# Facile method of quantification for oxidized tryptophan degradants of monoclonal antibody by mixed mode ultra performance liquid chromatography

Cintyu Wong [a,*], Camille Strachan-Mills [b], Sudhir Burman [a]

[a] *Analytical Development – Large Molecule Method Development, Janssen Pharmaceutical Companies of Johnson & Johnson, Raritan, NJ 08869, USA*
[b] *Analytical Development – Large Molecule Method Development, Janssen Pharmaceutical Companies of Johnson & Johnson, Radnor, PA 19087, USA*

## ABSTRACT

Oxidation in therapeutic monoclonal antibody is a common occurrence and it may affect potency. Thus controlling and monitoring the amount of oxidized variant in the drug product sample is important since it may impact the purity. Here, we present the development of a fast and easy method utilizing size exclusion – ultra performance liquid chromatography (SE-UPLC) run under moderate hydrophobic conditions (mixed mode) to monitor the heterogeneity in drug product samples. The best separation was obtained using Waters Acquity BEH200 size exclusion column along with a mobile phase consisting of sodium acetate and sodium sulfate that separates IgG into aggregate, monomer, and fragment. The moderate salt concentration resulted in a second mode of separation based on hydrophobicity, resolving a monomer pre-peak from the monomer main peak. Multi-angle light scattering (MALS) determined the pre-peak has a similar mass as the IgG monomer. Characterization of the purified pre-peak fraction using mass spectrometry (MS), and bioactivity revealed this degradant to be a Trp-oxidized IgG monomer with significantly reduced bioactivity. Method qualification of the mixed mode UPLC method showed good recovery for the spiked monomer pre-peak and Fab fragment. However, the recovery of spiked dimer was low. This method is suitable for determining the relative distribution of the oxidized monomer and the native monomer species.

© 2012 Elsevier B.V. All rights reserved.

## 1. Introduction

Therapeutic monoclonal antibodies are required to show high levels of potency, purity, and low level of structural heterogeneity. Structural heterogeneity often affects the bioactivity and efficacy of a drug. Therefore, characterizing and quantifying the therapeutic monoclonal antibody heterogeneity is important in pharmaceutical drug development. Such heterogeneity arises from post-translational modifications as well as inherent chemical modifications during manufacturing and storage conditions. Modifications that result in structural heterogeneity include glycosylation, C-terminal lysine processing, isomerization, deamidation, and oxidation to name a few [1]. Oxidation of labile amino acid is common in antibodies. For example, oxidation of methionine was found in OKT3 degradation [2], anti-respiratory syncytial virus human monoclonal antibody [3], and rhuMAb HER2 [4]. Recently, tryptophan oxidation has also been reported in monoclonal antibodies as well. Matamoros Fernandez et al. reported tryptophan oxidation in a humanized antibody specific against human epidermal growth

factor receptor [5]. It was also observed in the complementarity determining region (CDR) of an IgG1 [6], as well as IgG2 [7].

Measuring oxidative variants can be challenging due to its similarities to the native antibody molecule in size, charge, and hydrophobicity. Previous characterization methods for oxidative variants include liquid chromatography–mass spectrometry (LC–MS) [5,8–10], hydrophobic interaction chromatography (HIC) [6,11–13], and reverse phase liquid chromatography [7,14]. Each of these methods has its limitations and disadvantages in measuring tryptophan oxidation. For example, LC–MS sample preparation is lengthy, and the method can introduce oxidized tryptophan as an artifact during sample preparation. Separation on reverse phase and hydrophobic interaction liquid chromatography require putting samples through denaturing and high salt conditions during analysis, respectively. These conditions may cause protein precipitation and/or loss of activity. In this study, we present a mixed mode UPLC method to separate and quantitate an oxidized tryptophan monomeric IgG under moderate salt native condition.

During stability studies of a therapeutic monoclonal antibody, a monomer fronting shoulder (called pre-peak) was observed by size exclusion – high performance liquid chromatography (SE-HPLC). Further characterizations discovered that the fronting shoulder was an oxidized tryptophan variant with significantly reduced activity. In this case, oxidation affected the potency and purity of the final

* Corresponding author at: 1000 Route 202 S, Raritan, NJ 08869, USA.
Tel.: +1 908 927 6073.
*E-mail address:* cwong13@its.jnj.com (C. Wong).

drug product. Therefore, an accurate and efficient method of measuring this oxidized variant was necessary. Our goal was to develop a quick liquid chromatography method that did not require the use of denaturing solvent as in the reverse phase or high salt as in the hydrophobic interaction chromatography to separate IgG variants.

## 2. Experimental

The monoclonal antibody used in this study is a fully human immunoglobulin that belongs to the IgG2 subclass. Formulated bulk (FB) of the antibody was produced in Chinese hamster ovary (CHO) cells and purified using several column chromatography steps. Stability samples containing various levels of pre-peak were generated by storing at 5 °C for 10 months, and 40 °C for 2 or 3 months.

All SE-HPLC data presented in this study were performed using Agilent 1100 HPLC system equipped with a PDA detector (Agilent, Santa Clara, CA, USA), and EMPOWER software (Waters, Milford, MA, USA) for data analysis. All mixed-mode and size exclusion UPLC data were performed using Waters Acquity H-Class Bio UPLC system equipped with a PDA detector (Waters, Milford, MA, USA), and EMPOWER software (Waters, Milford, MA, USA) for data analysis. All data were collected at 214 nm and 280 nm. The aggregate and fragment were quantified at 214 nm where peptide bonds absorb, while the monomer and monomer pre-peak were quantified at 280 nm where aromatic amino acid residues absorb. A wavelength ratio was used to convert the aggregate and fragment areas at 214 nm to the corresponding areas at 280 nm for final calculation of area percent. The use of two wavelengths for data collection improved sensitivity to detect protein aggregates and fragments in high concentration IgG monoclonal antibody compared to single wavelength SE-HPLC [15].

### 2.1. Size exclusion HPLC (SE-HPLC) procedure

Size exclusion HPLC separation was carried out on a Tosoh TSKgel Bioassist G3SW$_{XL}$ (PEEK) 5 μm, 7.8 mm × 300 mm analytical column (Tosoh Bioscience, Montgomeryville, PA, USA) and Tosoh SW$_{XL}$ (PEEK) 7 μm, 6.0 × 40 mm guard column (Tosoh Bioscience, Montgomeryville, PA, USA). An isocratic elution with 20 mM sodium phosphate, 250 mM sodium chloride, pH 7.0 mobile phase at 0.4 mL/min flow rate was used. Gel filtration molecular weight standards (Bio-Rad Laboratories, Hercules, CA, USA), 20 μL of 1.8 mg/mL, were injected for system suitability, and 20 μL of 10 mg/mL protein samples were injected for analysis.

