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Automated tryptic digestion procedure for HPLC/MS/MS peptide mapping of immunoglobulin gamma antibodies in pharmaceutics

Dirk Chelius [a], Gang Xiao [a], Andrew C. Nichols [a], Alona Vizel [a], Bing He [a], Thomas M. Dillon [a], Douglas S. Rehder [b], Gary D. Pipes [a], Edmund Kraft [c], Asha Oroska [d], Michael J. Treuheit [a], Pavel V. Bondarenko [a,*]

[a] *Amgen, Process and Product Development One Amgen Center Drive, Thousand Oaks, CA 91320, United States*
[b] *Amgen, Process and Product Development 1201 Amgen Court West, Seattle, WA 98119, United States*
[c] *Tecan, PO Box 13953, Research Triangle Park, NC 27709, United States*
[d] *Orochem Technologies Inc., 331 Eisenhower Lane, Lombard, IL 60148, United States*

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Abstract

The rapid growth of antibody drugs and drug candidates in the biopharmaceutical industry has created a demand for automated proteolytic digestion to assist in pharmaceutical stability studies, identity assays and quality control of these therapeutic proteins. Here, we describe the development of a fully automated proteolytic digestion procedure for monoclonal antibodies in solution, which requires a high concentration of denaturants for unfolding. The antibody samples were placed in a 96-well plate or in 0.5-mL Eppendorf tubes. The proteins were then reduced and alkylated in a denaturing solution of 6 M guanidine HCl. The denaturing solution was replaced with a digestion buffer using a custom-designed 96-well size-exclusion plate for desalting. The sample was digested for 5 h with two additions of trypsin. The completeness and reproducibility of digestion were verified by reversed-phase high-performance liquid chromatography tandem mass spectrometry (HPLC/MS) analysis of the digestion products. The performance of the automatic digestion was comparable to the currently used manual digestion procedure, but saved time, reduced manual labor, and increased the reproducibility of the tryptic digests. Our method should be useful not only for high-throughput analysis of antibodies, but for other therapeutic protein samples as well. Other applications like gel-free proteomics, where the analysis of a large number of samples is often needed and the completeness of the liquid digestion is critical for the identification of a large number of different proteins, should also benefit from this fully automated liquid proteolytic digestion procedure.
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1. Introduction

In the mid 1990s, large-scale identification of proteomes of different organisms by mass spectrometry from two-dimensional gels [1,2] led to the development of automated in-gel digestion [3,4]. The robots and protocols for automated proteolytic digestion became available commercially and improvements in protocols continue to this day [5]. During the in-gel digestion, proteins are retained in a gel matrix during the unfolding, reduction and alkylation steps, simplifying the exchange from a denaturing buffer to a digestion buffer. Because digestion in solution was not in the main stream of proteomic development and it required a challenging unfolding-to-digesting buffer exchange step, fewer reports are available about in-solution digestion protocols [6]. The automated buffer exchange is technically difficult without the gel matrix and we addressed this challenge with the current article. Immunoglobulin gamma (IgG) antibodies from subclasses 1 and 2 (IgG1 and IgG2) have emerged as a popular modality in modern protein therapeutics. Each human IgG antibody molecule includes two identical heavy and two identical light chains linked by disulfide bonds. They are named according to the type of heavy

* Corresponding author at: Amgen, Process and Product Development One Amgen Center Drive, MS 8-1-C Thousand Oaks, CA 91320, United States. Tel.: +1 805 447 7215; fax: +1 805 447 3259.
E-mail address: pavel.bondarenko@amgen.com (P.V. Bondarenko).

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chain (1 or 2) and light chain (κ or λ) [7]. The IgG antibody molecules have significant homology in sequence, and tryptic digestion typically produces approximately 50–70% of identical peptides. A new modality, peptide-Fc fusion protein [8], consists of two peptides with specific affinity to a therapeutic target and a conserved fragment (Fc) of human IgG1. It has been recognized previously [9] that quantitative digestion of monoclonal antibodies is difficult because intrachain disulfide bonds, particularly in the CH_3 region of antibodies, are resistant to reduction and alkylation under native conditions. Antibodies are large, hydrophobic and strongly folded proteins, which are rather resistant to unfolding by denaturants, extremes of pH, heat or alcohols. Antibody denaturation in 6 M guanidine [10] led to complete unfolding of protein structure and facilitated complete reduction and alkylation. Many similar methods have been developed since and are widely used in the pharmaceutical industry [11–15] and in proteomic research [16]. Reduction and alkylation are typically done in 6–8 M guanidine or urea, followed by a buffer exchange step using size-exclusion (gel-filtration) cartridges or molecular weight cut-off membrane spin cartridges for tryptic digestion [11–17]. However, none of the reported methods are automated or easy to automate. Fully automated size-exclusion (gel-filtration) cartridges are difficult to control, and they require a vacuum use in most cases. On the other hand, the use of spin columns is almost impossible to automate utilizing current autosamplers. Other options like 96-well plate molecular weight cut-off membranes for desalting applications have not been explored in automatic mode to our knowledge.

In this report, we have evaluated different approaches in order to automate the tryptic digestion procedure for IgGs and have found that a custom-designed 96-well size-exclusion desalting plate was best suited for the desalting of protein samples. We have developed a fully automated digestion procedure, which is highly reproducible, reduces manual labor, and allows digestion of up to 96 samples at a time.

