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Accelerated tryptic digestion for the analysis of biopharmaceutical monoclonal antibodies in plasma by liquid chromatography with tandem mass spectrometric detection

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ABSTRACT

Accelerated tryptic digestion of a therapeutic protein including microwave irradiation and thermal transfer by convection at 60 °C and 37 °C was investigated. An analytical setup was devised to follow the protein digestion rate using 1D gel electrophoresis and liquid chromatography coupled a triple quadrupole linear ion trap mass spectrometer. The formation kinetic of its tryptic peptides was monitored in the selected monitoring mode (LC-SRM/MS). Different digestion end points (e.g. 2, 5, 10, 15, 30 and 60 min) as well as an overnight digestion were tested using a therapeutic human monoclonal antibody (mAb) with the goal of its LC-SRM/MS quantification in human plasma. The peptides from the human mAb were generated at different rates and were classified into three categories: (1) the fast forming peptides, (2) the slow forming peptides and (3) the peptides degrading over time. For many monitored peptides, a heating temperature of 37 °C with a 750 rpm mixing applied for at least 30 min provided equivalent results to microwave-assisted digestion and generally allowed the achievement of an equivalent peptide concentration as an overnight digestion carried out at 37 °C. The disappearance of the protein of the heavy and light chains can be monitored by 1D gel electrophoresis but was found not to be representative of the final tryptic peptide concentrations. For quantitative purposes a stable isotope labeled version ($^{13}C_4$, $^{15}N_1$) of the therapeutic protein was used. The labeled protein as internal standard was found to be very efficient to compensate for incomplete digestion or losses during sample preparation.

1. Introduction

The number of biotech drugs such as proteins or peptides has significantly increased over the last decade. Between 1994 and 2004, 19 therapeutics monoclonal antibodies (mAbs) were approved for different therapeutic indications [1]. Therefore the development of absolute quantitation strategies of those proteins in biofluids (plasma) is required for supporting the preclinical and clinical study workflow. Immunoassays are a commonly used technique for protein quantitation in plasma, but recently, mass spectrometric approaches have been developed as alternative methods without the need of antigen and antibodies production and development. However, quantitation of a protein in plasma by mass spectrometry (MS) remains very challenging due to the high complexity and dynamic range of this biological matrix [2]. While immunoassays quantify the intact form of the protein, LC–MS is generally used to monitor its specific tryptic peptides. Sample preparation and trypsin digestion remain the key challenges in LC–MS analysis of proteins, in particular when hundreds of samples need to be analyzed on a daily basis.

The enzymatic digestion of proteins followed by analysis of the resulting peptides by mass spectrometry is a recurrent approach in "bottom-up" proteomic workflows. Improvements in chromatographic separation power combined with more sensitive, accurate and fast scanning mass spectrometers result in accession of higher throughput analysis [3,4]. Due to the shortening of the LC–MS run time, sample preparation became the new bottleneck for high throughput analyses. In proteomic workflow, protein enzymatic digestion is one of the main time limiting factors and has led to the development of new protocols for accelerating this step. In such a context, the use of acid-labile surfactant [5–8], the addition of organic solvent (methanol, acetonitrile, acetone or 2-propanol) [9], the use of immobilized trypsin reactor [10], ultrasound [11], microwave [12] or more recently the application of high-pressure during digestion [13] have been described. Protocols based on microwave irradiation have emerged during the last decade; indeed, microwave irradiation is well known in the world

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of synthetic chemistry for improving the synthesis yields and shortening reaction times since the early 1980s [14,15]. In the field of proteomics microwave irradiation has been described for amino acids analysis, for peptides mapping after acidic cleavage [16,17] or as a tryptic digestion enhancer. Pramanik et al. have applied microwave irradiation to enzymatic digestion of proteins for peptides mapping using a temperature-controlled microwave oven with Lys-C or trypsin as protease [18]. Based on protein coverage, by peptide mass fingerprinting (PMF), they observed that only 10 min of microwave-assisted digestion or the use of a heating plate set at 60 °C gave comparable results to the classical overnight digestion. Further digestion protocols based on a combination of microwave irradiation and organic solvents [19] or magnetic beads or particles with immobilized enzymes [20–22] have been developed. In other hand microwave-assisted digestion has been successfully applied for in-gel [23–25] digestion. However, in most cases, the estimation of the digestion efficiency was essentially performed by monitoring the protein sequence coverage and the specificity of the cleavage sites by MS using peptide mass fingerprint or MS/MS sequencing strategies. This approach is suitable for qualitative proteomics studies where enhancement of the sequence coverage is essential for unambiguous protein identification.

Absolute quantification of proteins by LC–MS/MS is characterized by a different approach consisting in monitoring protein-specific peptides signals using selected reaction monitoring (SRM) experiments [26]. A study of microwave-assisted enzymatic digestion for quantitative proteomic using LC–MS/MS and synthetic peptides as internal standard, was performed by Vesper et al. on hemoglobin A1c [27]. The authors observed that digestion reach a plateau after 20 min and only a slight improvement using microwave energy as thermal transfer. In this study, we investigated the possibility to accelerate the tryptic digestion of a therapeutic mAb in view of its absolute quantification by LC-SRM/MS in plasma using either microwave irradiation or thermal transfer at various experimental conditions.