### 2.2. Size exclusion UPLC (SE-UPLC) procedure

Size exclusion UPLC separation was carried out on a Waters Acquity BEH200 1.7 μm 4.6 mm × 300 mm analytical column. An isocratic condition with the same mobile phase and flow rate as the SE-HPLC analysis (20 mM sodium phosphate, 250 mM sodium chloride, pH 7.0 at 0.4 mL/min) was used. Gel filtration molecular weight standards (Bio-Rad Laboratories, Hercules, CA, USA), 20 μL of 1.8 mg/mL, were injected for system suitability, and 20 μL of 10 mg/mL protein samples were injected for analysis.

### 2.3. Mixed mode UPLC procedure

A mixed mode condition was achieved by using a size exclusion column and moderate salt elution conditions to affect hydrophobic interactions. During method development, three size exclusion UPLC columns were evaluated: two Waters Acquity BEH200 1.7 μm analytical columns (4.6 mm × 150 mm and 4.6 mm × 300 mm) (Waters, Milford, MA, USA), and a Sepax Zenix SEC-300 3 μm 7.8 mm × 200 mm analytical column (Sepax Technologies, Inc., Newark, DE, USA). Various mobile phases evaluated

were 0.25–1.8 M sodium chloride in 20 mM sodium phosphate buffer, 0.8–1.2 M sodium acetate in 20 mM sodium phosphate buffer, 0.2–1.0 M sodium sulfate in 20 mM sodium phosphate buffer, and a combination of sodium acetate (0.1–0.5 M) and sodium sulfate (0.2–0.4 M) in 20 mM sodium phosphate buffer. Sodium phosphate at pH 6.0 and 7.0, and sodium acetate at pH 5.0 and 6.0 mobile phases were evaluated for the pH effect on separation of pre-peak and main peak. Flow rates of 0.1, 0.15, and 0.2 mL/min, as well as injection mass load of 50–200 μg total protein were also evaluated to achieve the best separation. The final mixed-mode UPLC method condition was obtained using the Waters Acquity BEH200 1.7 μm, 4.6 mm × 300 mm analytical column with 20 mM sodium phosphate, 0.1 M sodium acetate, 0.4 M sodium sulfate, pH 7.0 mobile phase at 0.15 mL/min flow rate. Gel filtration molecular weight standards (Bio-Rad Laboratories, Hercules, CA, USA), 10 μL of 1.8 mg/mL, were injected for system suitability, and 10 μL of 10 mg/mL protein samples were injected for analysis. Monomer material was purified from formulated bulk, and purified pre-peak material was obtained from 40 °C, 9 months stability sample using the final mixed-mode UPLC method for further characterization.

### 2.4. Multi-angle light scattering (MALS) analysis

A drug product stored at 40 °C for 3 months stability sample, and photo stress forced degraded sample (exposed to 150 h of white light, followed by 20 h of UV light at 25 °C) were analyzed using the final mixed mode UPLC condition with an online DAWN HELEOS MALS detector (Wyatt Technologies, Inc., Santa Barbara, CA, USA). In addition, a drug product stored at 40 °C for 6 months stability sample was analyzed using the SE-HPLC equipped with an online DAWN HELEOS MALS detector. Detector angles 3 through 18 at 280 nm were used for MALS analysis. Data were analyzed using ASTRA v5.3.4.20 software (Wyatt Technologies, Inc., Santa Barbara, CA, USA). A d$n$/d$c$ value of 0.185, a UV extinction coefficient of 1400 mL/g cm, and a first order Zimm fit was used for all data analysis.

### 2.5. Peptide map analysis

Peptide mapping of both the monomer and pre-peak materials purified from the mixed mode UPLC method was performed to characterize the differences between the samples. Both the purified monomer and pre-peak materials were denatured with a solution of 6 M Guanidine–HCl, 100 mM Tris, and 2.5 mM EDTA, pH 8.0. The denatured protein was reduced with 1 M dithiothreitol at 37 °C for 1 h, then alkylated with 1 M sodium iodoacetate for 40 min in the dark, followed by trypsin digestion for 2 h at 37 °C. The resulting peptides were analyzed by RP-HPLC/MS on an Agilent HPLC (Agilent, Santa Clara, CA, USA) with a capillary pump and diode array detector connected in-line to a Thermo ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The RP-HPLC separation was performed using a gradient of 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic in acetonitrile at a flow rate of 0.2 mL/min on a Vydac C18 2.1 mm × 150 mm column (Grace, Deerfield, IL, USA) at 40 °C. Elution of peptides was monitored by measuring absorbance at 214 and 280 nm. The chromatographic profiles were compared in terms of retention times, intensities, and presence or absence of peaks. Relative quantification was performed by using the total intensity of an extracted ion chromatogram of the peptides of interest. Post-translational modifications were calculated as a relative percent of the native peptide.
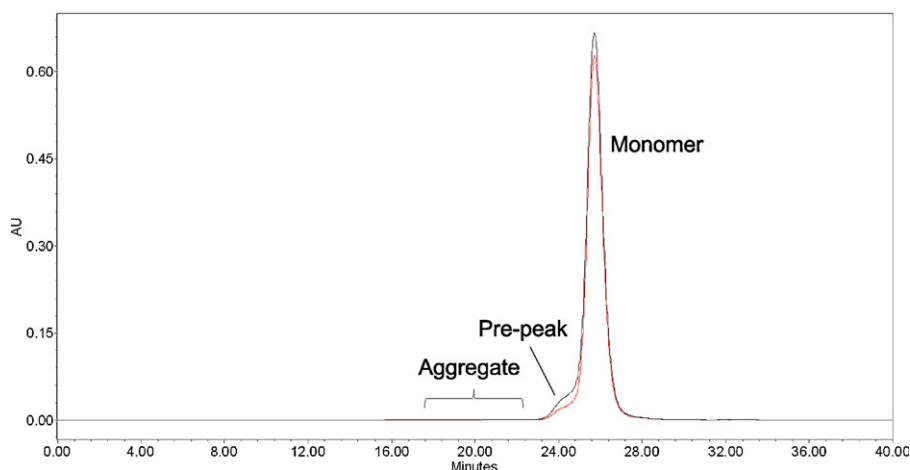
**Fig. 1.** SE-HPLC chromatogram profile at 280 nm showing fronting shoulder on monomer of a drug product at time zero (red) and 40 °C 2 months stability sample (black). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

