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Improved detection specificity of plasma proteins by targeting cysteine-containing peptides with photo-SRM

*Quentin Enjalbert^{a,b,c}, Marion Girod^{a,c}, Romain Simon^{a,c}, Jérémy Jeudy^{a,c}, Fabien Chirot^{a,c},
Arnaud Salvador^{a,c}, Rodolphe Antoine^{a,b}, Philippe Dugourd^{a,b*}, Jérôme Lemoine^{a,c*}.*

^a Université Lyon, F-69622, Lyon, France ;

^b CNRS et Université Lyon 1, UMR5306, ILM.

^c CNRS et Université Lyon 1 UMR 5280, ISA

⁴To whom correspondence should be addressed:

Jérôme Lemoine: jerome.lemoine@univ-lyon1.fr

ABSTRACT

Targeted mass spectrometry using Selected Reaction Monitoring (SRM) mode has emerged as an alternative technology to immuno-assay for protein quantification owing to faster development time and high multiplexing capability. However, SRM strategy faces the high complexity of peptide mixtures after trypsin digestion of whole plasma or cellular proteome that irretrievably causes most of the time contaminations by interfering compounds in the monitored transition channels. This event becomes more and more acute as the targeted protein is present at a low concentration. In the present work, the merit of laser induced photo-dissociation in the visible range at 473 nm implemented in an hybrid quadrupole linear ion trap mass spectrometer (photo-SRM) was evaluated towards the detection specificity of cysteine-containing peptides of a panel of plasma proteins after tagging with a DABCYL chromophore. Compared to conventional SRM, photo-SRM chromatograms depict improved detection specificity for most of monitored peptides. The comparison of the signals obtained for best proteotypic peptides in SRM mode and those recorded by photo-SRM on cysteine-containing peptides for the same proteins shows either increased (up to 10 fold) or similar signal with photo-SRM detection. Finally, photo-SRM demonstrates extended response linearity across a calibration curve obtained by diluting human plasma in rat plasma, down to the lowest concentrations. Hence, photo-SRM may advantageously complement conventional SRM in assaying proteins in complex biological matrices.

Keywords

Photo-SRM, cysteine-containing peptides quantification, chromophore derivatization, plasma proteins.

Introduction

Accurate quantification of proteins and peptides in complex matrices such as plasma, serum or urine is currently a key issue, particularly with the growing interest for clinical evaluation of candidate biomarkers identified during the proteomic discovery phases [1]. Until recently, this critical evaluation step relied primarily on immunoassays, such as ELISA, thanks to their specificity toward the target analyte and the sensitivity (pg of proteins per mL of plasma). However, the development of a new immunoassay is very expensive because it is often long and tightly dependent of specific antibody discovery [2,3]. Mass spectrometry has recently emerged

as an alternative method to immunoassay for quantitative proteomics approaches. Targeted quantification carried out with triple quadrupole instrument relies on the monitoring in the so-called Selected Reaction Monitoring mode (SRM) of surrogate peptides following trypsin digestion of the protein pool [4]. The value of SRM-based absolute quantification has been now widely illustrated for numerous relatively abundant plasma proteins or few weakly concentrated biomarkers and good correlations with the referent immunoassays have been demonstrated [5-10]. It remains that, unless extensive depletion of the more abundant protein, peptide fractionation or immuno enrichment [11,12], the current sensitivity of SRM does not provide the access to the lowest limit of quantification (LOQ) required for the reliable quantification of most clinically relevant biomarkers (pg of proteins per mL). This limitation is the consequence of the huge dynamic range of protein concentrations in biological extracts spanning 12 orders of magnitude in human plasma while only 4 to 6 orders are measurable in a whole sample. The second reason arises from the great complexity of a non-fractionated tryptic digest where the likelihood of co-eluted interfering compounds sharing similar SRM transitions with the targeted peptide is high.

Recently, instrumental and methodological developments brought partial responses to improve the monitoring of peptides in non-fractionated proteomic extracts. For instance, the ion funnel technology combined with a multi-capillary inlet enables in average a ten-fold increase of SRM peak intensities [13]. In an attempt to improve the detection specificity, we recently documented the value of a new technique called MRM-cubed (MRM³) [14,15]. By adding an additional MS³ filtering stage, an outstanding low LOQ of about 5 ng/mL was demonstrated for the Prostate Specific Antigen model of biomarker in clinical samples of plasma without any extensive fractionation. An alternative to overcome the hurdle of tryptic digest complexity and dynamic range would consist in targeting only a limited subset of the original peptide mixture, by considering only the rare cysteine-containing peptides. Indeed, cysteine (Cys, C) is present in almost all human proteins (89.3% harbor at least one), but only 17.2% of tryptic peptides contain one or more cysteine residues [16]. Beside, Cysteine is also among the most reactive amino acids through the strong reactivity of the side-chain thiol group towards oxidizing or alkylating reagents. This weak abundance has been judiciously exploited to help for proteomic discovery of differentially expressed and/or minor proteins [17]. The probably best accomplished well-known isotope coded affinity tag reagent (ICAT) [18] bears for instance a thiol reactive group and biotin to facilitate selective enrichment on avidin beads.

Natural or induced optical properties of biological molecules like fluorescence are also interesting filters to extract specific signals from the bulk compounds present in the surrounding cellular matrix [19].