2. Experimental

2.1. Reagents

Solutions (20 mM Tris–HCl, pH 5.5) of recombinant human monoclonal antibody (MW = 145,157 kDa – 53.8 μg/μL) and its version isotopically labeled on threonines ($^{13}C_4$, $^{15}N_1$, $\Delta m = 5$ u – 5.54 μg/μL), used as internal standard (IS), were obtained from Novartis Pharma AG (Basel, Switzerland). Ammonium bicarbonate and acetic acid were provided by Fluka (Fluka Chemie GmbH, Buchs, Switzerland). DL-Dithiothreitol, iodoacetamide, HPLC grade acetonitrile and Trypsin I-XS were purchased from Sigma–Aldrich (Sigma–Aldrich Chemie GmbH, Steinheim, Germany), formic acid was obtained from Merck (Merck KGaA, Damstadt, Germany), sequencing grade modified trypsin was provided by Promega (Promega AG, Dübendorf, Switzerland). Human plasma with citrate was obtained from the Geneva University Hospital (Geneva, Switzerland).

2.2. SDS-PAGE

SDS-PAGE was performed on Xcell system (Invitrogen AG, Basel, Switzerland) using precast 10 wells, 1.5 mm, 4–12% Bis–Tris Gel (NuPAGE, Invitrogen AG), migration was performed at a constant voltage of 170 V using MES SDS buffer kit (Invitrogen AG). Samples were prepared according to the manufacturer procedure except that the denaturation step was performed at 80 °C instead of 70 °C. Gels were stained with Colloidal Blue Stain kit (Invitrogen AG) according to the manufacturer protocol. Gels were de-stained in 5% acetic acid instead of water.

2.3. mAb characterization

2.3.1. Reduction and alkylation

1 μL of unlabeled mAb stock solution (53.8 μg/μL in 20 mM Tris-HCl, pH 5.5) was added into a 0.5 mL Protein LoBind tube (Vaudaux-Eppendorf, Schönenbuch, Switzerland) containing 89 μL of 50 mM ammonium bicarbonate and 10 μL of 50 mM dithiothreitol. LoBind tubes are useful for high temperature digestion because they avoid water condensation on the surface of the tube and consequently keep the reaction volume constant. The sample was reduced under agitation at 60 °C for 45 min (Thermomixer Comfort, Vaudaux-Eppendorf, Schönenbuch, Switzerland). After cooling the sample at room temperature, 11 μL of 300 mM iodoacetamide was added for the alkylation of the cysteines. The sample was briefly vortexed, centrifugated and then incubated for 30 min in the dark at room temperature.

2.3.2. In-solution tryptic digestion

The reduced and alkylated protein was enzymatically digested by adding 5.53 μL (0.5 μg/μL in 1 mM HCl) of proteomic grade trypsin solution (Promega), corresponding to a final trypsin/protein ratio of 1/20 (w/w). Sample was immediately vortexed and centrifugated prior incubation for 15 min in a 1400 W domestic microwave oven (Intertronic input power 1400 W), power was set to 30%. Directly after incubation, digestion was quenched by formic acid addition (6 μL); afterwards sample was briefly vortexed and cooled in ice water. The digest was stored at −20 °C until further analysis.

2.3.3. In silico digestion and collision induced fragmentation screening

Y-ions of predictable peptides were screened to establish a set of optimum SRM transitions required to further monitor the mAb's peptides. Therefore the sequences of the matured mAb's heavy and light chains were *in silico* digested using MS-Digest (Protein Prospector basic v 4.27.2, University of California, San Francisco, USA). Resulting peptides were selected according to the following criteria: peptides should possess: (i) a mass ranging from 400 to 4000 Da, (ii) a minimum length of five amino acids and (iii) no tryptic missed cleavage in their sequence. In addition, the candidate peptides must contain at least one threonine residue because the IS protein is isotopically labeled on threonine ($^{13}C_4$, $^{15}N_1$ – $\Delta m = 5$ u). In total, 24 peptide candidates were selected and submitted to *in silico* fragmentation via MS-Product (Protein Prospector) assuming all cysteines were alkylated by carboxyamidomethylation (+57 Da). For each selected peptide a set of SRM transitions was constructed specifically according to the following rules: (i) only y-ion series were used, (ii) for peptides with mass less than 1000 Da, only transitions from 1^+ and 2^+ precursors to 1^+ products ions were considered, (iii) for peptides with mass ranges between 1000 and 2500 Da, only transitions from 2^+ and 3^+ precursors to 1^+ products ions were considered and (iv) for peptides with mass above 2500 Da, transitions from 3^+ and 4^+ precursors to 1^+ and 2^+ fragments ions were considered. Several transitions were monitored for a single peptide. The dwell time ranged from 10 to 125 ms while the total cycle time did not exceed 1 s. The collision energies were defined by generic formulas according to the m/z ratio and the charge state of the precursor ion (i.e. rolling CE script): (i) $CE = m/z \times 0.05 + 9$ (for 1^+ precursors), (ii) $CE = m/z \times 0.05 + 5$ (for 2^+ precursors), (iii) $CE = m/z \times 0.044 + 3$ (for 3^+ precursors) and (iv) $CE = m/z \times 0.05 + 2$ (for 4^+ precursors).

A preliminary targeted experiment using LC-SRM/MS analysis has been devised to determine the best SRM transition (i.e. good intensity and selectivity) for monitoring each putative peptide.

Table 1
Selected peptides for the monitoring of the mAb's enzymatic digestion by LC-SRM/MS.