2. Experimental

2.1. Materials

The recombinant human monoclonal antibodies analyzed in this study were expressed at Amgen and purified using standard manufacturing procedures. They had been stored in formulation solutions at 4 °C or frozen at −80°C before analysis. Guanidine HCl and ammonium bicarbonate were from Mallinckrodt (St. Louis, MO). Dithiothreitol (DTT) was from MP Biomedicals (Irvine, CA), iodoacetamide (IAM) was from Sigma (St. Louis MO), and trypsin was from Roche (Sequencing Grade, Basel Switzerland).

2.2. Fully automated tryptic digestion procedure

The liquid handling system (Freedom EVO 100, Tecan US, Research Triangle Park, NC) was equipped with a 2-tip liquid handling (LiHa) arm with low volume washable fixed tips and 500-μL syringes. The system also had a robotic manipu-

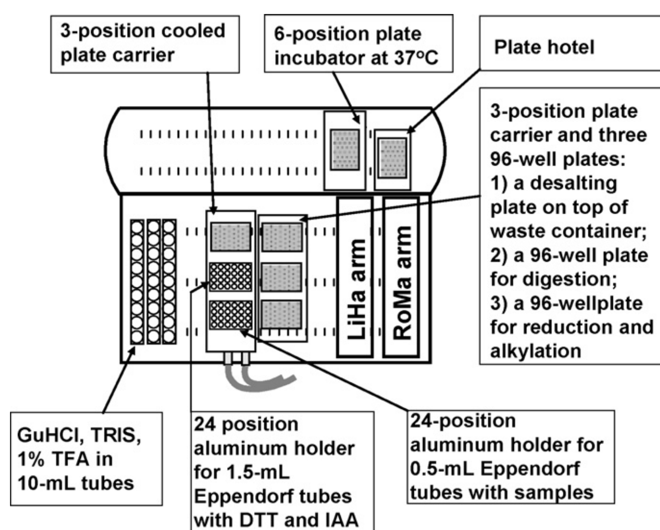

Fig. 1. Schematic of the Tecan Evo 100 system for fully automated tryptic digestions. The system was equipped with a 2-tip liquid handling (LiHa) arm with low volume washable fixed tips and 500-μL syringes. The system also had an integrated robotic manipulator (RoMa) arm, a microplate incubator, a cooled carrier for microplates and tubes, a microplate carrier and microplate hotel.

lator (RoMa) arm, a microplate incubator, a cooled carrier for microplates and tubes, a microplate carrier and a microplate hotel. The 96-well desalting plates were custom made and filled with silica-based size-exclusion (gel-filtration) medium by Orochem Technologies (Lombard, IL). A script using Freedom EVOware software had been written allowing the following steps to be performed by the Tecan liquid handling system. The protein samples (25 μL at 10 mg/mL) in 0.5-mL Eppendorf tubes were placed in a 24-position aluminum holder mounted on a cooled plate carrier at 4–8 °C (Fig. 1). If the number of samples was greater than 24, they were presented in a 96-well plate positioned in place of the aluminum holder. Trypsin solution at 0.5 mg/mL in water was located in the last position of the same aluminum holder or the plate. Other stock solutions were placed according to Fig. 1 and the automatic digestion program proceeded as follows: the samples (20 μL at 10 mg/mL) were automatically moved from their original location (0.5-mL Eppendorf tubes) into the wells of a 96-well plate for reduction and alkylation (Fig. 1). The samples were diluted in 100 μL denaturing buffer (7.5 M GuHCl, 2 mM EDTA, 100 mM Tris pH 7.5) and 5 μL of reducing solution (0.1 M DTT) and mixed. The 96-well plate with the solutions was moved to the plate incubator and incubated at 37 °C for 45 min. A 13-μL aliquot of alkylation solution (0.1 M iodoacetamide) was added to the reduced samples and incubated in the plate hotel at room temperature for 45 min. In parallel with the sample reduction and alkylation procedure, the 96-well size-exclusion desalting plate was equilibrated with digestion buffer (twice with 200 μL per well). Only the required number of desalting wells was used (one well per sample), while other wells were filled with a preserving buffer and sealed. Aliquots (100 μL) of the reduced and alkylated samples were loaded onto the desalting plate. After

5 min, the RoMa arm of the Tecan system moved the 96-well size-exclusion desalting plate, which was stored on top of a waste container, onto the top of the 96-well collection plate for elution (a plate for digestion in Fig. 1). Samples were eluted with 200-μL aliquots of digestion buffer (100 mM Tris pH 7.5) from the desalting plate into the plate for digestion. The elution volume (200 μL) was optimized to separate the earlier eluting large protein molecules from small molecules of GuHCl, DTT and iodoacetamide, which remained in the size-exclusion medium of the 96-well plate. After 5 min, the desalting plate was removed from the top of the collection plate, moved back onto the top of the waste container, and 50 μL of digestion buffer was added to the samples to bring the total solution volume to 250 μL per well. Then 4 μL of the trypsin solution at 0.5 mg/mL was added and mixed. The digestion solutions were incubated for 2.5 h in the plate incubator at 37 °C. A second aliquot of enzyme solution (4 μL) was added followed by incubation for additional 2.5 h at 37 °C. The tryptic digestion was stopped by adding 4 μL of quenching solution (1% TFA), and the 96-well plate was moved to the 3-position cooled plate carrier, covered with a plastic cover top and stored at 4 °C until analysis.