### 2.6. Bioassay

Antibody bioactivity was assessed by measuring the neutralization of proliferation of a nerve growth factor (NGF) sensitivity cell line (TF-1) in response to increasing concentration of therapeutic antibody. Various concentrations of therapeutic antibody (reference material, purified monomer, and purified pre-peak) were neutralized with recombinant human beta nerve growth factor in a 96-well plate for 60 min at room temperature. Approximately $3 \times 10^5$ cells/mL TF-1 cells were added to the neutralized antibody, and incubated at 37 °C, 5% $CO_2$ for 62 h. TF-1 cell incubated with recombinant human beta nerve grow factor, but no neutralized antibody was performed as a negative control. TF-1 cell incubated without human beta nerve growth factor and neutralized antibody was performed as a positive control. Alamar Blue (Invitrogen, Gran Island, NY, USA) was added to the cells and incubated for 6 h as a viability indicator. The absorbance of each well was measured using an excitation wavelength of 530 nm and emission wavelength of 590 nm. The relative potency of the purified monomer and pre-peak were calculated using a standard curve.

### 3. Results and discussion

A monomer fronting shoulder (pre-peak) was observed for a monoclonal antibody by size exclusion – high performance liquid chromatography (SE-HPLC) using a Tosoh TSKgel BioAsisst G3SW$_{XL}$ column (Fig. 1). The amount of monomer fronting shoulder (pre-peak) was found to increase in stability samples under recommended storage condition of 2–8 °C and accelerated conditions of 25 °C and 40 °C. The SE-HPLC method was not suitable for quantifying the amount of pre-peak in the drug product sample because the pre-peak was not well resolved from the monomer main peak. The pre-peak showed as a fronting shoulder on the monomer main peak (Fig. 1), which made the integration difficult and inconsistent across different samples, especially at low levels of pre-peak. Therefore, another fast analytical method that separates and quantifies the pre-peak species under non-denaturing and mild condition was needed.

### 3.1. Optimization of mixed mode UPLC conditions

Among the three columns evaluated, Waters Acquity BEH200 1.7 μm 4.6 × 300 mm column showed the best separation of the pre-peak from the monomer peak. The longer 30 cm Waters Acquity BEH200 column provided better overall resolution and still

had a reasonable run time compared to the same column at 15 cm length. The Sepax Zenix SEC-300 column had an interfering buffer peak in the aggregate elution region; therefore, it was unsuitable for further development.

High salts are often used to promote hydrophobic interactions between proteins and mildly hydrophobic surfaces [16]. Waters Acquity BEH200 column separates proteins based on size, but in this mixed mode method, the addition of salt to the mobile phase were employed to affect the separation of proteins based on size as well as on its hydrophobicity. For example, no monomer fronting shoulder (pre-peak) was observed in formulated bulk material under low salt, 0.25 M sodium chloride in 20 mM sodium phosphate buffer, pH 7.0, SE-UPLC condition (Fig. 2, black trace). However, a progression from no monomer shoulder to a separate pre-peak was observed as sodium chloride concentration was increased to 1.0 M (Fig. 2, blue – 0.5 M NaCl, green – 0.75 M NaCl, and cyan – 1.0 M NaCl). Furthermore, at 1.25–1.7 M NaCl, more than one pre-peak was well resolved from the monomer (Fig. 2, magenta – 1.25 M NaCl, purple – 1.5 M NaCl, and black – 1.7 M NaCl). At 1.8 M sodium chloride, the monomer co-eluted with the buffer peak (data not shown), and therefore this condition was unsuitable for analysis.

Even though by using mobile phases containing higher than 1.5 M sodium chloride was able to resolve the pre-peak species, this condition was not preferred for analysis because the high salt content may affect the pre-peak and monomer distribution in the sample during analysis. To avoid the extreme salt conditions similar to those used in HIC, other salt compositions were evaluated in order to provide the same hydrophobic interaction between IgG variants and column but at a lower ionic strength. A combination of 0.1 M sodium acetate and 0.4 M sodium sulfate in 20 mM sodium phosphate, pH 7.0 buffer was found to be the best salt composition to provide a good separation between aggregates, pre-peak, monomer, and fragments. Both acetate and sulfate are anions in the Hofmeister series that provide better salting-out effect than chloride [17,18]. Moreover, Senczuk et al. found that a dual salt system increases hydrophobic binding capacity up to two folds over the traditional single salt system [19]. Therefore, using a combination of sodium acetate and sodium sulfate, the pre-peak is well resolved from the monomer at an overall lower salt concentration compared to sodium chloride. When only sodium acetate or only sodium sulfate was used in the mobile phase, less than desirable separation was achieved.

Different buffer pH and flow rates were also evaluated to give the best separation among various IgG species. Mobile phase containing 20 mM sodium phosphate at pH 7.0 and a flow rate of
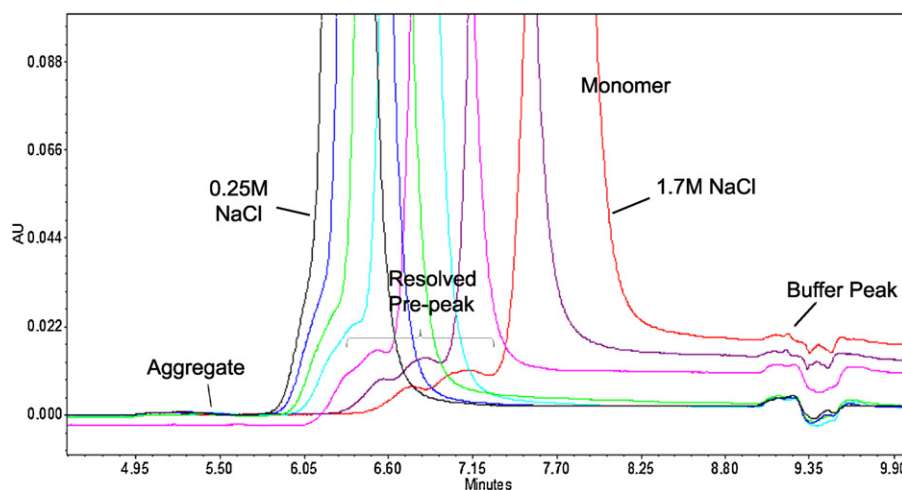
**Fig. 2.** Representative chromatograms of formulated bulk separation using 0.25 M NaCl (black), 0.5 M NaCl (blue), 0.75 M NaCl (green), 1.0 M NaCl (cyan), 1.25 M NaCl (magenta), 1.5 M NaCl (purple), and 1.7 M NaCl (red) sodium chloride in 20 mM sodium phosphate, pH 7.0 mobile phase, and Waters Acquity BHE200 4.6 mm × 300 mm column shown at 280 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

