30 to a suitable support, e.g. a membrane in a device, which is in the form of a card as described in WO/2012/141831. The same volume of the prepared

sample solution is applied to one or more spots, which maybe present on the same support or on a different but identical supports. The support is subsequently dried using one of the following techniques: heat (40 - 60 °C, 0.5 - 2 minutes); compressed air/nitrogen or any other inert gas (0.5 - 2 minutes) or a microwave oven. The dry support is subsequently inserted into the sample compartment of the IR instrument. The transmission/absorption spectrum of the blank solution is measured between 4000 and 400 cm⁻¹, or any other suitable part, portion or range of the mid infrared wavenumber range, in order to obtain a spectrum for the background followed by the measurement of the sample at the same wavenumber range. Figures 2 and 3 show parts of relevant wavenumber ranges to be used for protein aggregate quantification, depicting wavenumber ranges showing a correlation between protein aggregate amount and band area or band height.

Using a calibration curve which is inbuilt into the system and generated using standards at various protein aggregate concentrations, concentration of protein aggregates in an unknown sample is subsequently determined.

Example 3: Preparation of antibody aggregates

In a representative experiment, antibody aggregates were prepared by exposing a solution, either cell culture fluid solution, drug substance or drug product or solution after using affinity chromatography, containing an antibody, to repeated pH- shifts. pH- shifts used may exemplary be within the range of pH 5.0- pH 10.0 and last for 2 hours before changing to the respective other pH back. pH- shifts may be repeated several times to induce antibody aggregates. Aggregates are then measured using e.g. size exclusion chromatography before preparing samples with different amounts of antibody aggregates, spiking aggregated antibody to non- aggregated antibody, for example.

In one example, two biotherapeutic antibodies, mAb A and mAb B were used as model proteins. Repeated pH-shift of mAb A resulted in formation of aggregated antibody present as mostly dimers and to a small percentage

as multimers, as analyzed by SEC. Aggregated fractions were concentrated, using Eshmuno S to final levels of aggregated antibody between 53- 54%, compared to overall antibody amount. mAb B was used as a process sample that contained 7% aggregates. Both antibody
5 solutions were spiked into the respective non-aggregated antibody solution to reach various levels of aggregate content.

Example 4: Quantitation of antibody aggregates in different cell culture fluids and post protein A elution using one quantification method
10 In another representative experiment, it is demonstrated that the methods according to the present invention can be used for quantitating aggregates during different steps of purification within the production of therapeutic antibodies.

The present invention can thereby be used not only for aggregate
15 quantification in a bioreactor, but also aggregate quantification after using e.g. affinity chromatography as well as aggregate quantification using a different cell culture fluid types.

Different samples containing antibody with antibody aggregates at various levels, either taken from the bioreactor, using two different cell culture fluid
20 types of chinese hamster ovary cell culture fluid (CHO4, CHO-DG44), as well as samples after affinity chromatography were measured using the here described method, using either the total or parts of the wavenumber ranges $1695\text{-}1601\text{ cm}^{-1}$, $1579\text{-}1480\text{ cm}^{-1}$, $1478\text{-}1423\text{ cm}^{-1}$, $1360\text{-}1355\text{ cm}^{-1}$, and a quantification method established to be used as general
25 quantification method for the above mentioned different sample types. Calibration is shown in figure 6.

Example 5: Quantitation of aggregates using different antibodies
The method was used similar as described above but comprised
30 aggregates using samples with three different antibodies (in different subsets of these samples), using all samples as cell culture fluid samples. Calibration is shown in figure 7 and highlight the suitability of this method to

be used for aggregate quantification using different antibodies. Table I shows results for unknown samples, not being used for calibration before. Coefficients of variation for results are mostly below 20%, with > 60% of samples showing coefficients of variation less than 10% (table I).