In the field of mass spectrometry, significant contributions by numerous groups have highlighted for years the value of photon absorption in the UV or IR wavelength ranges to induce dissociation of peptides and proteins [20-24], such as the use of ultraviolet photo-dissociation at 266 nm to selectively cleave disulfide bonds in the gas phase [25], sugars [26-28], oligonucleotides [29-33] or small organic compounds [34]. Specific optical properties can be induced in proteins or peptides following chromophore-derivatization [35,36]. In a preliminary work, we have demonstrated that a new method called photo-SRM could be a valuable option to improve the detection specificity in SRM mode [37]. Following peptide derivatization with a chromophore and substituting the conventional collision-induced dissociation (CID) with laser-induced dissociation (LID), the limit of detection of chromophore-tagged oxytocin peptide spiked in a whole trypsin digest of human plasma was extended to lower concentration range. Similarly, the group of Brodbelt described favoured cleavages induced by 10.6 μm photons of phosphorylated species that help to discriminate between phosphorylated and non-phosphorylated peptides [38] or lipid A structures [39].

In the present work, we evaluate the merit of photo-SRM implemented in a hybrid quadrupole linear ion trap instrument towards proteins detection specificity. We report the detection and quantification of 6 proteins of high to medium abundances. For about half of the proteins, the signal intensity provided by photo-SRM targeting cysteine-containing peptides tagged with a dabcyl group is similar or increased when compared to the signal measured by CID-SRM tracking the best proteotypic peptide. Hence, the ease of photo-SRM implementation in a commercial triple quadrupole instrument makes this technique complementary for the challenges posed by multiplexed quantification of proteins in biological fluids or any other compounds with specific absorption properties.

Material and methods

Chemicals and reagents.

Acetonitrile (ACN), methanol (MeOH) and water (LC–MS grade) were obtained from Fisher Scientific (Strasbourg, France). Dithiothreitol (DTT), iodoacetamide (IAM), formic acid (FA) (LC–MS grade), trypsin (type IX-S from Porcine Pancreas), ammonium bicarbonate (AMBIC), tris(2-carboxyethyl)phosphine (TCEP), ammonium acetate (AA) were purchased from Sigma–Aldrich (St Quentin-Fallavier, France). DABCYL C2 maleimide was purchased from AnaSpec (Fremont, CA). Human serum was obtained from Etablissement Français du Sang. Rat plasma was obtained from Charles River Laboratories (L'Arbresle, France). Prostate Specific Antigen (PSA) proteotypic peptide (IVGGWECEK) was synthesized by Millegen (Labège, France).

Instrumentation.

SRM and Photo-SRM analyses were performed on an 4000 QTRAP® mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with a Turbo V™ ion source coupled to an Agilent 1290 series high pressure liquid chromatography (Agilent technologies, Waldbronn, Germany). A schematic of the photo-SRM set-up is given in Scheme S1 in Online Resource. A quartz window was fitted on the rear of the MS instrument chamber to allow the introduction of a laser beam. The laser is a 473 nm continuous wavelength laser (cw) (ACAL BFI, Evry, France). The output power of the laser is tunable from 0 to 500 mW and its beam diameter is 1.5 mm (divergence 1 mrad). The laser beam passes through one diaphragm and is injected in the triple quadrupole instrument using two mirrors. To avoid fragmentation in Q1 and Q3, the laser beam is slightly off-axis ($\sim 0.2^\circ$) as explained in the proof of concept article [37]. For photo-SRM analyses, the continuous laser is permanently turned on.

MS/MS experiments in data dependent scan were done using a 5500 QTRAP® mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with a Turbo V™ ion source coupled to an Agilent 1290 series high pressure liquid chromatography (Agilent technologies, Waldbronn, Germany).

HPLC separation.

For chromophore-derivatization-condition experiments in photo-SRM and SRM, the HPLC separation was carried out on a XBridge C₈ column (100 X 2.1 mm, 3.5 μ m) from Waters. The HPLC mobile phase consisted of water containing formic acid 0.1 % (v/v) as eluent A and ACN containing formic acid 0.1% (v/v) as eluent B. Elution was performed at a flow rate of 300 μ L/min with an analysis time of 65 min. The elution sequence included a plateau with 87 % of eluent A for 2 min followed by a linear gradient from 87 % to 62 % over 45 min, then a plateau at 0 % of eluent A for 8 min. The gradient was returned to the initial conditions and held there for 10 min. The injection volume was 20 μ L in order to inject 2 μ L of serum equivalent. For MS/MS experiments the same HPLC conditions as photo-SRM were applied.

For SRM analyses of common proteotypic peptides, the HPLC separation is described in the Electronic Supplementary Material (ESM).

Mass Spectrometry Operating Conditions.

For photo-SRM and SRM experiments on the cysteine-derivatized peptides, the following conditions were found to be a compromise in order to detect the largest number of tagged peptides from human serum. Ionization was achieved using electrospray in the positive ionization mode with an ion spray voltage of 5 500 V. The curtain gas flow (nitrogen), the ion source gas 1 and 2 (air) were respectively set at 10, 30 and 20 units. The Turbo ion spray source was operating at 250°C. Q1 and Q3 quadrupole resolution was adjusted $\sim 1.1 \pm 0.1$ amu. For CID experiments the collision energy was set to 40 eV for doubly charged ions and 20 eV for triply charged ions (shown in Table 1). For LID, the collision energy was reduced to 5 eV (to avoid CID). The laser power was adjusted at 500 mW. For discovery analyses, 120 SRM transitions (couple precursor ion/product ion) were simultaneously followed in SRM and photo-SRM experiments with a dwell time of 20 ms. For final analyses, 12 SRM transitions were followed in SRM and photo-SRM experiments with a dwell time of 50 ms.

For MS/MS experiments in data dependent scan, ionization was achieved using electrospray in the positive ionization mode using an ion spray voltage of 5 500 V. The curtain gas flow (nitrogen), the ion source gas 1 and 2 (air) were respectively set at 50, 50 and 40 units. The Turbo ion spray source was operating at 250°C. Q1 and Q3 quadrupole resolution was adjusted 0.7 ± 0.1 amu. Collision energy was set to 40 eV for doubly charged ions and 20 eV for triply charged ions.