2.3. High-performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) analysis of tryptic peptides

For the HPLC/MS/MS analysis, the samples were aliquoted to Agilent sample vials. Alternatively, the injection could be performed directly from the wells of the same 96-well plate placed into the Agilent HPLC autosampler. The method for the analysis of the tryptic peptides has been described previously [17]. Briefly, the tryptic peptides were separated on a Polaris C18 Ether column (250 mm × 2.0 mm Varian, Torrance, CA) using a linear gradient from 0% to 65% B over 195 min. Solvent A was 0.1% TFA in water, and solvent B was 0.085% TFA, 90% acetonitrile (Baker, Phillipsburg, NJ) in water. Before sample injection, the column was equilibrated with 100% solvent A. The column temperature was maintained at 50 °C. The flow rate was 0.2 mL/min and 40 μg of protein digest was injected onto the column for analysis.

The HPLC was directly coupled to a Finnigan LCQ Deca ion trap mass spectrometer (Thermo Electron, San Jose, CA) equipped with an electrospray ionization source. The spray voltage was 4.5 kV, and the capillary temperature was 250 °C. The fragmentation mass spectra were obtained using ion trap collision offset value of 35%. Peptides were identified automatically by two different computer programs. Sequest, an algorithm of BioWorks (version 3.1, Thermo Electron, San Jose, CA, USA) was used to correlate the experimental tandem mass spectra against theoretical tandem mass spectra from a FASTA database including the sequences of the analyzed IgG antibodies. A software program written in-house [18,19] was also employed to correlate the experimental tandem mass spectra against theoretical tandem mass spectra generated from the known antibody amino acid sequence for peptide identification.

3. Results and discussion

3.1. Development of a fully automated procedure

Tryptic digestion of IgG molecules requires reduction and alkylation in the presence of high concentration of denaturing reagents (6 M GuHCl) which have to be removed prior to the addition of trypsin, since trypsin is not active under these denaturing conditions [10–17]. Desalting and buffer exchange in our previous manual procedure were achieved using size-exclusion (gel-filtration) columns (NAP-5; Amersham Biosciences) [13,17]. This step was labor intensive and difficult to automate. Hence, finding an alternative to the desalting step was the major challenge during the development of an automated tryptic digestion procedure. The following alternatives to the NAP-5 column desalting were considered and evaluated: (1) desalting using molecular weight cut-off membranes, which are available in 96-well plate format; (2) unfolding and reduction/alkylation in organic solutions like acetonitrile, which are compatible with trypsin; (3) develop or find commercially available 96-well desalting plates. The results of the evaluation were as follows:

1) Molecular weight cut-off membranes from Pall for desalting were evaluated and quickly dismissed. Sufficient vacuum for buffer exchange was difficult to achieve and the recovery was less than optimal (∼10%, data not shown).
2) Reduction/alkylation using organic solvents was evaluated. This approach looked promising at first for some antibodies, but we were unable to achieve complete digestion for most of the molecules. Different organic solvents (acetonitrile, isopropanol, and methanol) under different concentrations and different temperatures (up to 60 °C for the unfolding/reduction/alkylation step) were evaluated without success. Precipitation of a fraction of antibody molecules did not allow complete digestion (data not shown).
3) Finally, converting the NAP-5 columns to a 96-well plate format was the best option. Unfortunately such plates were not commercially available. We made in-house and evaluated several desalting plates by using commercially available 96-well filter plates and loading them with Sephadex G-25, which is the material in NAP-5 columns. Some of our in-house-made desalting plates gave excellent results (for example, Whatmann MBPP filter plate), however they required wetting of the hydrophobic membrane with organic solutions like isopropanol or methanol in order to start the elution by gravity alone (data not shown). Orochem Technologies provided custom made 96-well desalting plates to our specification. The plates contained 500 μL of the desalting material and could accommodate an additional volume of 250 μL for equilibration solution, sample loading, and elution. Those plates did not require vacuum or wetting of the filter material to start the gravity flow. The plates were covered with foil at the bottom and the top and started dripping immediately after the foil was removed. Importantly, leaving the desalting plate sitting at room temperature for up to 2 h without foil did not seem to affect the desalting effi-