5

Table I: Results of here described method for aggregate quantification using three different antibodies. CV: coefficient of variation. All results in % (w/w).

	mAb A			mAb B			mAb C		
	True	Predicted	CV	True	Predicted	CV	True	Predicted	CV
10	14,8	15,5	3,5	7,0	7,0	0,5	6,7	5,2	14,9
	14,0	14,5	2,5	6,5	5,6	10,3	5,4	5,2	2,6
	12,0	10,2	10,7	6,0	4,7	14,8	4,8	4,2	9,2
	10,0	10,7	5,0	5,5	5,4	1,6	4,2	4,6	6,9
	8,0	8,5	4,1	5,0	4,6	5,9	3,5	3,4	2,8
15	6,0	5,6	4,5	4,5	5,0	8,4	2,9	3,5	15,0
	4,0	4,6	10,7	4,0	4,0	0,8	2,2	2,2	0,3
	2,0	1,5	18,8	3,5	4,3	15,5	1,5	2,4	44,4
	1,0	0,9	8,2	3,0	3,6	14,0	0,7	1,0	28,9
				2,5	2,6	3,5			
20				2,0	1,8	6,9			

Example 6: Quantitation of aggregates in low concentration

In a representative experiment, the methods according to the present invention were used for aggregate quantification in low concentration, using the wavenumber ranges 1663-1658 cm^{-1} , 1572-1567 cm^{-1} , 1542-1536 cm^{-1} , 1502-1497 cm^{-1} , 1473-1469 cm^{-1} , 1458-1450 cm^{-1} and 1360-1355 cm^{-1} .

Results show a concentration range which can be covered by the here described method, after adequate calibration, ranging down to 0.02 mg/ml aggregates in solution. Results are shown in figure 1.

30

Example 7: Comparison aggregate quantification using size exclusion chromatography and the here described invention and method

A comparison was done, using here described method for aggregate quantification and size exclusion chromatography for aggregate quantification, after preparing different sets of samples with different amounts of antibody aggregates. Results are shown in figure 5 and show that both techniques give similar results within the here shown aggregate concentration range of 0.1- 0.8 mg/ml.

Example 8: Distinguishing non-covalent aggregate types from covalent aggregate types in same sample, using the here described method

In a representative experiment, the methods according to the present invention were used for aggregate differentiation, using this method to distinguish non-covalent from covalent antibodies in the same sample. Wavenumber ranges showing a clear difference between aggregates from non-covalent and covalent type were used to establish selective quantification models. Suitable wavenumber ranges can be found in figure 4, but are not limited to the ones being shown in figure 4. Covalent antibody aggregates were obtained after pH- stress and non-covalent antibody aggregates obtained from a purification process after affinity chromatography. These different aggregate types were mixed at various ratios, also using non-aggregated antibody. Antibody concentration in all samples was kept constant. Results of selective quantification of different aggregate types are shown in table II. Additionally, overall antibody amount, composed of both aggregate types, was quantified as presented in table II. Coefficients of variation for results are mostly below 20%, with 2/3 of samples showing coefficients of variation less than 10% (table II).

Table II: Selective quantification of covalent and non-covalent aggregate types in same sample using here described method

aggregation type	true [%]	predicted [%]	% CV	aggregation type	true [%]	predicted [%]	% CV
---------------------	-------------	------------------	------	---------------------	-------------	------------------	------

5	both types	7,89	8,01	1,08	covalent	7,89	8,04	1,36
					covalent			
	both types	7,71	7,88	1,55		6,31	5,8	5,73
					covalent			
	both types	7,18	4,51	26,3		6,31	6,39	0,92
10					covalent			
	both types	7	6,81	1,95		5,92	5,94	0,31
					covalent			
	both types	6,97	5,9	10,82		5,52	5,07	5,78
					covalent			
15	both types	6,31	7,1	8,79		4,73	4,61	1,89
					covalent			
	both types	6,22	5,67	6,31		3,95	3,21	13,22
					covalent			
	both types	5,69	3,26	30,21		3,16	3,15	0,09
20					covalent			
	both types	5,6	6,83	15,47		2,76	2,89	3,31
					covalent			
	both types		4,31	13,69		1,97	2,12	5,11
25	both types		5,19	6,87	non-covalent	7	6,04	9,72
					non-			
	both types		3,46	3,29	covalent	5,6	5,28	3,99
					non-			
	both types		3,12	8,38	covalent	5,6	4,52	13,67
30					non-			
	both types		3,52	8,05	covalent	4,9	4,76	2,01
					non-			
	both types		3,86	17,09	covalent	4,2	4,53	5,62
					non-			
	both types		2,57	7,52	covalent	3,15	2,75	8,95
					non-			
	both types		1,74	11,98	covalent	2,8	2,03	19,5
					non-			
					covalent	2,45	1,95	14,54