Photo-SRM has been compared to classical SRM analyses on common proteotypic peptides. For these experiments, mass spectrometry operating conditions are described in the ESM.

Sample Preparations.

For experimental gas-phase photo-fragmentation spectrum and sample stability experiments, PSA proteotypic peptide was derivatized with a 3-fold molar excess solution of TCEP (0.1 mg/mL in a 60 mM AA solution, pH = 8-8.5) and a 5-fold molar excess of DABCYL C2 maleimide in MeOH (1.33 µg/mL in MeOH). The mixture was stirred in the sonic bath then stored in the dark for 4h at room temperature.

For SRM analyses of common proteotypic peptides, sample preparation is described in Online Resource.

For SRM and photo-SRM analyses, cysteine-containing proteins were derivatized with DABCYL C2 maleimide (structure shown in Fig. S1a in ESM). The whole human serum derivatization was realized as followed. 10 µL of human serum was dissolved in 3 000 µL of a 3-fold molar excess solution of TCEP (0.1 mg/mL in a 60 mM AA solution). Then, 3 000 µL of a 5-fold molar excess of DABCYL C2 maleimide in MeOH (1.33 µg/mL in MeOH) was added. The mixture was stirred in the sonic bath then stored in the dark for 4h at room temperature. The samples were diluted with 3 000 µL of a 60 mM AA solution prior to overnight digestion at 37°C with trypsin using a 1:30 (w/w) enzyme to substrate ratio. Digestion was stopped by addition of formic acid to a final concentration of 0.5%. All samples were desalted and concentrated using Oasis™ HLB 3cc (60mg) reversed phase cartridges (Waters, Milford, MA, USA). Before loading the tryptic digest onto the Oasis cartridges, all cartridges were conditioned with 1 mL of MeOH and then 1 mL of water containing 0.5% FA. After the loading, all cartridges were washed with 1 mL of MeOH/water (5/95, v/v) containing 0.5% FA and eluted with 2 mL of ACN containing 0.5% FA. All samples were evaporated to dryness and resuspended in 100 µL of water/MeOH (2:1, v/v) containing 0.5% FA. All solutions were stored at -18°C prior to use.

Standard samples for calibration curves were prepared by diluting human serum into rat plasma prior to the whole sample preparation. The human serum volume ratios were 0, 0.01, 0.02, 0.1, 0.20, 0.5 and 1.0. Each standard sample was separately derivatized, digested with trypsin and desalted as described above. Triplicates of each standard were realized. C4b Binding Protein Alpha Chain calibration curves (SRM and photo-SRM) were obtained by diluting human serum into rat plasma. The initial C4b Binding Protein Alpha Chain concentration was estimated to be 337.5 µg of protein per mL in human serum [40].

Results and discussion

The aim of the present work was to assess the value of photo-SRM, for the targeted quantification of proteins in plasma by tracking cysteine-containing peptides. In the experimental set-up, a continuous 473 nm laser beam was focused slightly off-axis in the collision cell of a commercial hybrid quadrupole linear ion trap mass spectrometer to allow analyses either in collision-induced (laser turned off) or laser-induced dissociation mode (laser turned on). The choice to irradiate in the visible range was governed by the non-absorbing property of the vast majority of natural molecules around 473 nm, which is the case for peptides and proteins. The LID mass spectrum of the unmodified PSA proteotypic peptide IVGGWECEK obtained following laser irradiation at 473 nm (see ESM, Fig. S1) does not show any product ion, which is relevant with the non-absorbing properties of proteins and peptides in the visible range. However, specific photo-dissociation is expected from the same peptide after derivatization with an adequate chromophore. As mentioned in the introduction section, the tagging of cysteine thiol group constitutes a key advantage in the quest for specificity and sensitivity considering that cysteine is a rare amino acid and that the thiol reactivity towards the maleimide functionalized chromophore is usually stoichiometric. Numerous chromophores are commercially available with a thiol-reactive group but a very few have quenching property. Among them, DABCYL C2-maleimide has a high photo-dissociation yield between 465 and 540 nm as shown by the optical spectrum of tagged PSA proteotypic peptide IVGGWECEK recorded on a linear ion trap coupled with an OPO laser [20,28] (Fig. S2a in ESM). When no product ions can be observed with the unmodified peptide after laser irradiation at 473 nm (Fig. S1 in ESM), several product ions are detected with the tagged peptide (Fig. S2b in ESM). Similar product ions are obtained with LID and CID fragmentation (Fig. S2c in ESM), and only ratios between fragments show some differences, thus there is no specific fragment obtained with either one or the other method. The most intense product ions derive from internal dissociation of the chromophore providing two complementary ions at m/z 252 and $[M+H-252]^+$ (Fig. S2d in ESM).

The completion of the chromophore-derivatization has been verified by analyzing a sample of PSA proteotypic peptide (Fig. S3 in ESM). After addition of the chromophore, any signal for the 3 SRM transitions (couple precursor ion/product ion) tracking the unmodified peptide (510.5/213.2, 510.5/693.3 and 510.5/750.3) could be observed while the 2 SRM transitions tracking the tagged peptide were detected providing that the tagging reaction is complete in these conditions. In order to assess the chemical stability of tagged IVGGWECEK peptide towards normal room conditions, a potential source of imprecision in case of spontaneous degradation, the intensity of SRM signals 707/252 and 707/1161 were monitored for three storage

conditions, i.e. room light/room temperature, room light/4°C regulated autosampler, darkness/4°C regulated autosampler. Fig. S4 in ESM shows the time course of the cysteine-tagged peptide signal stability sampled every 30 minutes over a 12h period. After 6h, the SRM signals slightly decrease by 5% and by 15% after 12h, whatever the storage conditions. This signal loss has been thus considered negligible for the following experiments in the present study. Nevertheless, the imprecision arising from this chemical degradation might alternatively be corrected for future assays by spiking an internal heavy peptide standard.