0.15 mL/min showed the best separation for the mixed mode UPLC method. The pre-peak and the monomer peak were fractionated using the final mixed mode UPLC method for characterization. Fig. 3A shows a typical resolution between pre-peak and monomer in the formulated bulk material (blue trace), and Fig. 3B shows a typical resolution between aggregate, two pre-peaks, monomer, and fragment in the 40 °C, 9 months stability sample (blue trace) that was enriched with impurities. Fig. 3A, black trace, shows the monomer purified from the formulated bulk material was free of aggregate, and fragment. There was however a small amount of pre-peak still remained in the monomer purified material, approximately 1.1%. The purified pre-peak fraction had a small amount of aggregate (Fig. 3B, black trace), which most likely arose from sample handling during concentration.

### 3.2. Multi-angle light scattering (MALS) analysis of Trp-Ox degradant

MALS analysis was employed to determine the molecular weight of each species observed in the chromatographic separation using the mixed mode UPLC method. A drug product stored at 40 °C for 3 months stability sample (Fig. 4, black trace) and photo stress forced degraded sample (Fig. 4, blue trace) were analyzed using the final mixed mode UPLC condition with an online MALS detector. Several pre-peaks were separated in both the stability and photo degraded samples using this method. The MALS analysis showed that peak 1 had a mass of approximately 145 kDa, corresponding to monomer species. Multiple peaks in region 2 had an average mass of 144–150 kDa (Table 1). The various pre-peaks have similar or slightly higher molecular weight than the monomer main peak. The pre-peaks eluted in region labeled Peak 3 had molecular weights of about 178–182 kDa, which is slightly higher than that of a monomer (Table 1). The MALS data confirmed that the pre-peaks were separated by the mixed mode UPLC chromatography and not co-eluted with the dimer or higher order aggregates. Peak eluting in region 4 corresponded to the dimer species with a molecular weight of about 297–333 kDa (Table 1). The MALS data from the mixed mode UPLC method also confirmed that this pre-peak correlated to the pre-peak observed under SE-HPLC condition, which also showed a slightly higher molecular weight than the monomer at about 155–160 kDa.

The pre-peak (peak 3, Fig. 4) with a slightly larger molecular weight compared to the monomer may be due to

conformational changes caused by the oxidized tryptophan in the molecule. These changes may reflect the larger hydrodynamic radius for the pre-peak (Table 1). The stability sample and the photodegraded material both have multiple pre-peaks, suggesting different tryptophan oxidized species present in the sample. One possibility for the presence of multiple pre-peak species is having one or both the tryptophan residues oxidized on the CDR regions in an IgG molecule. Another possibility is having different oxidized tryptophan byproducts. Previous studies have found four tryptophan oxidation products: hydroxytryptophan, N-formyl-kynurenine, kynurenine, and 3-hydroxykynurenine [20–23]. In addition, the hydroxytryptophan could have the hydroxyl group addition at any position of the indole ring [24], creating five different hydroxytryptophan structures. These different tryptophan oxidation products may contribute to the presence of multiple pre-peaks observed.

### 3.3. Peptide map analysis

In order to characterize the purified pre-peak material further and provide evidence for the hypothesis of multiple tryptophan oxidation byproducts presented above, peptide map analysis of the purified pre-peak material was performed. Both purified monomer and pre-peak fractions from the mixed mode UPLC method were reduced, alkylated, and digested using trypsin enzyme. The resultant peptides were separated using RP-HPLC with an in-line mass spectrometer. The peptide map analysis showed significant differences between the purified monomer and pre-peak material. The most notable difference was the relative percent increase in oxidation at the tryptophan 104 residue of the IgG. Fig. 5 shows UV chromatograms of tryptic peptide map analysis of the purified monomer with Trp104-containing peptide eluting around 140 min (top), and tryptic peptide map analysis of purified pre-peak (bottom). There was a marked decrease in the native peptide (Fig. 5A, 140 min), and a significant increase in the oxidized peptides spanning a 9 min window (Fig. 5B, 131–140 min). A variety of tryptophan oxidation byproducts were identified by tandem mass spectrometry including kynurenine (+4), N-formylkynurenine (+32), and hydroxytryptophan (+16), with the latter being the predominant form.