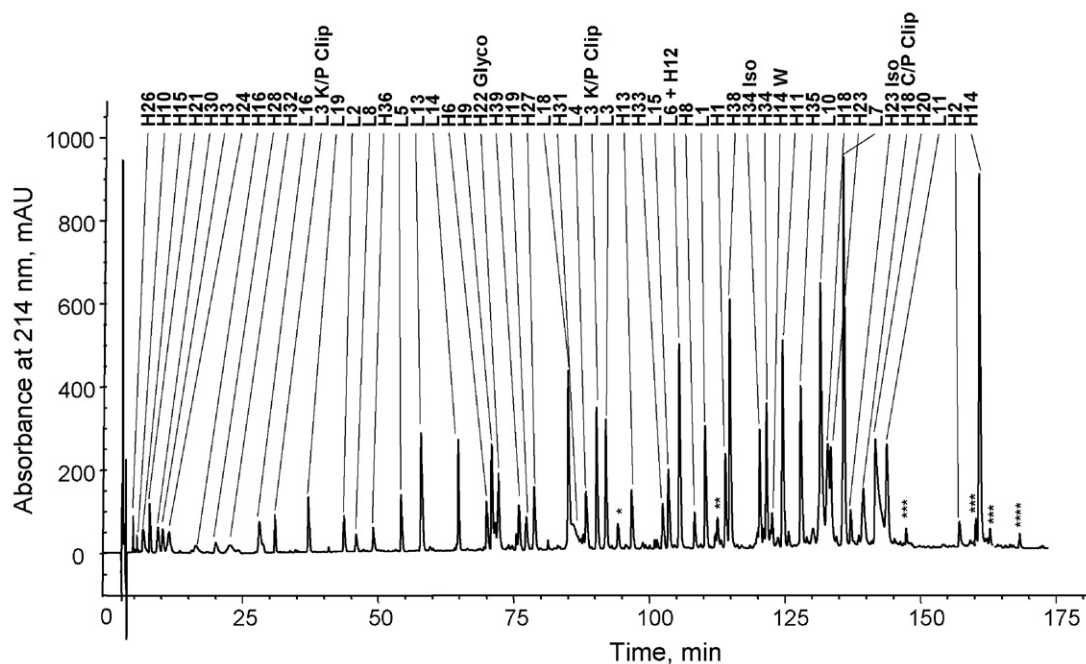

Fig. 2. Reversed-phase chromatogram with UV detection at 215 nm of a tryptic digest using the fully automated procedure for an IgG2 antibody. The eluting peptides were analyzed on-line by mass spectrometry for peptide identification, the results of which are summarized in Table 1. Other marked peaks were unidentified peak with *m*/*z* value of 996.3 (*), unidentified peak with *m*/*z* value 1044.4 (**), missed cleavages (***) and trypsin-related peaks (****).

ciency. Manual tryptic peptide maps using desalting plates provided by Orochem Technologies gave essentially identical results as compared to using NAP-5 columns for the desalting step (data not shown) and were therefore included in the automation process.

3.2. Fully automated digestion

After the decision to use Orochem desalting plates was made, we assembled a liquid handling system (Tecan Freedom EVO 100) according to desired specifications (Fig. 1). The following hardware was included: a cooling tray to keep samples and reagents at 4 °C, and an incubator for the reduction and digestion steps. Although programming of the script was relatively straightforward, several technical challenges needed to be overcome. Initially, great variability in the concentration of the digested protein was observed between samples. Upon careful examination, it was determined that the cause of the variability was that the jet from the dispensers perturbed the integrity of the gel bed of the desalting wells. After the flow was reduced to 25 mL/min, reproducibility and consistency in the concentration were improved without any significant loss in preparation time. Another challenge emerged during handling of the desalting plate. After conditioning the desalting wells and also after eluting the 200-μL aliquots of samples, occasional droplets of ~40 μL were hanging from the bottoms of some of the wells, thereby changing the intended volume (200 μL) of the eluted sample. The droplets were automatically shaken off into the waste container after the conditioning of the 96-well desalting plate and then into the 96-well plate for digestion after eluting the sample. By using the shaking steps, the droplets were removed, and the precision of the final volumes and concentrations of the digested samples was improved.

Fig. 2 shows the UV chromatogram of the tryptic digest of an IgG2 antibody as an example of the performance of the automated tryptic digestion procedure. An 80-μL aliquot of the digest (40 μg of protein) was injected and peak intensities were sufficient for identification by mass spectrometry. The results of the identification are summarized in Table 1. The peaks were identified by correlating fragmentation mass spectra to sequences of light and heavy chains of this IgG2 antibody in a FASTA database. The automatically derived correlation coefficients of Mass Analyzer [18] and Sequest (Xcorr) and, in some cases, manual interpretation of mass spectra were used to verify correctness of peptide identification in all peaks. *De novo* algorithm of the Mass Analyzer [19] was also employed to identify peptides with unexpected modifications. The antibody sequence coverage was over 97% based on the number of amino acids identified (638 out of 658). Only very short peptides could not be identified. This is not surprising, since it is well known that short hydrophilic peptides are not retained by the reversed-phase column and eluted in the injection volume together with the TRIS digestion buffer, which suppresses ionization.

3.3. Comparison with manual digestion procedure

A comparison of the automated digestion with the manual tryptic digestion procedure described in detail in [13,17] is shown in Fig. 3 for an IgG2κ antibody as an example. Both chromatograms overlay very well, demonstrating that the fully automated tryptic digestion procedure resulted in very similar digestions compared to the manual procedure using NAP-5

Table 1
An IgG2κ antibody was digested using the fully automated procedure and analyzed by HPLC/MS/MS as described in Section 2