Relative performance of photo-SRM versus SRM.

We first extracted all tryptic peptides containing between 5 and 15 amino-acids residues and a single cysteine from a list of 30 concentrated human plasma proteins spanning a dynamic range of 4 orders of magnitude with the open source Skyline software (<http://proteome.gs.washington.edu/software/skyline>) [40-42]. These features filtered a set of 70 peptides. By extrapolating the above fragmentation pattern of tagged PSA peptide, a list of theoretical transitions was build considering doubly or triply protonated precursor ions for each peptide, the chromophore fragment at m/z 252 and its complementary charge reduced product ion retaining the peptide moiety, see Table S1 in Online Resource. All putative transitions were next monitored by photo-SRM towards a whole trypsin digest of human plasma sample (10 μ L) derivatized with DABCYL C2-maleimide. The derivatization was performed at the protein level in order to cleave disulfide bonds and allow a complete tryptic digestion. The analysis leads to the detection of 23 peaks showing significant signals within the two targeted transition channels. This low number of detected peptides could be explained by the low ionization yield of these peptides and the matrix complexity. The Fig. 1 shows the superimposed reconstructed ion chromatograms of most intense peaks recorded either in photo-SRM (Fig. 1a) or SRM modes (Fig. 1b) for the same derivatized sample. Compared to SRM, fewer signals are observed in photo-SRM, especially in the first half of the chromatogram where non-derivatized (more hydrophilic) peptides are expected to elute. Note that owing to a marked increase in peptide hydrophobicity after chromophore tagging, we moved from a classical C₁₈ reversed phase column to a C₈ reversed phase in order to keep enough peak capacity during the chromatographic separation. In turn, a large fraction of non-derivatized peptides are early eluted even at the weakest percentage of organic solvent, which advantageously contributes to minor both matrix effect and probability of co-eluting interferences. This first result clearly indicates that tracking cysteine-containing peptides derivatized with the DABCYL chromophore is univocally more specific when applying photo-SRM instead of SRM, as expected.

In order to validate the peptide identities, tandem CID mass spectra were recorded in data dependent scan triggered tandem mass spectrometry on all putative transitions. This experiment has been carried out on a more recent unmodified hybrid quadrupole linear ion trap mass spectrometer (5500 QTRAP® mass spectrometer) owing to its increased sensitivity in acquiring product ion spectra. Among the multiplicity of detected peaks, 7 targeted peptide sequences were confidently attributed to proteins of high to medium abundances i.e. albumin, afamin, C4b binding protein alpha chain, complement C3, isoform 1 of gelsolin, beta chain of haptoglobin and plasminogen. Based on the values reported by Anderson et al. [40], the concentrations of these proteins range from 150 µg of protein per mL for the lowest concentrated afamin protein to 50 mg of protein per mL for albumin. CID MS/MS spectra of 4 from the 7 corresponding proteotypic cysteine-containing peptides are shown in Figures 2a-d and correspond to the dissociation spectra of triply charged ions (see Fig. S5 for the 3 other CID MS/MS spectra). Unidentified ions could probably come from co-eluted compound with the same precursor ion. Though the chromophore-tagged peptides were also detected as doubly charged species in SRM-triggered MS/MS experiments, $[M+3H]^{3+}$ were mostly detected as the predominant pseudo-molecular ions. This feature likely results from the high proton affinity of the amine group carried by the DABCYL C2-maleimide quencher (Fig. S2) that introduces an additional favoured protonation site. Furthermore, the triply charged ions showed a better fragmentation efficiency and provide more informative fragmentation patterns than their doubly charged homologues and were consequently only considered for the succeeding experiments. Beside the systematic detection of a fragment ion at m/z 252 arising from the dissociation within the chromophore group, peptide sequences were established according to b_n and y_n fragment ions (y ions are much more intense than b ions). These backbone fragment ions retaining the chromophore group have been chosen to reconstruct the ion chromatograms owing to increase specificity with regard to the non-specific product ion at m/z 252 available with every tagged peptide. Note that the fragmentation parameters were not optimized to record optimal SRM signals since we did not have an access to synthetic peptides. Hence, an average collision energy of 20 eV has been chosen for all triply charged peptides while 40 eV has been chosen for doubly charged peptides for subsequent monitoring in SRM on the dedicated instrument. On the other side, we cannot neither optimize the laser power for each peptide so a value of 500 mW has been chosen for doubly and triply charged peptides.

The comparison of the relative merits of photo-SRM and CID-SRM detection selectivity for cysteine-containing peptides has been made for these 7 proteins. Figure 2e-h shows, as an example, superimposed extracted ion chromatograms of the most specific transition defined for afamin (2a), C4b binding protein alpha