Peptide sequence	Mw [Da]	y-ion	Precursor ion (m/z for Q_1)	Product ion (m/z for Q_3)
mAb's heavy chain peptides				
FTISR	622.4	y4	312.2	476.3
LTVDK	574.3	y4	288.2	462.3
NQVSLTCLVK	1160.6	y7	581.3	820.5
DTLMISR	834.4	y4	418.2	506.3
AEDTAVYYCAR	1317.6	y8	659.8	1003.5
NTLYLQMNGLR	1321.7	y8	661.8	994.5
STSGGTAALGCLVK	1320.7	y9	661.3	932.5
TGPFDYWGQGTLVTVSSASTK	2201.1	y8	734.7	780.4
THTCPPCPAPELLGGPSVFLFPPKPK	2843.5	y5	711.9	566.4
TPEVTCVVVDVSHEDPEVK	2138.0	y4	713.7	472.3
TTPPVLDSDGSFFLYSK	1872.9	y5	625.3	657.4
VVSVLTVLHQDWLNGK	1807.0	y11	603.3	1310.7
mAb's light chain peptides				
VYACEVTHQGLSSPVTK	1874.9	y8	620.0	788.5
TVAAPSVFIFPPSDEQLK	1945.0	y8	649.3	913.5
VTITCR	748.4	y4	375.2	549.3
SGTASVVCLLNNFYPR	1796.9	y6	600.0	810.4
DSTYSLSSTLTLSK	1501.8	y8	751.9	836.5
EIVLTQSPDFQSVTPK	1787.9	y5	597.0	531.3

Among the 24 candidates, only 18 were retained for their SRM transition specificity (i.e. several transitions observed at the same retention time). Table 1 shows the selected peptides and their SRM transitions for the monitoring of the mAb enzymatic digestion.

2.3.4. LC-SRM/MS analysis

The tryptic digest was diluted three times with 0.1% formic acid prior to injection, corresponding to a protein concentration of approximately 1 pmol/μL. The LC-SRM/MS system consisted of a Ultimate 3000 micro-LC (Dionex Softron GmbH, Germering, Germany) configured in a binary high-pressure gradient mode, 1 μL of digest was injected onto a 1.0 mm × 150 mm, 3.5 μm Zorbax SB-C18 reversed-phase column (Agilent Technologies, Switzerland). The column oven was set at 25 °C and the flow rate was fixed at 80 μL/min. Eluent A consisted of 0.1% formic acid and eluent B of 0.1% formic acid in acetonitrile. Peptides were eluted by a linear gradient starting from 15% B to 50% B in 15 min. Electrospray ionization (Turbo V ion source) was operated in positive mode. MS data were collected using a 4000 Q TRAP hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) with Q1 and Q3 quadrupoles operating at unit mass resolution.

2.4. mAb digestion rate study

2.4.1. In-solution tryptic digestion

The protein was reduced and alkylated as described previously. Prior digestion the samples were thermalized at 20 °C in the dark in order to start proteolysis of all samples at the same temperature. In total, four heating methods were tested at several time points and each experiment was performed in triplicate (except for 5 min at 100% microwave irradiation power which was performed in duplicate):

(i) Digestion at 37 °C (thermal transfer) under agitation (750 rpm – Thermomixer) for 2, 5, 10, 15, 30, 60 min and overnight.
(ii) Digestion at 60 °C (thermal transfer) under agitation (750 rpm) for 2, 5, 10, 15 min and overnight.
(iii) Digestion assisted by microwave irradiation at a power of 40% for 2, 5, 10, 15, 30, and 60 min.
(iv) Digestion assisted by microwave irradiation at a power of 100% for 2, 5, 10, and 15 min, in this case a flask of 500 mL of water was put in the microwave oven for absorbing the excess of energy and to avoid the trigger of the oven security switch.

Each digestion time point was prepared independently (i.e. three tubes by time point). Proteomic grade trypsin was used at a trypsin/protein ratio of approximately 1/30 (w/w). Digestions were stopped by adding 1 μL of pure formic acid with a briefly vortex-mixing and then directly cooled in ice water. Prior to the LC-SRM/MS analysis, digests were diluted three times with formic acid 1% and spiked with the same volume of a tryptic digest of the isotopically labeled internal standard. The ratio between unlabeled and labeled protein was of 5. The mAb internal standard digest was generated by an overnight digestion at 37 °C under agitation with a trypsin/protein ratio of 1/30 (w/w), this digestion was stopped by adding formic acid to final concentration of 1%. The digests were analyzed twice, 24 h and 1 month post digestion.

2.4.2. LC-SRM/MS analysis

Samples were analyzed onto a 2.1 mm ID × 100 mm, 3.5 μm XTerra MS C18 column (Waters) with the same LC–MS/MS system as described previously. Elution was performed by a gradient of 5–50% B in 20 min and the flow rate was set at 250 μL/min. Peptides generation was monitored using a SRM set built with the best transitions previously screened. Data were acquired with a 4000 Q TRAP using Q1 and Q3 operating at unit resolution and a dwell time per transition set to 20 ms. Data processing was performed using Analyst 1.4.2 software and peptide signals were integrated using Analyst classical algorithm.

2.5. Evaluation of the isotopically labeled mAb as internal standard

2.5.1. Sample preparation

Sample preparation was similar as previously described (see Section 2.4) but instead of spiking a digest of the labeled mAb, the labeled and the unlabeled mAb were digested together. In each tube the ratio between unlabeled and labeled protein was of 4.9 (53.8 μg of unlabeled and 11.08 μg of labeled). Trypsin/protein ratio was kept to 1/30 by adding 4.33 μL of a 0.5 μg/μL of proteomic grade trypsin solution. Digestion by thermal transfer at 37 °C was compared with digestion assisted by microwave irradiation at 40% of power. Six time points (2, 5, 10, 15, 30, and 60 min) were analyzed in triplicate. Digestions were stopped by adding 1 μL of pure formic acid. The sample was immediately placed in ice water and subsequently frozen at −20 °C.