The native Trp104 peptide was observed as both doubly and triply charged states of 1621 and 1081 *m/z* (Fig. 6), respectively. The oxidized Trp104 peptide was mostly observed as doubly and
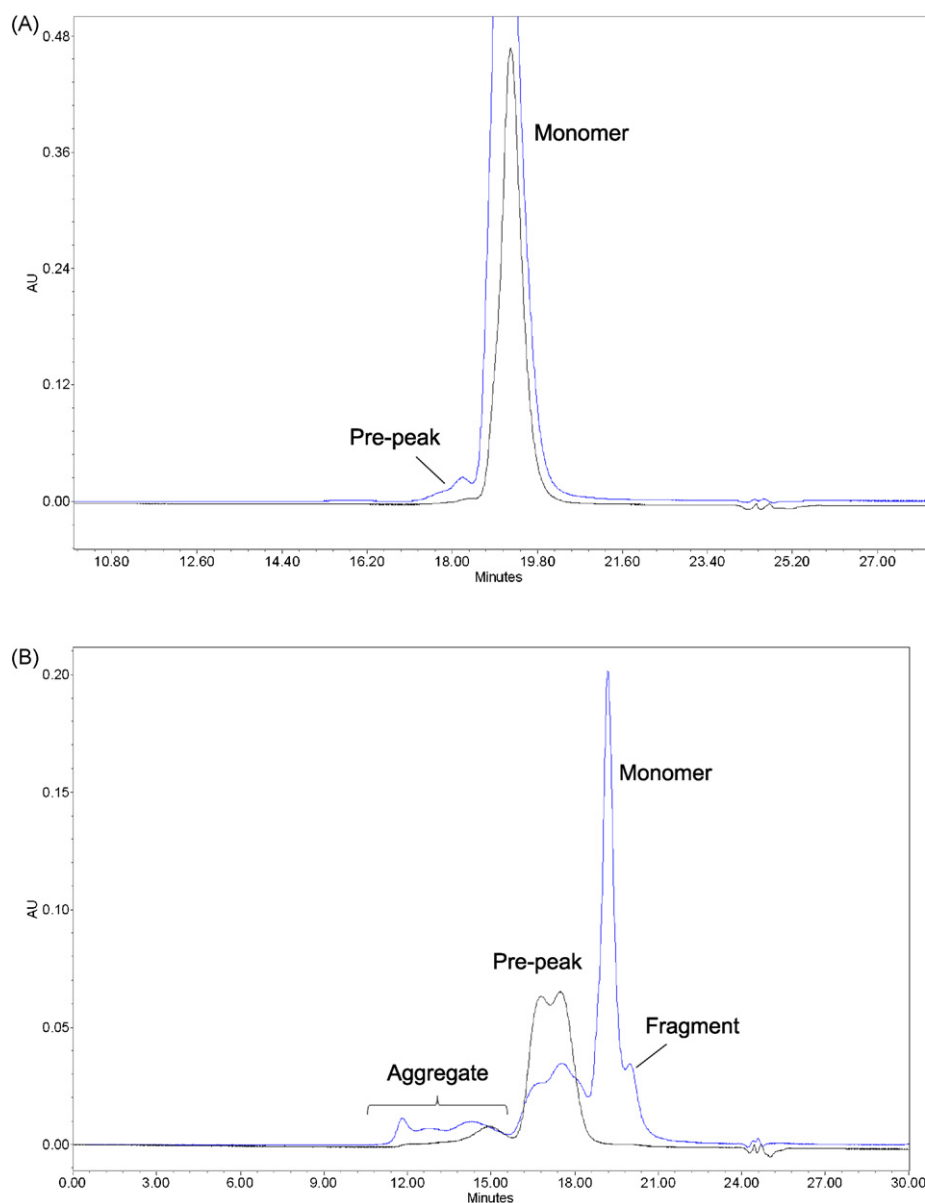
**Fig. 3.** Representative chromatogram of (A) formulated bulk material (blue) and purified monomer fraction (black), and (B) 40 °C, 9 months stability sample (blue) and purified pre-peak fraction (black) using the final mixed mode UPLC method at 280 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

triply charged state of hydroxytrptophan with 1629 and 1087 $m/z$ (Fig. 7), respectively. The estimated ratio of oxidized Trp104 peptide in the purified pre-peak material calculated from the reconstructed ion chromatogram intensities was about 84%. Based on this value, it is possible that some IgG have one oxidized tryptophan on the CDR region, whereas some IgG have both tryptophan oxidized on the CDR, one on each Fab. Since the location of the oxidized Trp104 was in the CDR region, it affected the potency of the IgG molecule. Bioactivity assay confirmed that the purified pre-peak fraction had 40–50% lower activity relative to the reference standard. Whereas, the purified monomer

material had about the same level of activity as the reference standard.

### 3.4. Comparison of mixed mode UPLC, SE-UPLC, and SE-HPLC analysis of stability samples

The SE-HPLC has been the gold standard for measuring the amount of impurities in a therapeutic IgG samples throughout the pharmaceutical and biotech industry. Since the mixed mode UPLC method may also quantify aggregate and fragment impurities, we want to compare the percent impurities obtained by the mixed

**Table 1**
Molecular mass and hydrodynamic radius (in parenthesis) of mixed mode UPLC species eluting in regions labeled peak 1–4 for both 40 °C, 3 months stability and photo stressed samples obtained by MALS.

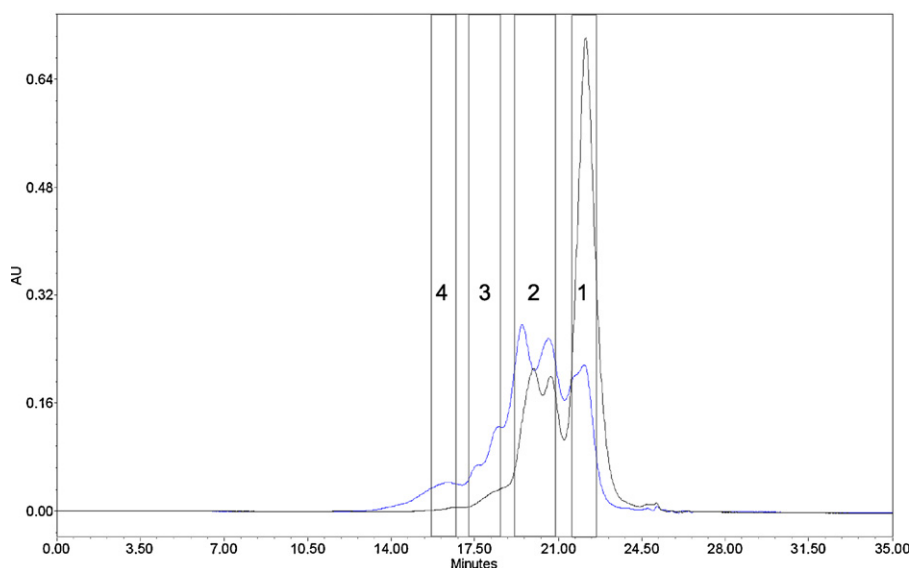| Sample | Peak 1 (21.7–22.4 min) | Peak 2 (19.2–20.9 min) | Peak 3 (17.5–18.5 min) | Peak 4 (16.2–16.8 min) |
|---|---|---|---|---|
| Drug product Lot A 40 °C, 3 months | 145 kDa (30.1 nm) | 144 kDa (29.0 nm) | 182 kDa (31.8 nm) | 333 kDa (32.8 nm) |
| Photo stress | 147 kDa (35.3 nm) | 150 kDa (36.3 nm) | 178 kDa (38.0 nm) | 297 kDa (37.6 nm) |

**Fig. 4.** Chromatogram profiles of drug product 40 °C, 3 months stability (black) and photo stressed (blue) samples using the final mixed mode UPLC method. The numbered sections represent peak regions used in the MALS analysis. Table 1 reports the molecular weight of each peak. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

mode UPLC method to those obtained by SE-UPLC and SE-HPLC. The SE-UPLC method was performed with the same column as used in the mixed mode UPLC method, but run under the SE-HPLC mobile phase and flow rate conditions. The comparison of results from the mixed mode UPLC and SE-UPLC showed the additional hydrophobic separation due to the use of salt in the mixed mode method. The comparison of results from the SE-UPLC and SE-HPLC methods showed the variation in results obtained from different size exclusion columns.