chain (2b), complement C3 (2c) and gelsolin (2d) proteins by photo-SRM (black line) or SRM (red line) monitoring. Except for C4b binding protein alpha chain, all photo-SRM chromatograms (black) exhibit less interfering compounds than SRM ones (red). This is especially spectacular in the case of cysteine-tagged peptide of afamin. For this latter, it is clearly detected at $t_r = 25.04$ min in the human plasma sample (mean endogenous concentration comprises between 75 and 250 $\mu\text{g/mL}$) using photo-SRM, whilst only interfering ions are detected in SRM mode (Fig. 2e). Together, these chromatograms unambiguously highlight the higher specificity of LID mode in detecting cysteine-containing peptides grafted with a chromophore group. Having demonstrated the superior detection specificity of photo-SRM, we next wanted to compare the overall sensitivity of photo-SRM tracking cysteine peptides with regard to the sensitivity of SRM monitoring the best proteotypic peptides for the same set of proteins. From a list of SRM transitions generated by MRM pilot software tool and consistent with the literature [43], the most sensitive transition for each peptide was monitored on the same instrument used for photo-SRM for the 7 targeted proteins. Figure S6 in Online Resource shows the extracted ion chromatograms of the most intense SRM transitions obtained for each proteotypic peptide. None of the most sensitive peptides followed in SRM contain a cysteine residue thus they all differ from the cysteine-tagged peptides monitored in photo-SRM. The spectral counts of best proteotypic peptides monitored in SRM and cysteine-containing peptides tracked by photo-SRM for the 7 targeted proteins starting from 2 μL of serum are reported in Table 1. Taken as a whole, the area and the height of the different signals are in a close range of value in photo-SRM and in SRM. Nonetheless, while we can noticed a slight decrease of sensitivity for afamin, albumin, gelsolin and plasminogen moving from SRM to photo-SRM, signals are increased in turn for C4b binding protein alpha chain, haptoglobin and more especially for complement C3 showing a nearly 10 fold signal enhancement in photo-SRM mode. The retention times of tagged-peptides are much greater than the non-modified ones. Therefore, they elute in a portion of the chromatogram where the ratio of water/organic eluent is lower. Greater percentages of organic solvent lead to higher ionization efficiencies and result in greater signal. Thus, the improvement in sensitivity obtained by the photo-SRM analysis of the tagged-peptides against SRM analysis of the best proteotypic peptides could be attributed to the whole method (sample preparation, liquid chromatography and laser-fragmentation) and not only to the laser-fragmentation.

These results indicate that quantification of some cysteine-containing proteins could be achieved by photo-SRM as a comparable or even better level to classical SRM for some peptides. In other words, we cannot claim that photo-SRM is a universal substitute to conventional SRM but it should be considered in the future as a

powerful alternative or more sensitive strategy for the quantification of some candidates and should be thus evaluated during the assay development.

Response linearity.

In order to assess the linearity of the complete sample workflow including the chromophore derivatization step we diluted human serum into rat plasma prior to sample preparation. Amongst 7 validated peptides only 6 were finally monitored in the dilution experiments due to the presence of the same endogenous cysteine-containing proteotypic peptide in rat serum coming from haptoglobin. From mean concentration values for the pure human serum, six different dilutions of human serum into rat plasma, over 2 decades, were prepared and analyzed in SRM and photo-SRM modes. The specificity of all SRM transitions was first assessed for both activation modes across the analysis of blank rat plasma hydrolysates i.e. not spiked with human serum). Figure 3 shows the ion chromatograms reconstructed on the most intense transition (732/1212) monitoring the cysteine-tagged peptide of the C4b binding protein alpha chain recorded in photo-SRM (Fig. 3a) and CID-SRM (Fig. 3c). The redundancy within the monitored transition channel displayed by the chromatograms between 25 and 30 min corresponds to interfering endogenous rat serum compounds. Using CID-SRM, a weak but noticeable interference is detected at the elution time expected (indicated by an arrow) for the targeted peptide tagged with DABCYL C2-maleimide quencher (insert in Fig. 3c). In contrast, no interference is detected at this retention time moving to photo-SRM mode, which features a higher specificity of 473 nm laser excitation process with regard with CID mode.

Figures 3b and 3c show the extracted ion chromatograms recorded in photo-SRM and CID-SRM of the same SRM transition for the lowest detected concentration of the calibration curve, i.e. 6.75 µg/mL. First, as foreseen by the analysis of the blank rat serum sample, the occurrence of a co-eluted interference at 26.4 min on the CID-SRM chromatogram precludes the detection of the targeted peptide at this lowest concentration range. This is illustrated by the Y-intercept value of the chromophore tagged peptide SRM calibration curve, which is consistent with a systematic peak contamination below 67.5 µg/mL (insert in Fig. 3e). In contrast, the zero- noise baseline level of blank rat serum monitored by photo-SRM allows the detection of the targeted peptide even at this lowest concentration, hence extends the response linearity all over the investigated range of concentrations ($r^2 = 0.999$) i.e. between 6.75 and 337.5 µg/mL.

By analyzing the results from a triplicate of the full experimental sample workflow (i.e. chromophore tagging, sample digestion, SPE), accuracy for measuring the ratio between nominal and back-calculated concentrations was in the range 80-115 over all the proteins, except for one concentration point (Table 2). Similarly, imprecision values expressed by the coefficient of variation (CV) were nearly all better than 20%. This shows that the robustness and the repeatability for the whole analytical process can be validated. These statistics are somewhat acceptable in a first attempt and in line with previous reports [44-47] if one considers that we did not use in the present study heavy internal standard usually employed to adjust for instance variation in the trypsin digestion step of different matrix effect from one sample to another one.

Conclusions

In the present work, we explored the potential interest of photo-SRM for the targeted detection of cysteine-containing peptides in a whole human plasma digest after tagging with a DABCYL group absorbing at the laser wavelength i.e. 473nm. The conferred selective optical absorption property allows the introduction of a new specificity at the dissociation step by substituting the conventional collisional process by laser-induced dissociation mode in SRM. This increased specificity has been here clearly demonstrated by the dramatic decrease of interfering peaks originating from isobaric peptides and product ions. As a consequence, linearity of photo-SRM signal was conserved even at the lowest concentrations of human C4b binding protein alpha chain when human plasma was diluted in rat plasma. Across a panel of 7 human plasma proteins, the overall sensitivity of photo-SRM monitoring cysteine-containing peptides compared well with conventional SRM when tracking the best responding proteotypic peptides and was markedly improved for one of them. Hence, photo-SRM appears not as an alternative to replace conventional SRM but more like a powerful complement that in many instances equals or may improve the limit of quantitation of protein targets.