Peptide	Retention time[a]	Calcul. mass	Observed mass	MS/MS data[b]
L1	108.2	1884.1	1883.7	√
L2	43.4	706.8	706.4	√
L3	92.8	2413.6	2413.4	√
L4	90.4	979.1	980.0	√
L5	53.6	728.8	728.7	√
L6	103.5	1632.47	1632.4	√
L7	133.1	3059.3	3059.0	√
L8	45.6	487.6	487.5	√
L9: R	No ID	174.2		
L10: TVAAPSVFIFPPSDEQLK	129.0	1946.2	1945.8	√
L11: SGTASVVCLLNNFYPR	145.15	1798.0	1797.7	√
L12: EAK	No ID	346.4		
L13: VQWK	57.3	559.7	559.5	√
L14: VDNALQSGNSQESVTEQDSK	66.49	2136.2	2134.0	
L15: DSTYSLSSTLTLSK	101.6	1502.6	1502.3	√
L16: ADYEK	34.36	624.7	624.5	√
L17: HK	No ID	283.3		
L18: VYACEVTHQGLSSPVTK	83.6	1876.1	1876.1	√
L19: SFNR	50	522.7	522.5	√
L20: GEC	No ID	364.4		
H1	111.8	1896.1	1896.1	√
H2	155.9	2170.5	2071.1	
H3	10.8	499.6	499.4	√
H4	129.0	2262.5	2262.3	
H5	No ID	231.3		
H6	68.9	622.7	622.9	√
H7	No ID	462.5		
H8	107.2	1352.6	1352.5	√
H9	69.9	1290.4	1290.6	√
H10	6.6	511.5	511.5	√
H11	122.1	2101.3	2101.3	√
H12: GPSVFPLAPCSR	103.5	1287.5	1287.7	√
H13: STSESTAALGCLVK	95.0	1423.6	1423.5	√
H14: DYFPEPVTVSWNSGALTSGVHTF-PAVLQSSGLYSLSSVVTVPSSNFGTQ-TYTCNVDHKPSNTK	159.96	6766.4	6765.6	√
H15: VDK	7.5	360.4	360.4	√
H16: TVER	16.8	503.6	503.4	
H17: K	No ID	146.2		
H18: CCVECPPCPAPPVAGPSVFLFPPKPK	130.3	2909.5	2909.3	√
H19: DTLMISR	76.1	835.0	834.8	√
H20: TPEVTCVVVDVSHEDPEVQFN-WYVDGVEVHNAK	138.9	3799.1	3798.5	√
H21: TKPR	8.5	500.6	500.5	√
H22: EEQFNSTFR	78.81	2602.2[a]	2602.0[a]	√
H23: VVSVLTVVHQDWLNGK	134.4	1794.1	1794.6	√
H24: EYK	11.9	438.5	438.4	√
H25: CK	No ID	306.4		
H26: VSNK	6.2	446.5	446.4	√
H27: GLPAPIEK	77.5	824.0	823.7	√
H28: TISK	20.4	447.5	447.4	√
H29: TK	No ID	247.3		
H30: GQPR	10.0	456.5	456.4	√
H31: EPQVYTLPPSR	83.3	1286.5	1286.2	√
H32: EEMTK	22.3	636.7	636.7	√
H33: NQVSLTCLVK	100.5	1161.4	1161.5	√
H34: GFYPSDIAVEWESNGQPENNYK	119.2	2544.7	2544.4	√
H35: TTPPMLDSDGSFFLYSK	125.3	1906.1	1905.9	√
H36: LTVDK	48.6	574.7	574.5	√
H37: SR	No ID	261.3		

Table 1 (*Continued*)

Peptide	Retention time[a]	Calcul. mass	Observed mass	MS/MS data[b]
H38: WQQGNVFSCSVMHEALHNHYTQK	112.6	2802.1	2801.8	√
H39: SLSLSPG	74.7	659.7	659.5	√

Peptides were identified manually or using an in-house developed software program as described. The protein coverage was calculated to be 97% based on the number of amino acids identified (638 from a total of 658). The numbering of the tryptic peptides was sequential from the N-terminus, labeling the heavy chain peptides "H" and the light chain peptides "L". The amino acid sequence of the conserved region shared by all IgG2 molecules is shown for reference.

[a] Retention time at the time of identification.

[b] MS/MS data available for identification. All other peptides were identified based on the molecular weight only.

columns. This experiment indicated that during the automated procedure the monoclonal antibody molecules were successfully unfolded, completely reduced, alkylated and digested without the loss of the sample due to precipitation or adsorption to the walls of the containers or during the desalting step. It is also important to note that no undigested protein was detected in manual or in automated digestion. In case of incomplete digestion, several large fragments eluted around 180 min (data not shown). This was not observed when using the optimized procedure illustrated in this article.

3.4. Reproducibility

Fig. 4 shows the UV chromatograms of four digests of an IgG2κ antibody performed on two different days. The samples were frozen at −80 °C after digestions, then thawed and analyzed by HPLC/MS/MS in a series of runs afterwards. The UV chromatograms of the four digests overlaid very well, indicating the good reproducibility of the automated digestion and high precision of the peak area measurements by the HPLC analysis with UV detection. Five peptides from the conserved region of this antibody were chosen to measure the peak areas of those peptides and the standard deviation among the four samples. The results summarized in Table 2 show that the fully automated digestion is highly reproducible with standard deviations of the peak areas for the four analyses of less than 10%. It was observed by others [10] and us, that two peptides containing complementarity-determining region 3 (CDR3) of heavy chain (H11, residues 99–121) and CDR3 of light chain (L7, residues 77–103 according to Edelman numbering) decrease upon extended storage (data not shown). These are relatively large and hydrophobic peptides with unique sequences, therefore their elution times slightly shift from one monoclonal antibody to another. The disappearance of these peptides was probably due to the precipitation or adsorption to the walls of vials and wells. The only practical solution found so far was to analyze a series of samples of interest within 1 day, and even during this period a significant decrease was sometimes observed. The peptides were labeled in Fig. 4. These two peptides represent a special case, which requires further investigation and, for this reason, they were not used for comparison in Table 2.