Comparison of the mixed mode UPLC, SE-UPLC, and SE-HPLC showed that the mixed mode UPLC method yielded less aggregate and more fragment (Table 2). This may be due to the use of moderate salt condition in the mixed mode UPLC method breaking some of the non-covalent aggregates, and creating more fragment during analysis. Based on the comparison between the mixed mode UPLC and SE-HPLC, the mixed mode UPLC method was found unsuitable in quantifying aggregate and fragment due to the salt condition. In general, the amount of % aggregate and % fragment obtained were comparable between SE-UPLC and SE-HPLC, indicating little variation in results obtained from different size exclusion columns. The results of the % pre-peak and % monomer cannot be compared because the pre-peak was
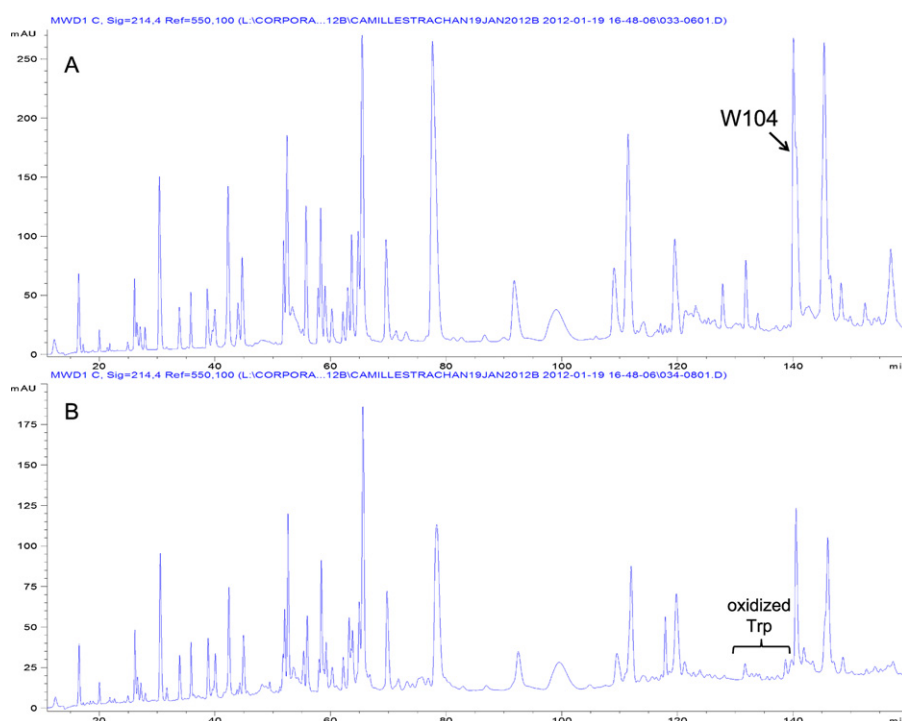


**Fig. 5.** UV chromatograms of tryptic peptide map analysis of (A) purified monomer material with arrow pointing to the Trp-containing peptide, and (B) purified pre-peak material with bracket indicating the oxidized Trp-containing peptides.
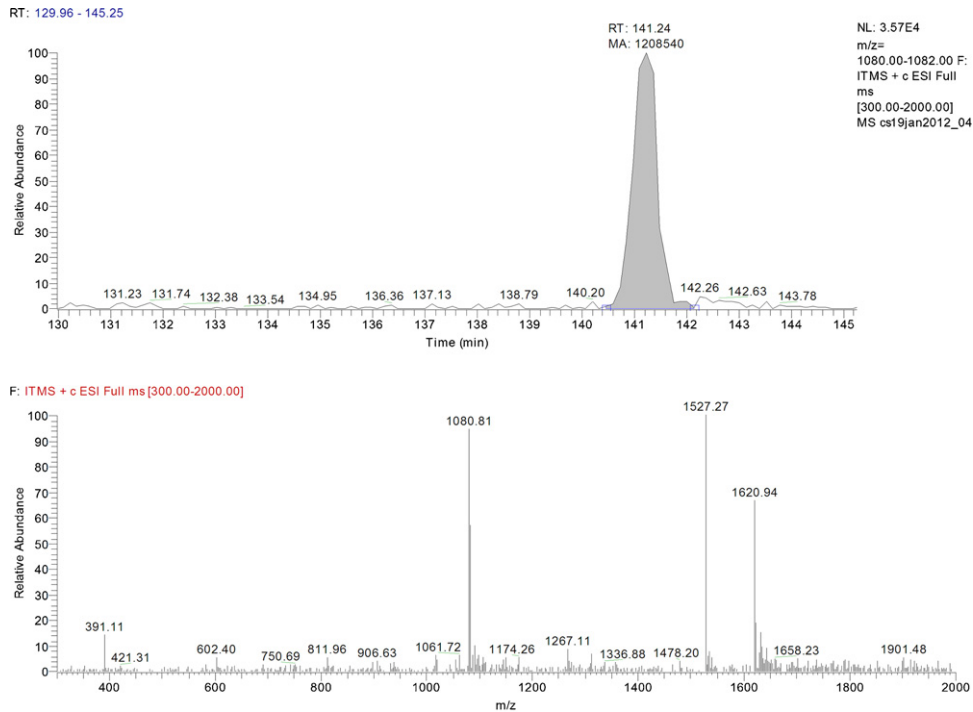
**Fig. 6.** Reconstructed ion chromatogram of native peptide in the purified pre-peak material with corresponding parent mass spectrometry data.

not resolved from the monomer using SE-UPLC and SE-HPLC methods.

### 3.5. Assay performance and qualification

For a method to be used as a release and stability assay in quantifying the amount of pre-peak in a drug product sample, it needs to demonstrate the performance requirements as described in the ICH 2Q(R1) guidelines on validation of analytical procedures.