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Figure 1. LC-MS/MS chromatograms of 12 SRM transitions of cysteine-containing peptides from human serum tagged with DABCYL C2 maleimide. The photo-SRM experiment (a) shows a decrease of interferences (▼) compare to the SRM one (b). All * show the peaks corresponding to the target molecules.

Figure 2. a)-d) CID MS/MS spectra were used to confirm the sequence of the different peptides (afamin a), C4b binding protein alpha chain b), complement C3 c), gelsolin d)). All * show triply charged precursor ions of the target molecules, while the fragment ion in italic and bold are the ones followed in photo-SRM and SRM experiments (not visible for Fig. 2b) because this product ion is out of range). e)-h) LC-MS/MS chromatograms obtained by photo-SRM (black) and SRM (red) analysis of most intense specific transitions for four cysteine-containing peptides from human serum tagged with DABCYL C2 maleimide (afamin m/z 494/372 e), C4b binding protein alpha chain m/z 732/1212 f), complement C3 m/z 344/443 g), gelsolin m/z 552/784 h)). All ▼ show the peaks corresponding to the target molecules.

Figure 3. LC-MS/MS chromatograms of the most specific intense transition tracking a proteotypic peptide from the C4b binding protein Alpha chain m/z 732/1212. Photo-SRM a) and SRM c) analyses of chromophore-derivatized rat plasma (C4b binding protein Alpha chain concentration = 0 μ g/mL). Photo-SRM b) and SRM d) analyses of chromophore-derivatized human serum spiked into rat plasma (C4b binding protein Alpha chain concentration = 6.75 μ g/mL). e) Calibration curves of the most intense specific transition obtained following chromophore-derivatized proteotypic C4b binding protein Alpha chain peptide in photo-SRM (■) and SRM (▲) modes. Inset shows low concentrations data (0, 6.75, 33.75, 67.5 μ g/mL).

Protein name	Peptide SRM	Peptide photo-SRM	Collision energy (SRM/photo-SRM)	Area in SRM	Area in photo-SRM	Height in SRM	Height in photo-SRM	Retention Time in SRM (min)	Retention Time in photo-SRM (min)	Transition in SRM	Transition in photo-SRM
Afamin	DADPDTFFAK	ICAMEGLPQK	24/5+LID	1.80E+04	1.16E+04	1.08E+03	1.10E+03	7.44	25.04	564/825	494/372
Albumin	LVNEVTEFAK	CASLQK	35/5+LID	3.97E+07	5.60E+06	2.41E+06	2.00E+06	5.94	17.26	575/937	347/475
C4b Binding Protein Alpha Chain	GYILVGQAK	YTCLPGYVR	29/5+LID	1.39E+05	2.60E+05	1.05E+04	1.20E+04	5.03	26.4	629/1015	732/1212
Complement C3	TGLQEVEVK	FSCQR	25/5+LID	2.63E+05	1.60E+06	2.42E+04	1.20E+05	4.69	18.38	502/731	344/443
Gelsolin, isoform 1	TGAQELLR	SEDCFILDHGK	25/5+LID	6.51E+04	2.50E+04	5.12E+03	1.60E+03	4.7	27.6	444/530	552/784
Haptoglobin beta chain	VGIVSGWGR	VMPICLPSK	25/5+LID	1.38E+06	2.20E+06	1.01E+05	1.1E+05	5.48	28.68	491/562	460/575
Plasminogen	LFLEPTR	QLGAGSIEECAK	18/5+LID	5.78E+05	3.50E+05	4.00E+04	2.50E+04	5.86	24.05	438/615	557/242

Table 1. Data for proteotypic peptides analyzed by common SRM and photo-SRM experiments. Values show in bold are the ones better in photo-SRM than in SRM.

Afamin			Albumin			C4b binding protein alpha chain		
µg/mL	Accuracies	CV (n=3)	µg/mL	Accuracies	CV (n=3)	µg/mL	Accuracies	CV (n=3)
0	NA	NA	0	NA	NA	0	NA	NA
1.13	NA	NA	410	107	21	3.38	NA	NA
2.25	NA	NA	820	118	6	6.75	82.1	17
11.25	NA	NA	4100	109	6	33.75	95	19
22.5	104	16	8200	112	5	67.5	113	9
56.25	89	13	20500	101	4	168.75	101	14
112.5	102	10	41000	96	12	337.5	105	16
Complement C3			Gelsolin			Plasminogen		
µg/mL	Accuracies	CV (n=3)	µg/mL	Accuracies	CV (n=3)	µg/mL	Accuracies	CV (n=3)
0	NA	NA	0	NA	NA	0	NA	NA
8.25	104	9	32	NA	NA	2.7	NA	NA
16.5	94.5	18	6.4	NA	NA	13.5	NA	NA
82.5	90.4	14	32	NA	NA	27	109	8
165	88.1	10	64	102	15	54	85	21
412.5	110	4	160	99	10	135	107	6
825	113	13	320	100	20	270	98.4	15

Table 2. Analytical performance levels by photo-SRM (precision and accuracy) calculated for replicated calibration curves of cystein-containing peptides tagged with a dansyl chromophore tracking six proteins of human serum diluted into rat plasma. Accuracies are expressed as percent difference: ((concentration found - concentration added) / concentration added) X 100.