Since the five chosen peptides were in the conserved region of the IgG molecule, reproducibility of the automated digestion using these peptides was also evaluated for different IgG antibodies. We analyzed four different antibodies and the results of the peak area calculations and the standard deviations are

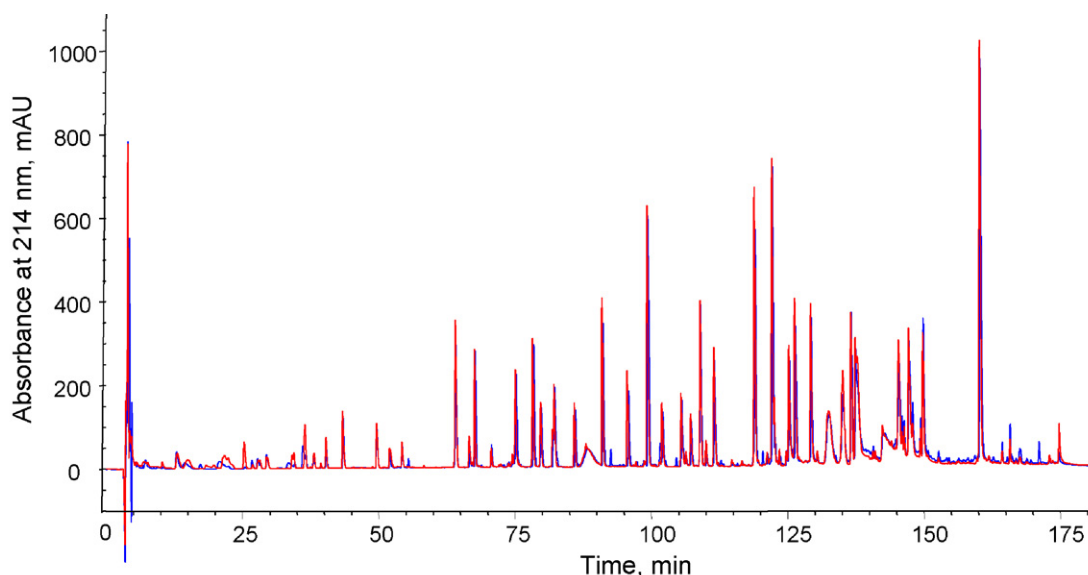

Fig. 3. Comparison of the reversed-phase chromatograms for digests of one IgG2 antibody using the fully automated TECAN procedure with the manual NAP-5 method. Forty micrograms of digest was injected for the TECAN procedure and 20 μg was injected for the manual NAP-5 method. The samples were analyzed using different columns on different days and the chromatograms were manually aligned.

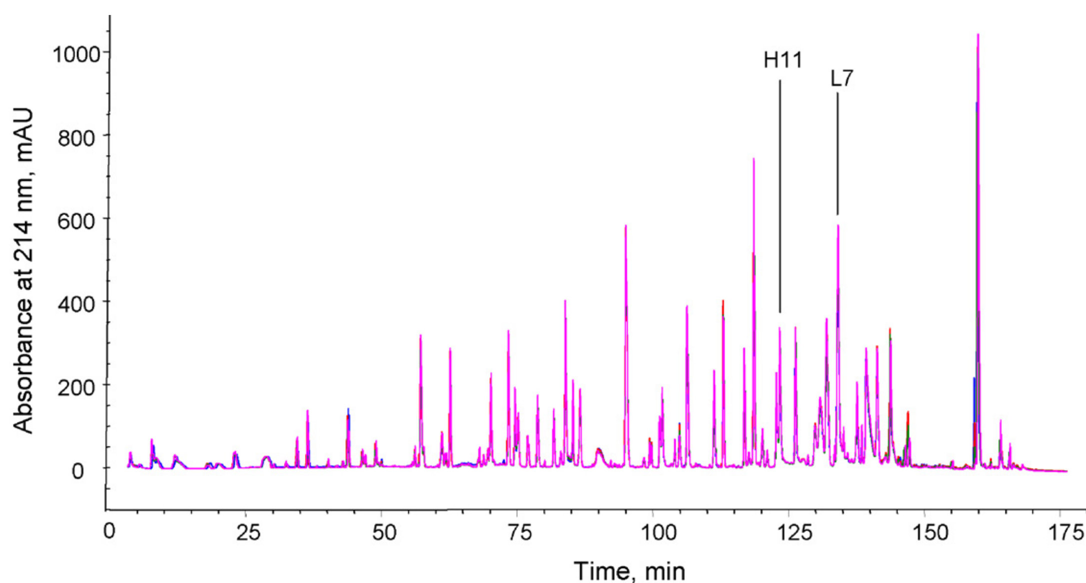

Fig. 4. Comparison of the reversed-phase chromatograms of tryptic digests of an IgG2 antibody performed using the fully automated procedure on different days.