Spiking experiments to demonstrate linearity and accuracy of aggregate, pre-peak, and fragment using the mixed mode SE-UPLC method were performed. Controls containing small amount of pre-peak and enriched pre-peak materials were mixed to produce samples with a range of different amounts of pre-peak, and same mass of IgG was injected for each sample. The theoretical amount of pre-peak in each sample was calculated using the dilution of each control during sample preparation, and the amount of pre-peak in each control measured by the mixed mode UPLC method. Same
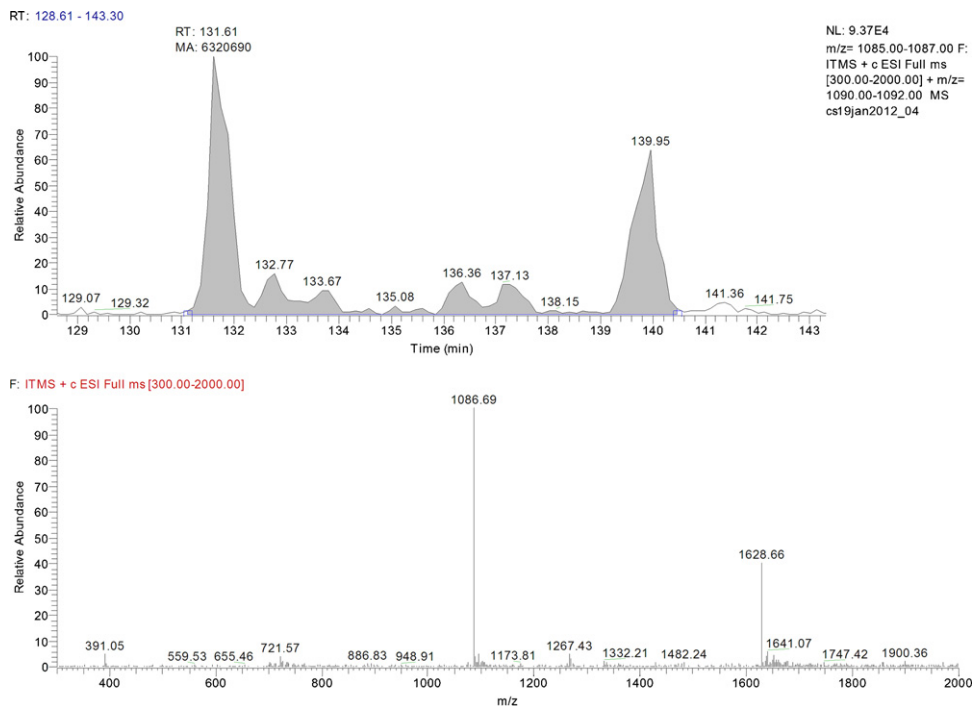


**Fig. 7.** Reconstructed ion chromatogram of oxidized Trp104 peptide in the purified pre-peak material with corresponding parent mass spectrometry data.

**Table 2**
Comparison of results obtained from mixed mode UPLC, SE-UPLC, and SE-HPLC methods on two lots of drug product stability samples. Refer to Section 2 for the separation conditions used.

| | Aggregate (%) | | | Pre-peak (%) | | | Monomer (%) | | | Fragment (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mixed mode UPLC | SE-UPLC | SE-HPLC | Mixed mode UPLC | SE-UPLC | SE-HPLC | Mixed mode UPLC | SE-UPLC | SE-HPLC | Mixed mode UPLC | SE-UPLC | SE-HPLC |
| Lot A 5C 10M | 0.0 | 0.1 | 0.1 | 1.7 | 0.0 | 0.0 | 95.0 | 99.9 | 99.9 | 3.4 | 0.0 | 0.0 |
| Lot A 40C 2M | 0.0 | 0.5 | 0.4 | 4.6 | 0.0 | 5.5 | 92.5 | 99.5 | 94.0 | 3.0 | 0.0 | 0.0 |
| Lot A 40C 3M | 0.7 | 2.2 | 2.2 | 27.9 | 26.4 | 32.3 | 69.6 | 71.4 | 65.5 | 1.8 | 0.1 | 0.1 |
| Lot B 5C 10M | 0.1 | 0.1 | 0.1 | 1.7 | 0.0 | 0.0 | 94.8 | 99.9 | 99.9 | 3.4 | 0.0 | 0.0 |
| Lot B 40C 2M | 0.7 | 1.7 | 1.7 | 25.2 | 22.2 | 28.7 | 72.2 | 76.0 | 69.6 | 2.0 | 0.1 | 0.0 |
| Lot B 40C 3M | 2.2 | 4.6 | 4.5 | 37.1 | 38.0 | 43.3 | 59.6 | 57.3 | 52.2 | 1.2 | 0.1 | 0.1 |

**Table 3**
Summary of % pre-peak and % fragment accuracy in mixed mode UPLC method by spiking experiments.

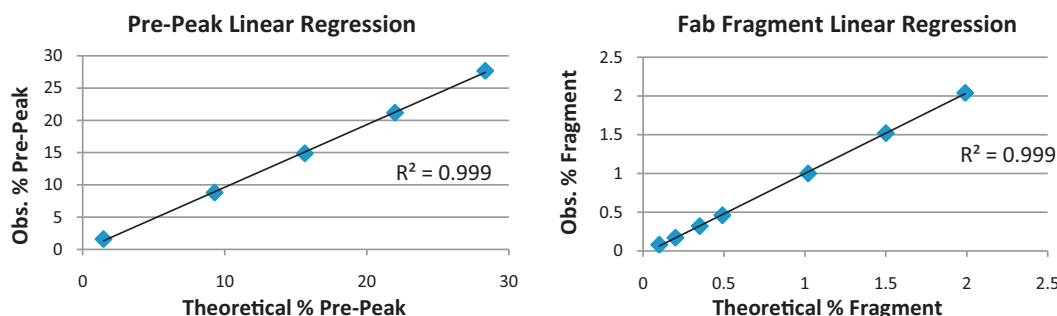| Theoretical % pre-peak | Observed % pre-peak | Pre-peak accuracy (%) | Theoretical % fragment | Observed % fragment | Fragment accuracy (%) |
|---|---|---|---|---|---|
| 28.3 | 27.7 | 97.8 | 2.0 | 2.0 | 100.0 |
| 22.0 | 21.2 | 96.4 | 1.5 | 1.5 | 100.0 |
| 15.6 | 14.9 | 95.5 | 1.0 | 1.0 | 100.0 |
| 9.3 | 8.8 | 94.6 | 0.5 | 0.5 | 100.0 |
| 1.5 | 1.6 | 106.7 | 0.4 | 0.3 | 75.0 |
| | | | 0.2 | 0.2 | 100.0 |
| | | | 0.1 | 0.1 | 100.0 |



**Fig. 8.** Mixed mode UPLC linearity regression plots of % pre-peak (left) and % Fab fragment (right).

approach was done for aggregate and Fab fragment linearity and accuracy studies. Qualification of the mixed mode UPLC method showed good accuracy for both the pre-peak and the fragment; however, the accuracy of aggregate was less than-optimal. Table 3 shows the pre-peak recovery was between 94.6 and 106.7% for IgG spiked with 1.5–28.3% pre-peak, and Fab fragment recovery was 75.0–100.0% for IgG spiked with 0.1–2.0% fragment. Fig. 8 shows the linear regression plots for pre-peak and Fab fragment recovery; both with determination of coefficient ($R^2$) closed to 1. The aggregate recovery was lower, ranging from 75.0 to 88.1% for IgG spiked with 2.0–5.0% aggregate. No linear least square regression was performed for aggregate recovery due to insufficient data.