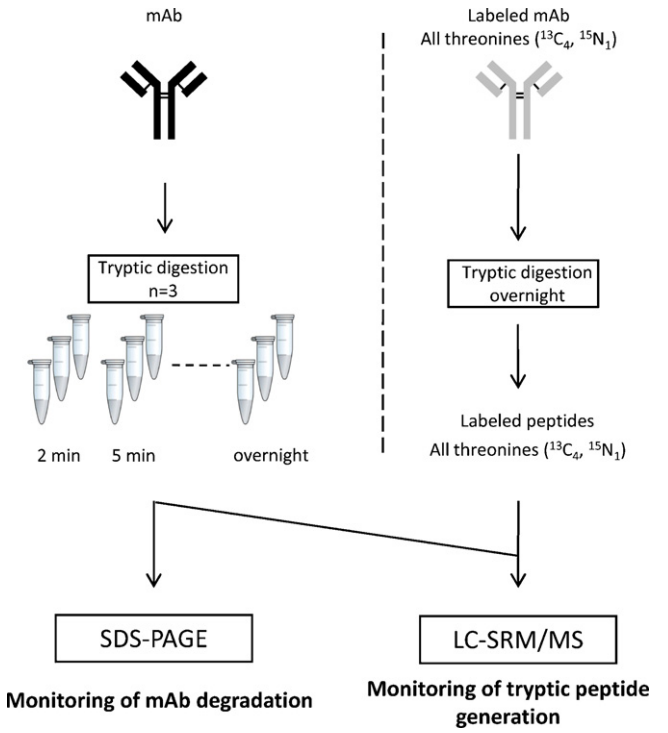


Fig. 1. Analytical setup for monitoring the disappearance of the protein intact form and the formation of the tryptic peptides.

2.5.2. LC-SRM/MS analysis

Digests were diluted three times with formic acid 0.1% prior to injection. Samples were injected onto a 1.0 mm ID × 150 mm, 3.5 μm Zorbax SB-C18 column (Agilent Technologies) with the same LC–MS/MS system described previously. Elution was performed by a gradient of 5–50% B in 20 min and flow rate was set at 80 μL/min. Peptides were monitored by the same SRM method previously built.

2.6. Evaluation of rapid digestion of the mAb spiked in human plasma

2.6.1. Sample preparation

Human plasma was centrifuged before spiking for 10 min at 14,171 × *g* for removing lipids and insoluble particles. 50 μL of this centrifuged plasma was then spiked with the isotopically labeled IS (intact form) at a constant concentration of 50 μg/mL together with the unlabeled mAb, which was spiked at various concentrations ranging from 20 to 1000 μg/mL. Aliquots of 125 μL of 50 mM ammonium bicarbonate buffer and 25 μL of 100 mM dithiothreitol were added. After agitation and brief centrifugation, spiked plasmas were reduced for 45 min at 60 °C. After cooling the samples at room temperature, 25 μL of 200 mM iodoacetamide was added. The reduced plasmas were incubated in the dark for 30 min at room temperature for the alkylation step.

2.6.2. Microwave-assisted tryptic digestion

Non-proteomic grade Trypsin X-IS was dissolved in 1 mM HCl to a final concentration of 10 μg/μL. A volume of 12 μL of trypsin solution was added to the reduced/alkylated plasma samples, corresponding to a ratio trypsin/protein (w/w) of approximately 1/30 (considering an average protein concentration in human plasma at 70 mg/mL). Samples were agitated, briefly centrifuged and immediately submitted to microwave irradiation for 15 min at 40% of power. After incubation, digestion was stopped by the addition of 2.5 μL of pure formic acid and samples were cooled in ice.

2.6.3. Sample clean-up by acetonitrile precipitation

A volume of 400 μL of acetonitrile was added in each sample in order to precipitate the remaining undigested protein. After vortex-mixing, the tubes were submitted to centrifugation for 10 min at 14,171 × *g*. Supernatants were collected and evaporated in a vacuum centrifuge (Uniequip, Germany) until a volume of approximately 5–10 μL. 70 μL of acetonitrile/water/formic acid (15/85/0.1; v/v/v) were added in each tube. Samples were agitated for 5 min and subsequently sonicated for 5 min in a water bath. Samples were finally centrifugated for 5 min at 14,171 × *g* in order to remove any residual insoluble materials.