Table 2
Four samples of a recombinant monoclonal IgG2 antibody were digested on different days (1 and 2 day one, 3 and 4 day two) using the fully automated procedure

Peptide	Peak area 1	Peak area 2	Peak area 3	Peak area 4	Average	S.D.	Error (%)
H14	18,240	21,050	19,900	20,660	19,960	1240	6
H18	9,668	10,606	10,061	11,728	10,515	895	8
H19	1,317	1,457	1,364	1,503	1,410	85	6
H34	11,747	12,601	11,865	13,049	12,315	618	5
H38	6,205	6,246	5,537	5,414	5,850	436	7
L11	7,029	7,587	6,956	7,496	7,267	320	4

The samples were frozen at −80 °C after digestion and analyzed afterwards. Peptides were separated as described in Section 2 and the peak areas of selected peptides were calculated from the UV absorbance at 214 nm using Agilent ChemStation software.

summarized in Table 3. The standard deviations of the peak areas of the five conserved peptides across all four IgGs were larger compared to the same IgG antibody analyzed several times. This observation was not surprising, since the peak area of the peptides could be affected by co-eluting peptides or peptides with similar retention time. Other factors like precipitation of hydrophobic peptides and their absorption to the walls of containers, incorrect starting concentrations or pipetting errors could also explain the results. However, the data demonstrated the high reproducibility of the automated digestion procedure even across different antibodies.

An increased methionine oxidation in one of the IgGs was detected. The peak area of the H19 and H38 peptides for IgG number 4 was significantly lower compared to the other antibodies. A closer look at the UV and MS/MS data revealed that this antibody was indeed experiencing oxidation due to storage at room temperature for one year time. The combined peak area of the oxidized species and the native peptides matched very well with the other, non-oxidized IgGs (peak areas of H19: 390 oxidized + 122 native = 512; peak areas of H38: 2597 oxidized + 922 native = 3519), indicating that the tryptic peptide mapping procedure can be used to monitor covalent modifications.

Table 3
Samples of four different recombinant monoclonal IgG antibodies (one IgG1κ and three IgG2κ) were digested using the fully automated procedure

Peptide	Peak area 1	Peak area 2	Peak area 3	Peak area 4	Average	S.D.	Error (%)
H14	8644	9084	7658	7552	8234	750	9
H18	3801	3146	2423	2809	3044	584	19
H19	823	607	503	122[a]	644	163	25
H34	3082	2886	2291	2273	2633	413	16
H38	5416	4629	4212	922[a]	4752	611	12
L11	3759	3408	2479	2656	3075	608	20

Peptides were separated as described in Section 2 and the peak areas of selected peptides were calculated from the UV absorbance at 214 nm using Agilent ChemStation software.

[a] This peptide underwent oxidation and it was not included in the calculations of the average and standard deviation.