The mixed mode UPLC method provided good separation for pre-peak, and accurately quantitated pre-peak and fragment impurities. However, this method was not suitable for quantifying aggregate due to its narrow range, 2.0–5.0% aggregate. Although the mixed mode UPLC method resulted in accurate and linear determination of fragment, the use of this method for fragment quantitation was not recommended because its value was not comparable to the values obtained from SE-HPLC as mentioned above. Therefore, the use of this method should be limited to the determination of the relative distribution of pre-peak and monomer. Other impurities, especially aggregates, required a separate SE-HPLC method for analysis.

The 1.5% pre-peak was the lowest level of pre-peak accurately determined using the mixed mode UPLC method; this was confirmed as the pre-peak limit of quantitation (LOQ) by determining the precision of six replicates. The relative standard deviation (RSD) for repeatability of six replicates of pre-peak LOQ (1.5%) was determined as 1.2% on day 1, and 0.5% on day 2 with a different lot of column. The RSD for reproducibility of twelve replicates of pre-peak LOQ (1.5%) over two days were determined as 3.2%. The mixed mode UPLC method demonstrated good precision and reproducibility at 1.5% pre-peak (LOQ), and the method was robust with different lots of column. Since this method was limited to determine the relative distribution of pre-peak and monomer, and not intended for aggregate and fragment quantitation, the LOQ for aggregate and fragment was not determined.

## 4. Conclusion

Our goal to develop a quick liquid chromatography method that does not require the use of denaturing solvent as in the reverse phase, or high salt as in the hydrophobic interaction chromatography to separate the oxidized IgG variants was achieved. This was done by developing a mixed mode UPLC method. This method offers advantages over traditional SE-HPLC method in its ability to separate and quantitate different variants of similar molecular weight IgGs. However, the moderate amount of salt may affect the % aggregate and % fragment observed in the mixed mode UPLC method compared to those observed in SE-HPLC or SE-UPLC. Although a trade-off may exist between the mixed mode and traditional

SE-UPLC/HPLC in the quantification of aggregate, we have demonstrated a proof of concept that this method is able to analyze variant monomeric IgG species.

## Acknowledgment

## References

[1] H. Liu, G. Gaza-Bulseco, D. Faldu, C. Chumsae, J. Sun, J. Pharm. Sci. 97 (2008) 2426.
[2] D.J. Kroon, A. Baldwin-Ferro, P. Lalan, Pharm. Res. 9 (1992) 1386.
[3] G.D. Roberts, W.P. Johnson, S. Burman, K.R. Anumula, S.A. Carr, Anal. Chem. 67 (1995) 3613.
[4] X.M. Lam, J.Y. Yang, J.L. Cleland, J. Pharm. Sci. 86 (1997) 1250.
[5] L.E. Matamoros Fernandez, D.E. Kalume, L. Calvo, M. Fernandez Mallo, A. Vallin, P. Roepstorff, J. Chromatogr. B: Biomed. Sci. Appl. 752 (2001) 247.
[6] D. Boyd, T. Kaschak, B. Yan, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 879 (2011) 955.
[7] J. Yang, S. Wang, J. Liu, A. Raghani, J. Chromatogr. A 1156 (2007) 174.
[8] V. Timm, P. Gruber, M. Wasiliu, H. Lindhofer, D. Chelius, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 878 (2010) 777.
[9] D. Houde, P. Kauppinen, R. Mhatre, Y. Lyubarskaya, J. Chromatogr. A 1123 (2006) 189.
[10] M. Hensel, R. Steurer, J. Fichtl, C. Elger, F. Wedekind, A. Petzold, T. Schlothauer, M. Molhoj, D. Reusch, P. Bulau, PLoS ONE 6 (2011) e17708.
[11] J. Valliere-Douglass, A. Wallace, A. Balland, J. Chromatogr. A 1214 (2008) 81.
[12] J. Valliere-Douglass, L. Jones, D. Shpektor, P. Kodama, A. Wallace, A. Balland, R. Bailey, Y. Zhang, Anal. Chem. 80 (2008) 3168.
[13] Y. Zhang, T. Martinez, B. Woodruff, A. Goetze, R. Bailey, D. Pettit, A. Balland, Anal. Chem. 80 (2008) 7022.
[14] D. Chelius, P. Ruf, P. Gruber, M. Ploscher, R. Liedtke, E. Gansberger, J. Hess, M. Wasiliu, H. Lindhofer, MAbs 2 (2010) 309.
[15] M.D. Bond, M.E. Panek, Z. Zhang, D. Wang, P. Mehndiratta, H. Zhao, K. Gunton, A. Ni, M.L. Nedved, S. Burman, D.B. Volkin, J. Pharm. Sci. 99 (2010) 2582.
[16] B.F. Roettger, M.R. Ladisch, Biotechnol. Adv. 7 (1989) 15.
[17] R.L. Baldwin, Biophys. J. 71 (1996) 2056.
[18] Y. Zhang, P.S. Cremer, Curr. Opin. Chem. Biol. 10 (2006) 658.
[19] A.M. Senczuk, R. Klinke, T. Arakawa, G. Vedantham, Y. Yigzaw, Biotechnol. Bioeng. 103 (2009) 930.
[20] R. Van Heyningen, Biochem. J. 123 (1971) 30P.
[21] L.A. Holt, B. Milligan, D.E. Rivett, F.H. Stewart, Biochim. Biophys. Acta 499 (1977) 131.
[22] Z. Maskos, J.D. Rush, W.H. Koppenol, Arch. Biochem. Biophys. 296 (1992) 514.
[23] A.C. Sen, N. Ueno, B. Chakrabarti, Photochem. Photobiol. 55 (1992) 753.
[24] E.L. Finley, J. Dillon, R.K. Crouch, K.L. Schey, Protein Sci. 7 (1998) 2391.