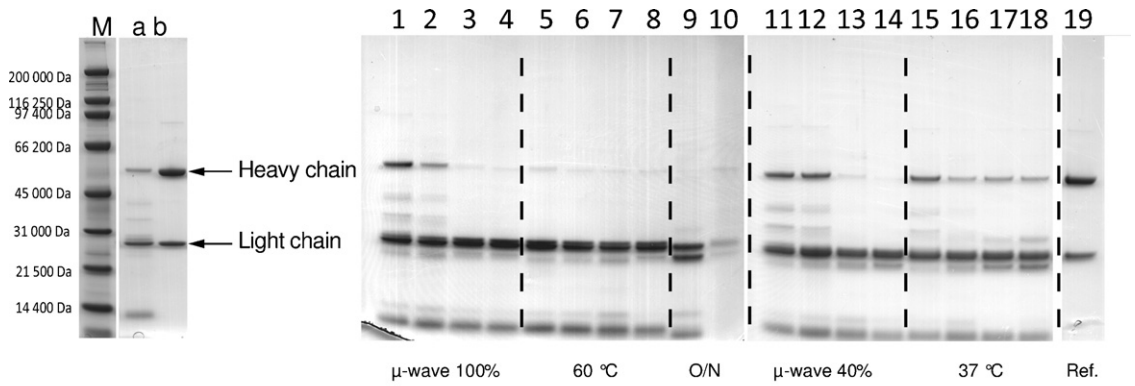
2.6.4. LC-SRM/MS analysis

25 μL of samples was analyzed on the previously described LC–MS/MS system with a separation on a 2.1 mm ID × 50 mm, 3 μm YMC-Pack Octyl C8 column (YMC). Elution consisted in 5.2 min isocratic mode at 15% B, followed by a linear gradient of 15–50% B in 16 min at a flow rate of 250 μL/min. The four most intense transitions of the triply charged ion of the signature peptide (CDR3-HC; $[M+3H]^{3+}$, *m/z* 734.7) were used for the detection and the quantification of the protein; (*m/z* 734.7 → *m/z* 879.5; *m/z* 734.7 → *m/z* 780.4; *m/z* 734.7 → *m/z* 679.4; *m/z* 734.7 > *m/z* 580.3). Collision energy was set to 105 eV and the transition dwell time at 150 ms. Data were acquired and processed by Analyst 1.4.2 using Analyst classic algorithm.

3. Results and discussion

The benefit of the use of accelerated microwave-assisted tryptic digestion has already been extensively reported [25]. In most of the described studies the aim was to reduce the digestion time as well as to increase the protein sequence coverage. Performance of the improved procedures was mainly monitored using peptide mass fingerprint (PMF) or LC–MS/MS. In this work, we have investigated from a bioanalytical perspective, the tryptic digestion kinetic of a therapeutic mAb at 37 °C and 60 °C using a heating plate device (Thermomixer) and a domestic microwave oven. The ultimate goal was to investigate accelerated digestion of large plasma sample sets for therapeutic and biomarkers proteins. The mAb intact form disappearance was monitored by SDS-PAGE while the peptides formation was followed by LC-SRM/MS. Because the digestion process is not fully quantitative the use of an internal standard is mandatory. A homolog of the protein, an isotopically labeled form of the protein (^{13}C, ^{15}N) or a tryptic cleavable peptide [28,29] can be used as internal standard. Isotopically labeled internal standards have been used for a long time for the quantitation of pharmaceuticals compounds by LC–MS/MS and have demonstrated their benefits to compensate for sample preparation losses and matrix effects. For the quantitative analysis of proteins by LC-SRM/MS, one or two signature peptides are selected as surrogates for the analyte and generally a stable labeled form of signature peptide is used as IS. In our case a stable isotope (i.e. $^{13}C_4$, $^{15}N_1$ threonine) labeled version of the protein was utilized as internal standard [30].

In the case of the LC–MS analysis endogenous compounds are known to affect the ionization. Because, the sample background may vary at different digestion time points an approach was applied, which uses the labeled form of the peptides as internal standard to compensate the potential matrix effects (Fig. 1).



Lanes

M: molecular weight marker	**1** µwave 100% pw ; 2 min	**11** µwave 40% pw ; 2 min
a: partially digested mAb	**2** µwave 100% pw ; 5 min	**12** µwave 40% pw ; 5 min
b: undigested mAb	**3** µwave 100% pw ; 10 min	**13** µwave 40% pw ; 15 min
	4 µwave 100% pw ; 15 min	**14** µwave 40% pw ; 30 min
	5 heating plate 60°C; 2 min	**15** heating plate 37°C; 2 min
	6 heating plate 60°C; 5 min	**16** heating plate 37°C; 10 min
	7 heating plate 60°C; 10 min	**17** heating plate 37°C; 30 min
	8 heating plate 60°C; 15 min	**18** heating plate 37°C; 60 min
	9 heating plate 37°C; overnight	**19** undigested mAb (ref)
	10 heating plate 60°C; overnight	

Fig. 2. Influence of the digestion duration for microwave-assisted and thermal transfer digestion methods monitored by SDS-PAGE.

3.1. Evaluation of the mAb intact form disappearance by SDS-PAGE

Four representative time points (from 2 min to overnight) for each digestion protocol were analyzed by SDS-PAGE (Fig. 2). At a first glance, strong bands are observed around the 25–30 kDa region corresponding to the mAb light chain migration. This suggests that a large amount of mAb remains incompletely digested. Similar observations have been reported with the detection of intermediate fragments of BSA after tryptic digest [25]. While disappearance of the heavy chain is observed, no significant decrease of the signal intensity is measured in the light chain mass range (Fig. 2). Parts of the degraded heavy chain may co-migrate with the remaining light chain impeding the evaluation of its own degradation. However considering only the digestion of the mAb heavy chain, the microwave-assisted digestion (at 40% and 100% of the power) shows similar efficiency within 15 min as an overnight digestion at 37 °C. Concerning the thermal transfer digestion approach, a temperature of 60 °C (lanes 5–8) is obviously more effective than 37 °C (lanes 15–18) since the heavy chain band has nearly disappeared after 2 min at 60 °C whereas it remains present after 1 h at 37 °C. High temperature digestion (60 °C) has been described as a good compromise between rapid proteolysis and a low denaturation rate of the trypsin [18] but this approach has been rarely used in routine protocols. As a matter of fact, SDS-PAGE allows to follow the disappearance of the protein but does not provide reliable information about the digestion performance because unspecific cleavage (i.e. non-tryptic cleavage) of trypsin fragments such as pseudotrypsin [31,32] or even degradation of the generated protein fragments in non-tryptic peptides cannot be excluded.

In addition, the relevance of the information provided by the gel could be affected by the losses of analytes occurring during sample preparation [33] particularly when high temperatures are applied. Therefore, we suggest monitoring the enzymatic digestion efficiency by a direct measurement of the generated tryptic peptides by LC-SRM/MS technique instead of monitoring the protein degradation.