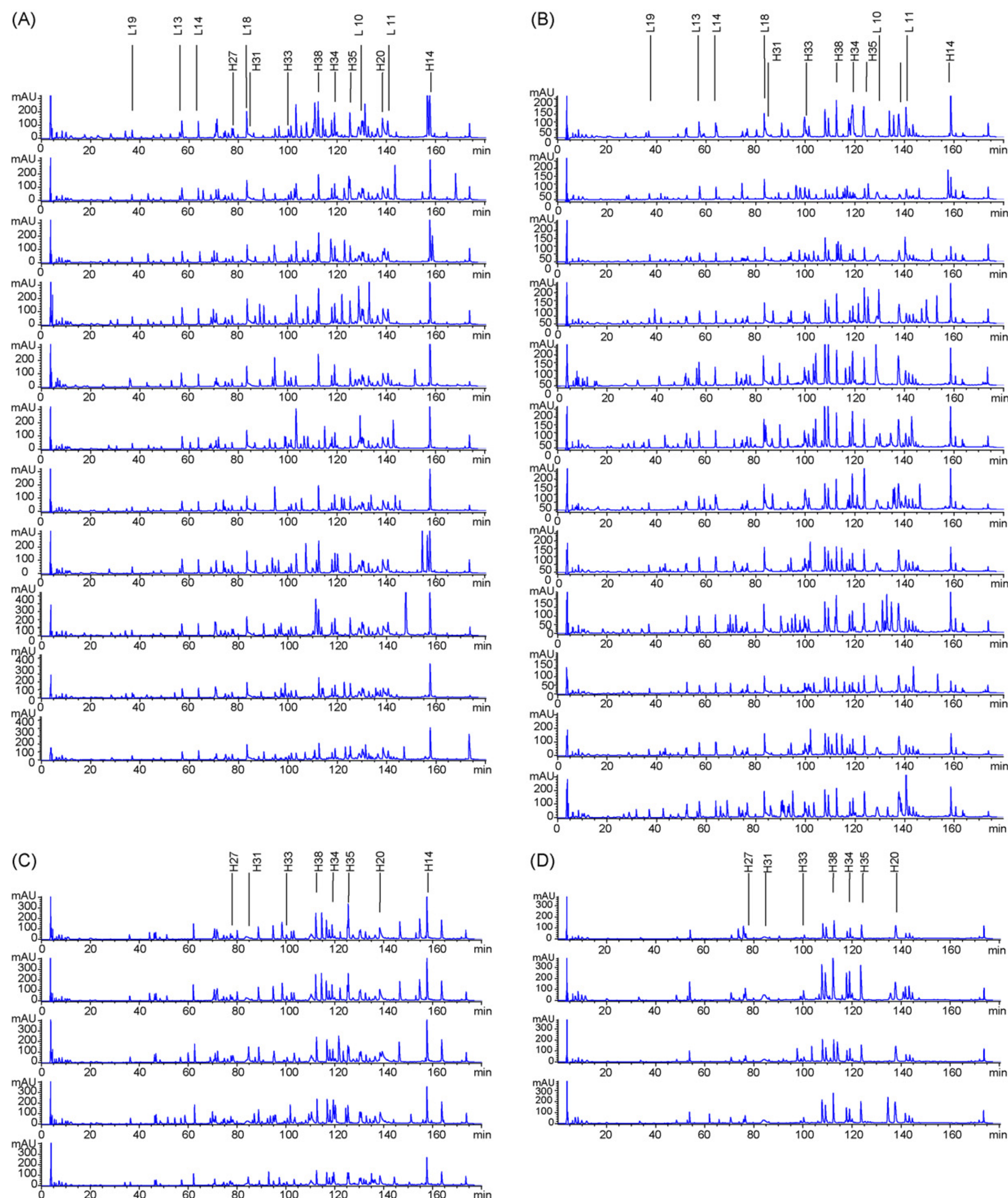

Fig. 5. Reversed-phase chromatograms with UV detection at 215 nm for tryptic digests of several monoclonal recombinant antibodies, including IgG2κ (A); IgG1κ (B); IgG2λ (C); peptide-Fc fusion proteins (D).

The mobile phase gradient and other parameters of the HPLC method were optimized to achieve complete chromatographic separation for most peptides in the map. Several peptides still co-eluted though, and reconstructed ion chromatograms (plots of *m/z* values of peptides *vs*. elution time) near the time of elution and MS/MS assisted identification were utilized for accurate quantification of each peptide. The reconstructed ion chromatograms were found especially useful for quantifying small sample-to-sample variations of peptide modifications such as oxidation, deamidation [17], N-terminal cyclization [20], glycosylation [13], etc.

3.5. Applications to different subclasses of IgG molecules

A total of 32 proteins, including human monoclonal IgG1κ, IgG2κ, IgG2λ and peptide-Fc fusion proteins have been digested

using the automated procedure followed by the reversed-phase HPLC/MS analysis of the digestion products (Fig. 5). The peptide-Fc fusion proteins included two active peptides and a conserved fragment (Fc) of human IgG1 comprising two heavy chain C-terminal sections including peptides H18-H39. Several common peptides from conserved regions of light and heavy chains were labeled in Fig. 5 to highlight the high degree of homology among the antibody molecules. IgG2κ (Fig. 5A) and IgG1κ (Fig. 5B) molecules have similar conserved regions, with the exception of the hinge peptides H15, H16, H17, H18 and a few other residues. IgG2λ molecules (Fig. 5C) contain λ light chains, which have a greatly different sequence of the conserved region as compare to the κ light chains. The conserved region of peptide-Fc fusion proteins includes only the C-terminal part of heavy chains interconnected at the hinge (Fig. 5D). In addition to the conserved regions, the peptide maps contained the unique peptides enabling unambiguous identification of the compounds. The automated digestion approach described here for pharmaceutical applications increased throughput and improved reproducibility of assays for monoclonal antibody identity and identification and quantification of covalent modifications during formulation stability studies.

The rate of protein modification (degradation) is an important parameter for optimization of production, purification, formulation and storage conditions for therapeutic monoclonal antibodies, and its accurate elucidation in a large number of samples requires high-throughput quantitative peptide mapping. Although the HPLC/MS/MS runs were relatively long (3 h) in our settings, they were fully automated and typically trouble-free. However, the front and back ends, sample preparation and data processing, have been the two bottle necks of the peptide mapping process. This report offers a fully automated peptide mapping protocol for monoclonal antibodies in pharmaceutics. The data processing procedure however, including identification and quantification of peptides and their degradation products, has not been fully automated in this study. This manuscript demonstrated our current semi-automatic data processing protocol utilizing Sequest or Mass Analyzer algorithm for identification and chromatograms with UV detection for quantification. The overlapping and minor peptide species were manually reconstructed using their *m*/*z* and elution time values and quantified using QualBrowser tool of Xcalibur software. Relative quantification of modified peptides across several samples is an important task of peptide mapping in protein pharmaceutics, and it is somewhat similar to the label-free relative quantification in proteomics. Two approaches are currently utilized for label-free quantitative mass spectrometry in proteomics, which could be adopted for the fully automated data processing of peptide mapping in protein pharmaceutics: (1) identify first—then compare [21,22] and (2) compare first—then identify [23,24]. The advantage of the second approach is that small changes in the signal intensity of a peptide can be detected by matching 3D (peptide *m*/*z*, elution time and intensity) topology maps for different HPLC/MS experiments and using a novel statistical image analysis [23,24]. This way, the peptide species with changed abundances will be highlighted by the software regardless if they were identified or not. If the automatic identification algorithm failed to identify a modified peptide due to the poor quality of its fragmentation spectra or because the chemical modification was not "anticipated" by the identification algorithm, the modified peptide can be identified off-line by trying different chemical modifications or using *de novo* approach. This approach minimizes the chance to miss an unknown modification. Fully automated mass spectrometric identification and quantification of modifications of pharmaceutical proteins are underway.

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