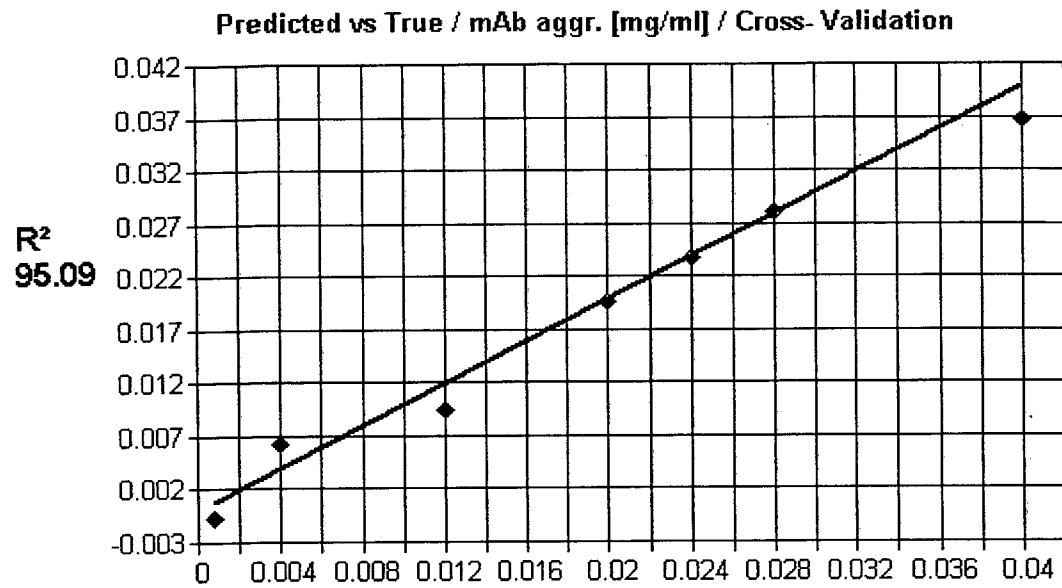
Claims

1. A method for quantitation of protein aggregates in a sample, the method comprising the steps of:
- 5 (a) providing a support
(b) applying the sample to the support
(c) optionally drying the sample
(d) exposing the sample on the support to an infrared beam comprising a wavelength in the spectral range of $4000\text{--}400\text{ cm}^{-1}$ or any portion of the
- 10 spectral range of $4000\text{--}400\text{ cm}^{-1}$, thereby to obtain an infrared absorption spectrum; wherein two or more absorption band areas and/or heights in the infrared absorption spectrum correlate with protein aggregate quantity within the sample.
- 15 2. The method of claim 1, wherein the absorption band areas and/or heights for protein aggregate quantification are selected from the ranges between $2000\text{--}800\text{ cm}^{-1}$.
- 20 3. The method of claims 1 or 2, wherein the absorption band areas and/or heights for protein aggregate quantification are selected from the ranges $1695\text{--}1601\text{ cm}^{-1}$, $1579\text{--}1480\text{ cm}^{-1}$, $1478\text{--}1423\text{ cm}^{-1}$ and $1360\text{--}1355\text{ cm}^{-1}$.
- 25 4. Method according to one or more of claims 1 to 3, wherein 3 to 12 absorption band areas and/or heights are used for protein aggregate quantification.
- 30 5. Method according to one or more of claims 1 to 3, wherein 3 to 7 absorption band areas and/or heights are used for protein aggregate quantification.