3.2. Monitoring of peptides formation by LC-SRM/MS

3.2.1. Signature peptide selection for quantification in human plasma

IgGs are among the most abundant proteins (10 mg/mL) in plasma with albumin [2] and possess a very conserved domain. The uniqueness of peptide sequences chosen for quantification is a critical factor and particularly for IgGs in complex biological matrices such as plasma. A sequence alignment approach using BLAST-P on predicted tryptic peptides was chosen for the evaluation of peptides uniqueness. The alignment was performed without any taxonomic restriction against all non-redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF excluding environmental samples from WGS projects database (January 24, 2008). Only three peptides were identified as unique and all come from the hypervariable region including at least a part of the complementary determining region (CDR) of the heavy chain: [CDR1] 20-LSCAASGFTFS [VYGMN]WVR-37, [CDR2] 44-GLEWVA[IIWYDGDNQYYADSVK-65 and [CDR3] 102-TGP]FDYWGQGTLVTVSSASTK-132. The best candidate must include at least one threonine residue because the internal standard is isotopically labeled on this amino acid. Moreover peptides containing amino acids such as C and M, which are subjected to chemical modifications, are rejected. Regarding those criteria 102-TGPFDYWGQGTLVTVSSASTK-132 remains the best peptide for further quantification of the mAb in plasma.

3.2.2. Comparison of the tryptic peptides generation under microwave irradiation or thermal transfer

Ideal conditions using proteomics grade trypsin and purified mAb were selected for providing the ability to monitor most of peptides with the less specificity or enzyme degradation issues as possible. Fig. 3 shows the formation of the signature peptide under microwave irradiation or by thermal transfer. A plateau was reached after 15 min with both methods. At this time point the peptide signature concentration was equivalent to the concentration obtained for the 37 °C overnight digestion. However, an overnight

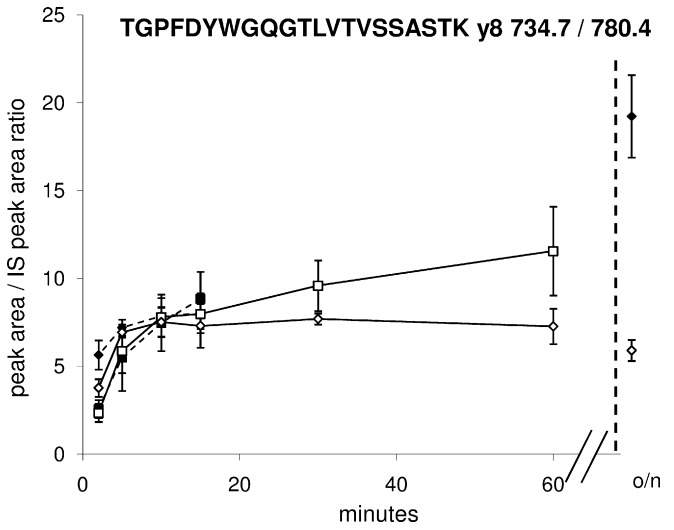


Fig. 3. Influence of the mAb tryptic digestion duration of the signature peptide with microwave-assisted (40% of power in white squares solid line, 100% of power in black squares dashed line) and with thermal transfer (37 °C in white diamonds solid line, 60 °C in black diamonds dashed line). Each point represents the average and standard deviation of three independent digestions (peak area ratio between the peptide and its isotopically labeled form of the peptide).

digestion at a higher temperature (60 °C) increases the peptide concentration of about 70% in comparison with 15 min digestion periods. For quantitative LC-SRM/MS purposes, the extended digestion duration would affect the limit of quantitation by a factor of only 2–3, but the sample preparation time would increase by a factor of about 36 and would be detrimental for the analytical throughput.

The heating mechanism is different for the domestic microwave oven and for the thermal transfer (e.g. by using a Thermomixer). In one case the energy transfer is done by irradiation and in the other case it is done by conduction/convection. For the microwave technique it is quite difficult to differentiate between the temperature effect and the microwave irradiation effect in the digestion of the protein. Therefore, temperature measurements have been performed in triplicate on a sample (115 μL of 50 mM ammonium bicarbonate) previously thermalyzed at 21 °C. After 30 min of microwave irradiation (40% power) the temperature in the tubes rise to 65 °C while only 10 min were necessary (100% power) to raise the temperature up to 70 °C. Also with the microwave irradiation, the sample volume is critical and the conditions need to be optimized for each situation. For example with the same irradiation power a 1 mL sample is heated more quickly than a 100 μL sample. With small sample volumes (115 μL), heating plates under agitation remains the best way for thermal transfer. A little more than one minute is sufficient to reach 60 °C or 37 °C with the Thermomixer. With the heating plate the control of the sample temperature is much faster and is not volume dependant as for the microwave irradiation technique.

The formation of the various tryptic peptides, as illustrated in Fig. 4, shows different patterns that can be classified into three main categories. The first category is represented in Fig. 4a where the digestion assisted by microwave irradiation (40% of the power) or by thermal transfer (37 °C) generates a maximum peptide concentration after 30 min. Extended digestion duration in particular for the microwave irradiation would degrade the peptide. This trend of degradation at longer digestion duration was also observed for the thermal transfer at 60 °C and for the microwave-assisted digestion at 100% of power. For an overnight digestion at 60 °C no peptide could be measured suggesting a total degradation.

The second category (Fig. 4b) includes peptides where higher temperatures significantly accelerate the peptide formation.