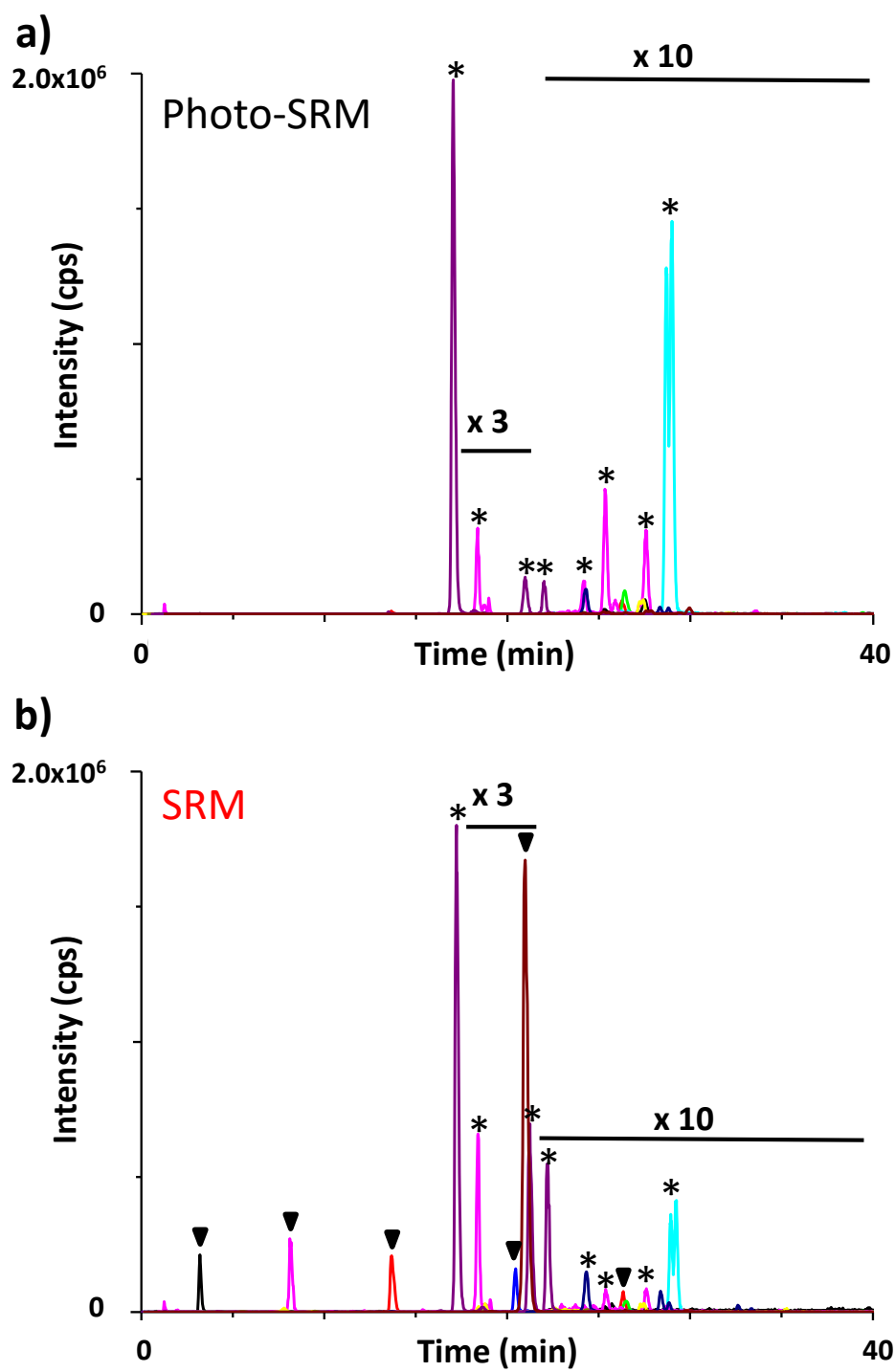


Figure 1.