6. Method according to one or more of claims 1 to 5, wherein the one or more protein aggregates is selected from the group of monoclonal, polyclonal or other forms of antibodies.
- 5 7. Method according to one or more of claims 1 to 6, wherein the samples applied to the support in step b) have undergone filtration, stirring, aeration, lyophilization, agitation and/or a pH shift.
- 10 8. Method according to one or more of claims 1 to 7, wherein in step d) two or more absorption band areas and/or heights correlate with non-covalent protein aggregates and two or more different absorption band areas and/or heights correlate with covalent protein aggregates.
- 15 9. Method according to one or more of claims 1 to 8, wherein the support provided in step a) is within a device.
- 20 10. Method according to one or more of claims 1 to 9, wherein the sample applied to the support in step b) comprises cell culture fluid, clarified cell culture fluid, drug product, drug substance, blood, plasma, serum and/or urine.
- 25 11. Method according to one or more of claims 1 to 10, wherein the sample applied in step b) comprises cell culture fluid originating from a bioreactor or biotank, directly or after one or more purification steps, such as chromatography, centrifugation, filtration, precipitation, virus filtration, pH-treatment and/or crystallization.

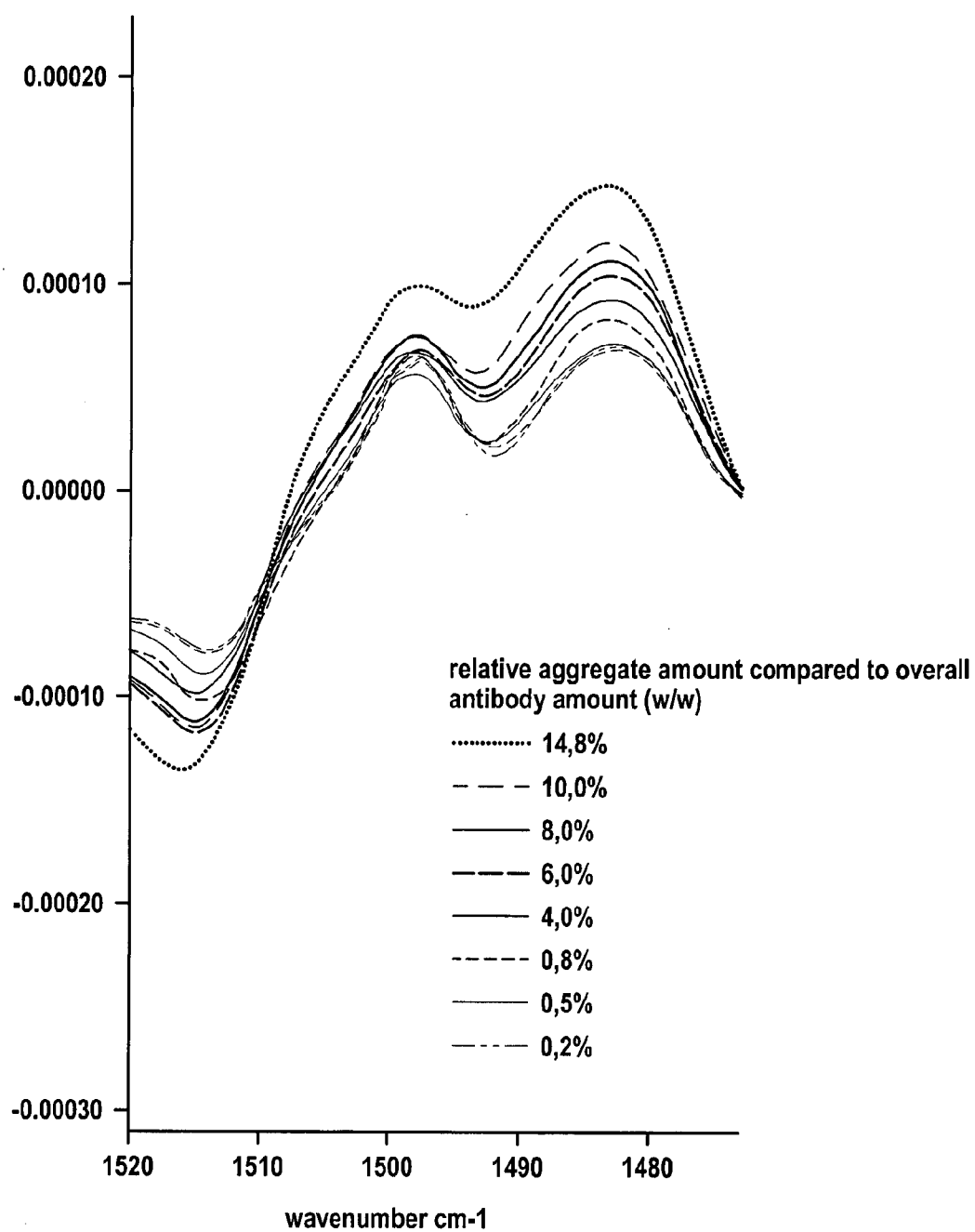
1/7

Fig. 1



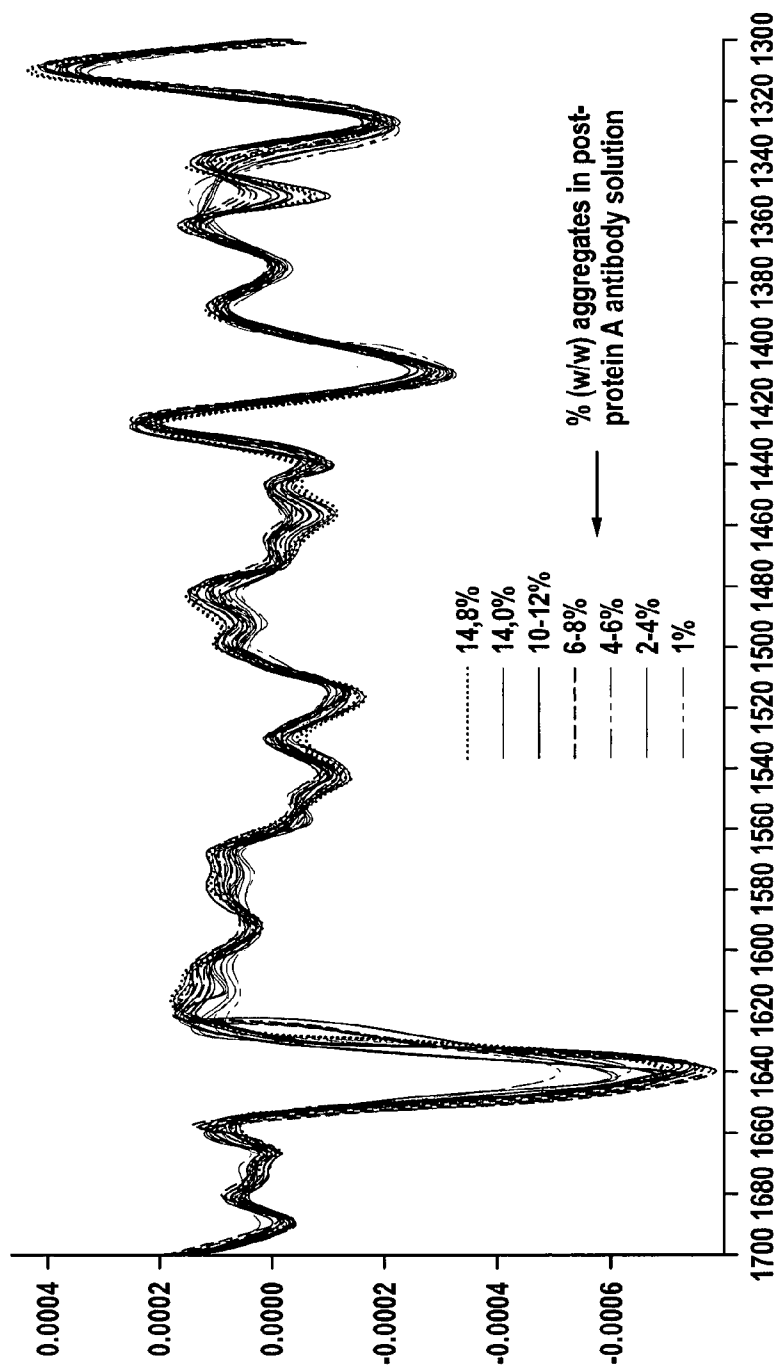
2 / 7

Fig. 2



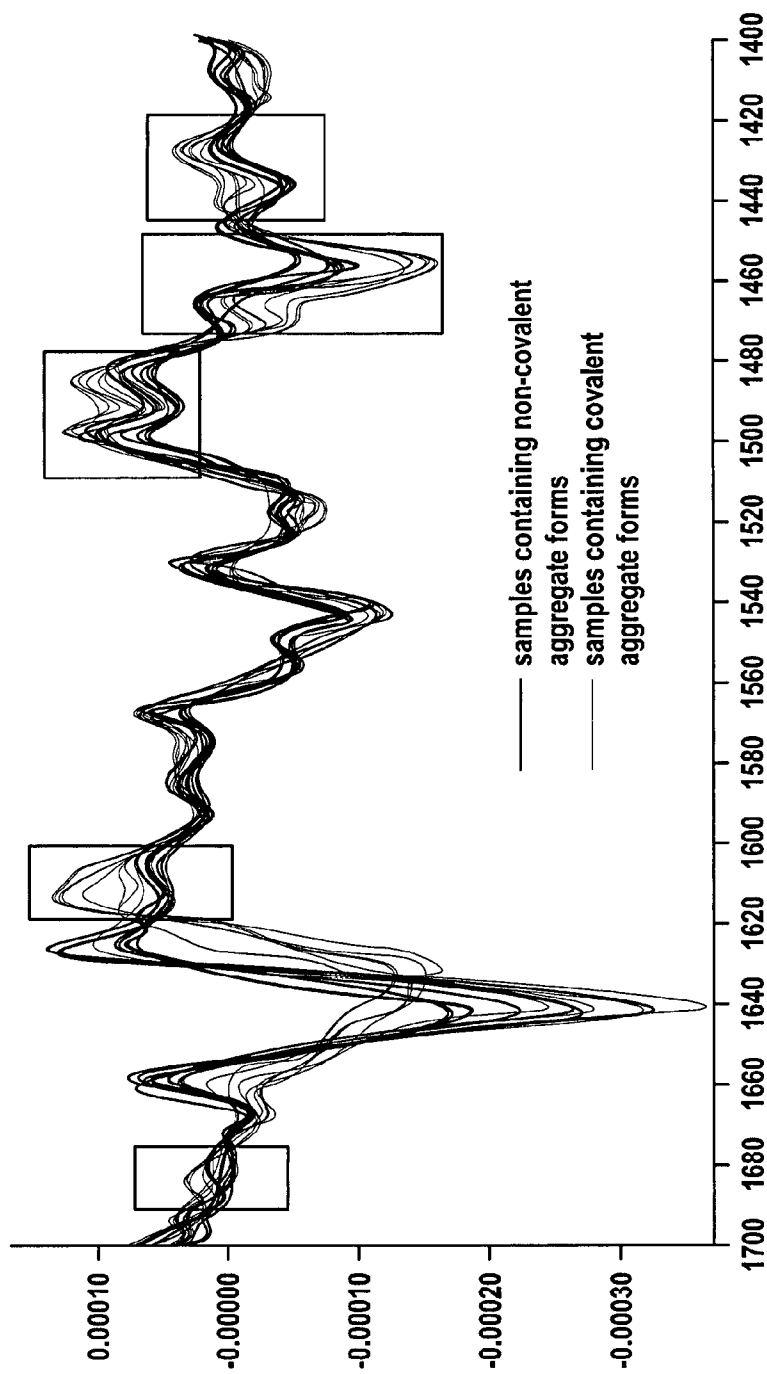
3 / 7

Fig. 3



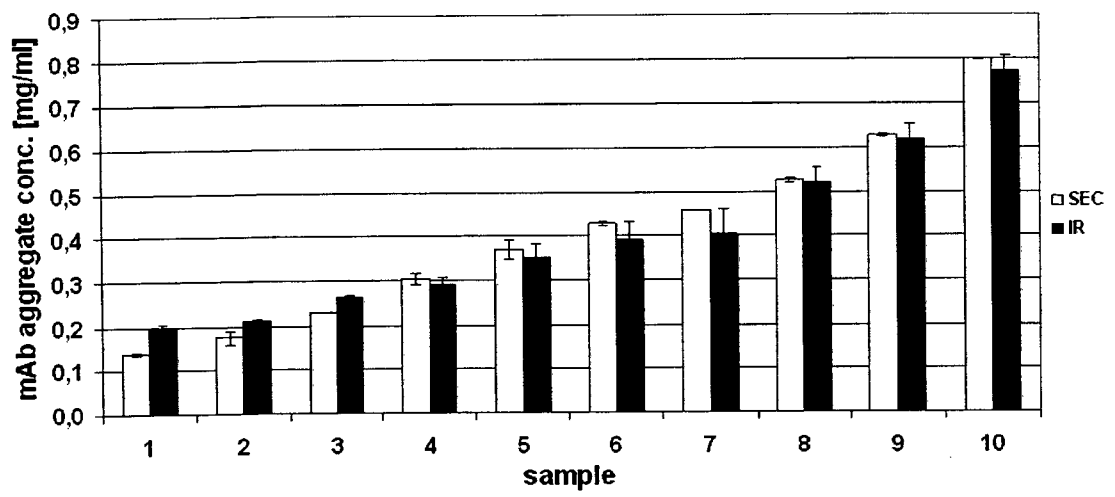
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Fig. 4



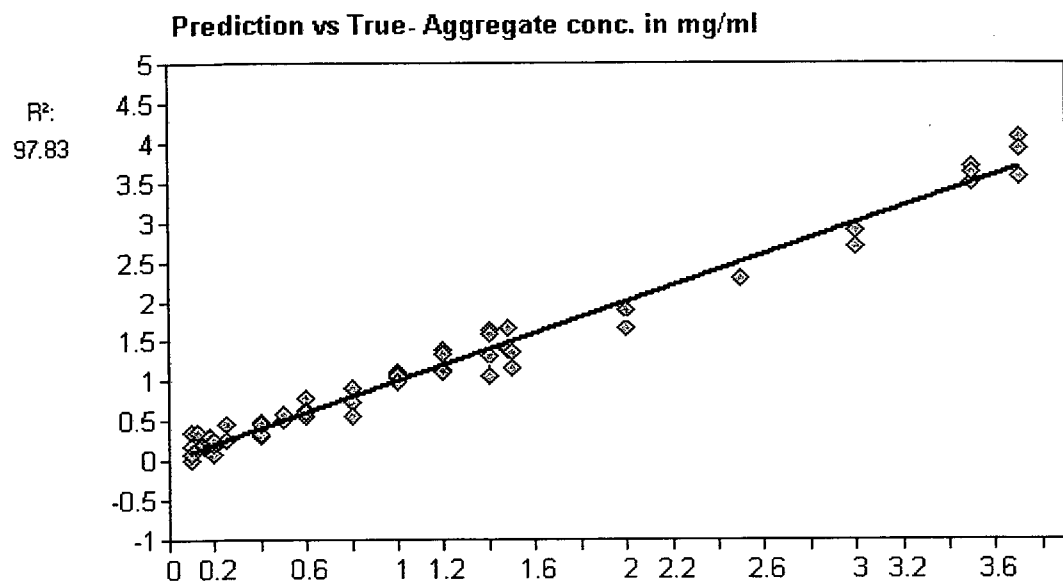
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Fig. 5