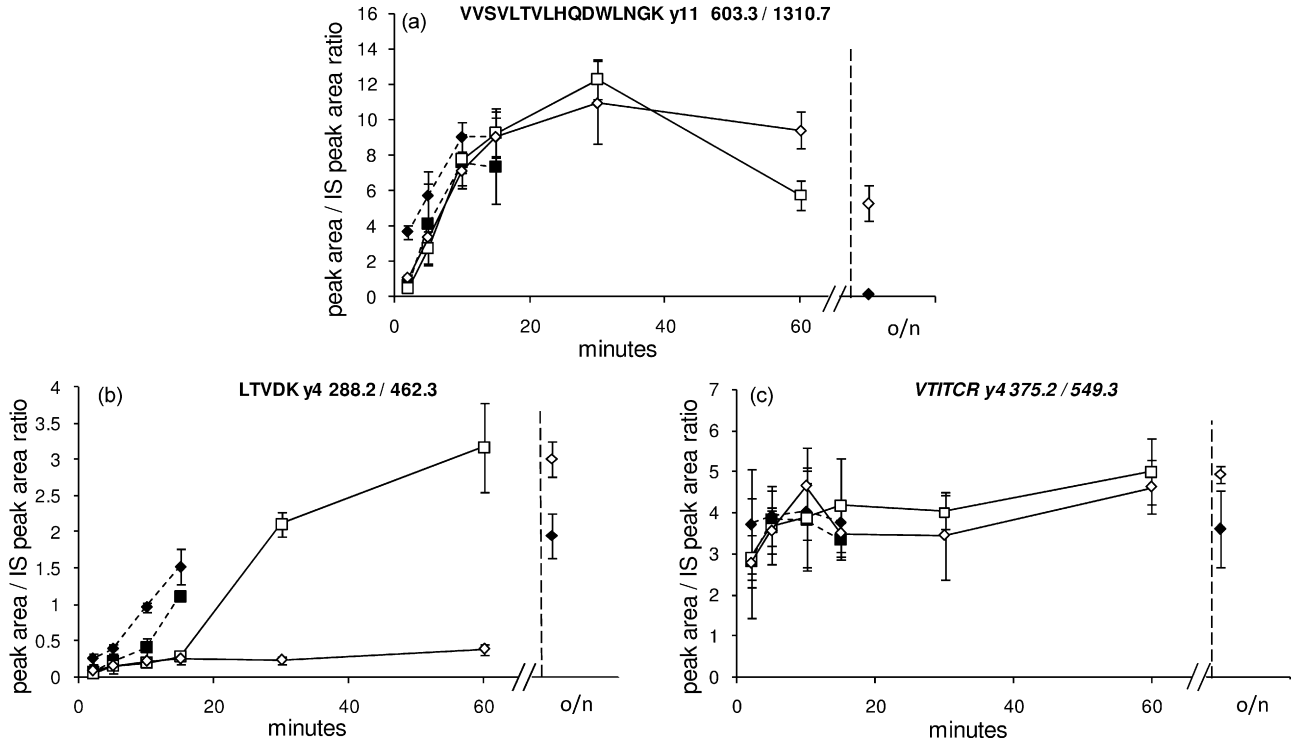


Fig. 4. Influence of the mAb tryptic digestion for the generation of three different peptides with microwave-assisted and thermal transfer (40% of power in white squares solid line, 100% of power in black squares dashed line) and on thermal transfer digestion (37 °C in white diamonds solid line, 60 °C in black diamonds dashed line) of tryptic digestion of mAb. Each point represents the average and standard deviation of three independent digestions (peak area ratio between the peptide and its isotopically labeled form of the peptide) (peptides in italic are from the light chain).

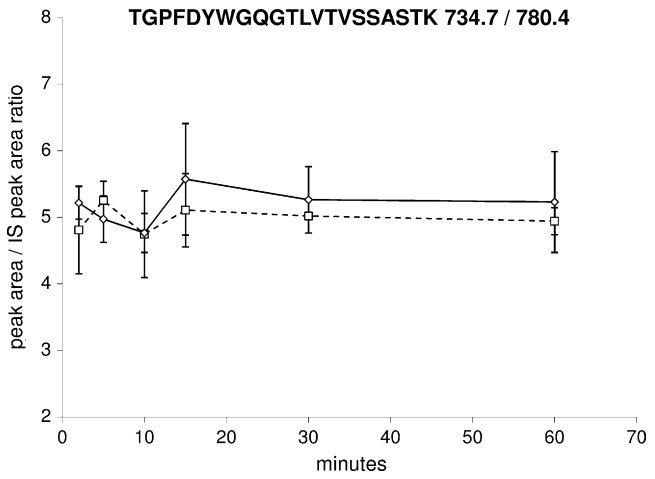


Fig. 5. Influence of the digestion duration on the signature peptide area/IS peptide area ratio when unlabeled and isotopically labeled mAb were digested together. White squares dashed line represent microwave-assisted digestion at 40% of power and white diamonds solid line correspond to the heating plate set to 37 °C. Each point represents the average and standard deviation of three replicates of peak area ratio between the peptide and IS (labeled form of the peptide). The theoretical ratio between the unlabeled and the labeled protein is of 4.9.