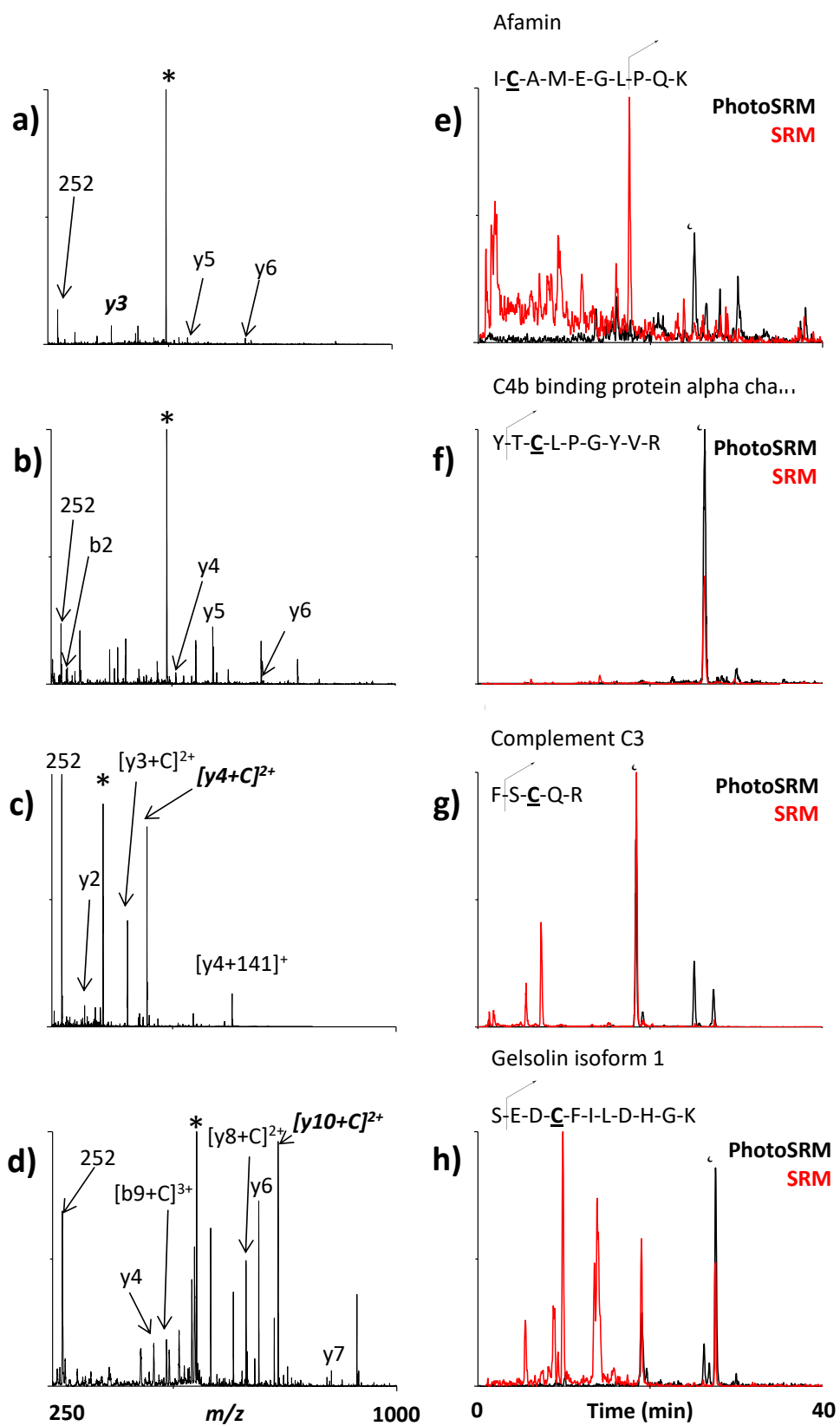


Figure 2.

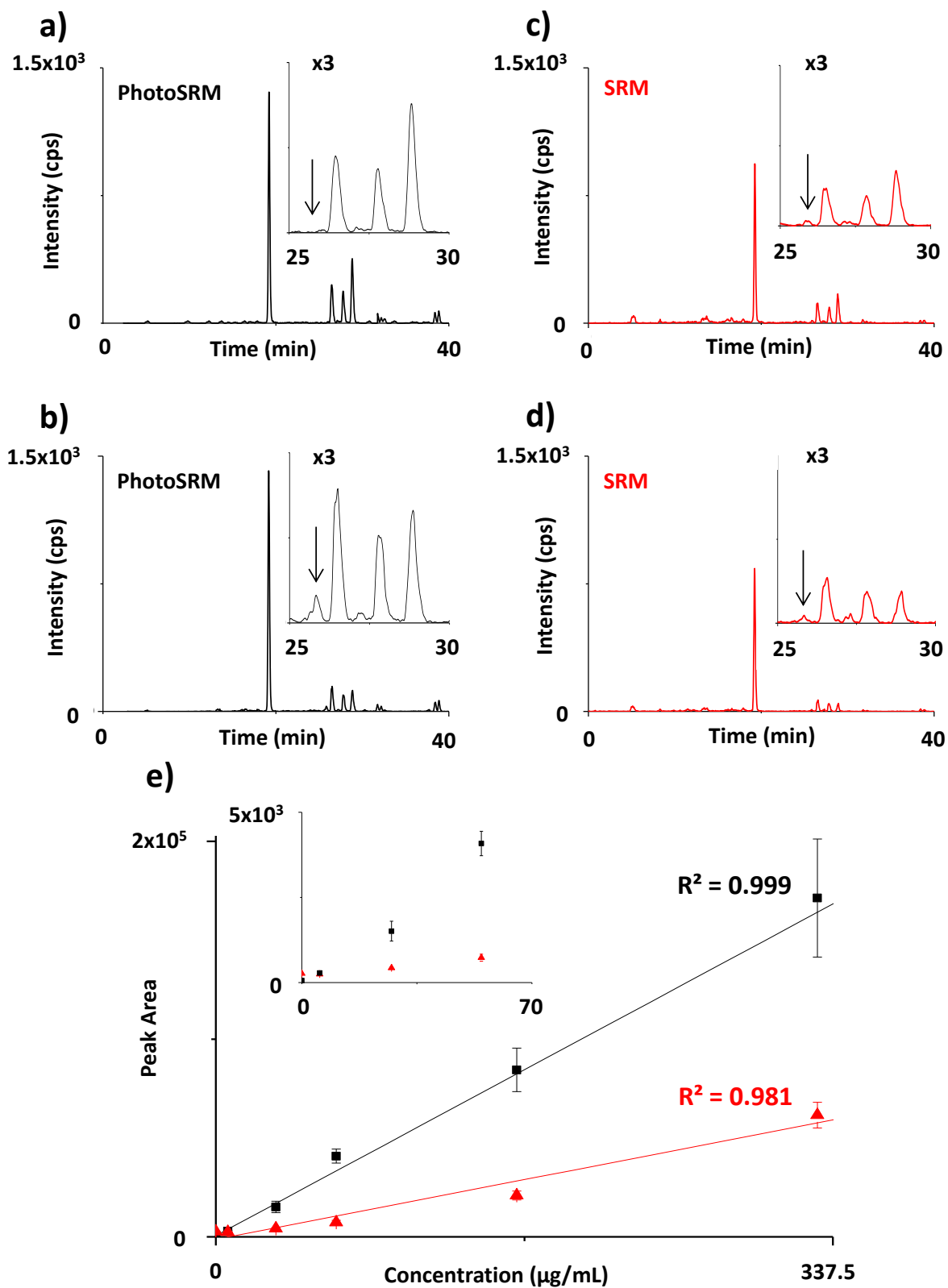


Figure 3.