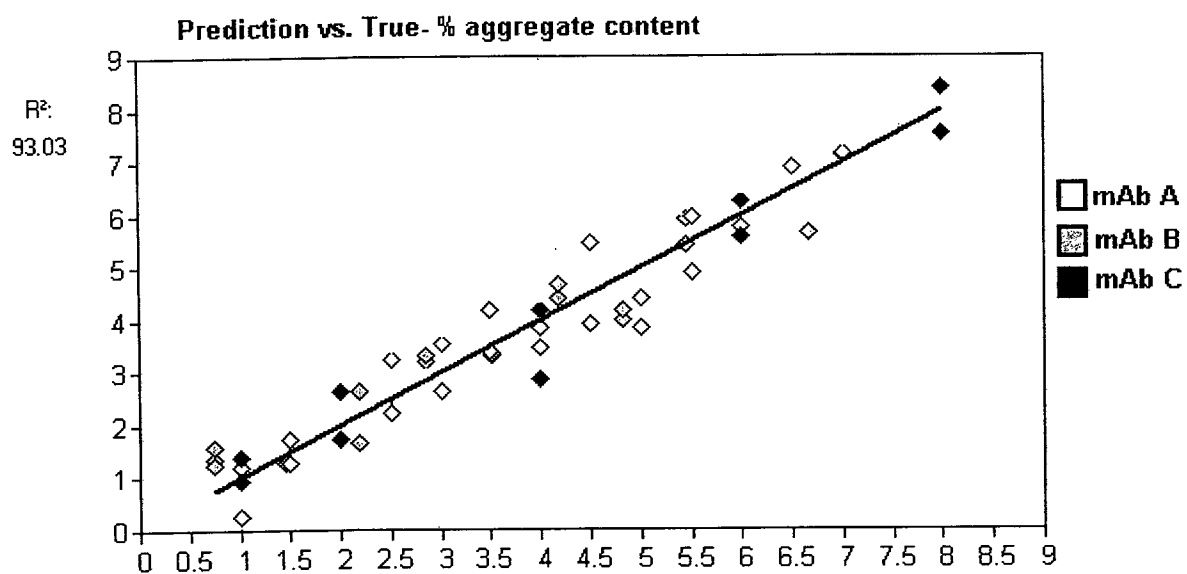
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Fig. 6



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Fig. 7



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/000679

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N21/35 G01N33/68
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)

G01N

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, FSTA, PAJ, 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	US 8 268 628 B1 (PASTRANA-RIOS BELINDA [US] ET AL) 18 September 2012 (2012-09-18) example 1	1-11
X	----- IDOIA MARTÍ-ALUJA ET AL: "Quantitative analysis of the effect of zidovudine, efavirenz, and ritonavir on insulin aggregation by multivariate curve resolution alternating least squares of infrared spectra", ANALYTICA CHIMICA ACTA, vol. 760, 21 November 2012 (2012-11-21), pages 16-24, XP055122408, ISSN: 0003-2670, DOI: 10.1016/j.aca.2012.10.057 abstract ----- -/--	1-11



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

10 June 2014

Date of mailing of the international search report

25/06/2014

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/000679

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A. DONG: "Entrapping intermediates of thermal aggregation in alpha-helical proteins with low concentration of guanidine hydrochloride", JOURNAL OF BIOLOGICAL CHEMISTRY, 27 June 2000 (2000-06-27), XP055122410, ISSN: 0021-9258, DOI: 10.1074/jbc.M005374200 abstract; figure 6	1-11
X	----- SILVIA MARIA DOGLIA ET AL: "Fourier transform infrared spectroscopy analysis of the conformational quality of recombinant proteins within inclusion bodies", BIOTECHNOLOGY JOURNAL, vol. 3, no. 2, 22 January 2008 (2008-01-22), pages 193-201, XP055122412, ISSN: 1860-6768, DOI: 10.1002/biot.200700238 abstract page 196; figure 3	1-11
X,P	----- FLORIAN CAPITO ET AL: "Mid-infrared spectroscopy-based antibody aggregate quantification in cell culture fluids", BIOTECHNOLOGY JOURNAL, vol. 8, no. 8, 21 June 2013 (2013-06-21), pages 912-917, XP055122416, ISSN: 1860-6768, DOI: 10.1002/biot.201300164 the whole document	1-11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2014/000679

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 8268628	B1	18-09-2012	NONE