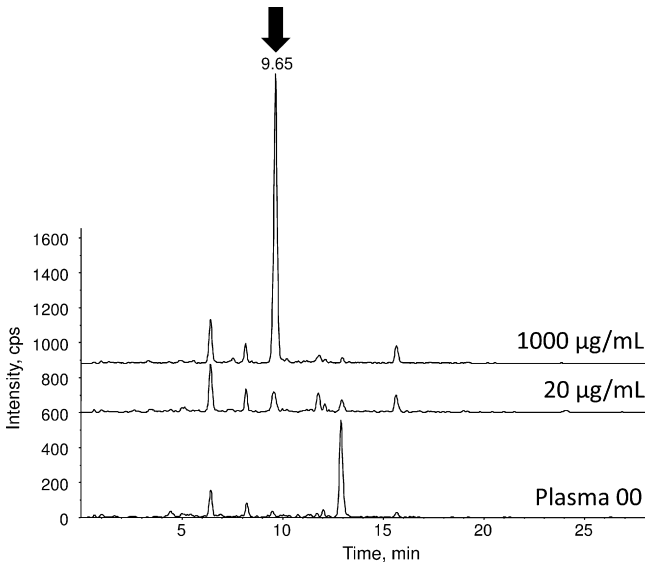


Fig. 6. SRM traces showing the *m*/*z* transition 734.7/879.5 from the 3⁺-ion of the signatures peptides for 20 and 1000 µg/mL and double blank plasma sample. The arrow indicates the signature peptide retention time.

Indeed, microwave-assisted digestion (40% of the power) shows the similar kinetic than the thermal transfer digestion at 37 °C up to 15 min, afterwards the concentration rise significantly. This increase was already observed after 10 min for the microwave irradiation at 100% of power and after 5 min for the thermal transfer at 60 °C, confirming that temperature accelerates the digestion kinetics.

The last category (Fig. 4c) includes peptides, where the formation kinetic of the peptide is not dependent on the digestion protocol. The peptide is quantitatively formed within 5–10 min and its concentration remains stable over time. The kinetic profiles of the other peptides are illustrated in supporting information Fig. C1.

High temperatures provided by convection or microwave irradiation can accelerate significantly the generation of tryptic peptides. Equivalent or slightly better peptide concentrations could be obtained within the first 30 min of digestion independently of the heating method compared with overnight digestion.

3.3. Isotopically labeled protein as internal standard for digestion

In most LC-SRM/MS quantitative assays one measures the ratio between an analyte and its internal standard. The co-digestion of mAb with its isotopically labeled form, used as internal standard, should compensate for protein losses during sample preparation and uncompleted digestion [30]. A screening of the accuracy and the precision of peak area/IS peak area ratios for selected peptides was performed. Most of the peptides including the signature peptide (Fig. 5), provide independently of the heating method a stable expected ratio of 4.9 throughout the digestion process. This finding confirms that peptides are generated from both the labeled and the unlabeled version of the mAb at the same rate. This is particularly important because it provides the possibility to quantify the mAb independently of the digestion yields. However, for ca. 20% of the peptides (i.e. 4 out of 18) higher or lower ratios than 4.9 were measured. This observation may be explained by interferences in the SRM transitions of these peptides from digestion by-products (see supporting information Fig. C2). The precision (variation of peak area ratios) of the 15 min microwave-assisted digestion (at 40% power) was investigated five times at three occasions. For the signature peptide, the accuracy of the peak area ratios in the different set (three times, $n = 5$) was of 97.4% and the relative coefficient of variation between was of 10.5%.

3.4. Feasibility of quantification in human plasma

Finally, as proof of concept, a calibration curve of spiked plasma samples (50 µL, 20–1000 µg/mL) was analyzed with the accelerated microwave-assisted digestion protocol using the protein labeled IS. The application of the labeled mAb as IS for LC-SRM/MS absolute quantification of the mAb was previously described by Heudi et al. [34] using a classical overnight digestion. In the present investigation 15 min of microwave-assisted digestion with non-proteomic grade trypsin were applied. The "non-proteomic grade" trypsin is not protected against autolysis by dimethylation and the manufacturer does not claim any TPCK treatment against chymotryptic activity. This "non-proteomic grade" trypsin is economically advantageous (12,000-folds cheaper than the proteomics grade trypsin) indeed, the high amount of protein in plasma required significantly more trypsin than a classical proteomics sample. Regardless of using autolysis unprotected trypsin, the digestion of the mAb spiked in human plasma at five concentrations levels (20, 100, 400, 800 and 1000 µg/mL) showed a linear response. Representative selected reaction monitoring chromatograms are shown in Fig. 6. The numerical analysis was performed using a linear area calibration ($y = ax + b$) with $R^2 = 0.997$. The accuracy of each standard is comprised between 89 and 110%. It is clear that sensitivity of the assay can be enhanced with better chromatographic conditions and further sample clean-up. In addition, the SRM channel used, *m*/*z* 734.7 → *m*/*z* 879.5 was less intense (at least twice in term of peak height) than the SRM channel *m*/*z* 734.7 → *m*/*z* 780.4 which showed inferences in the present chromatographic conditions. Heudi et al. have described that a reduction of sample complexity after digestion using solid phase extraction is essential for minimizing ion suppression during the ionization process. In the other hand, the removal of the most abundant plasma proteins, such as albumin could also reduce the complexity of the sample before digestion and reduce the amount of trypsin needed [35].

4. Conclusion

For the analysis of large batches of samples (hundred) overnight digestion of the mAb could be considered as time overkill. In most

case conventional digestion conditions (37 °C provided by thermal transfer or microwave irradiation) are sufficient to generate a peptide concentration equivalent to the overnight digestion within the first 30 min. Nevertheless some peptides benefit of high temperatures provided by microwave irradiation or by convection heating for a rapid digestion. Regarding the choice of the digestion system, temperatures measurements have shown that the thermal transfer (e.g. with a Thermomixer system) may be for small sample volumes more advantageous than a domestic microwave oven. The accelerated digestion protocol using a low cost trypsin could be applied to measure by LC-SRM/MS a calibration curve of mAb spiked in human plasma using the isotopically labeled protein labeled as IS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2009.11.011.

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