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A quality control of proteomic experiments based on multiple isotopologous internal standards

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ABSTRACT

The harmonization of proteomics experiments facilitates the exchange and comparison of results. The definition of standards and metrics ensures reliable and consistent data quality. An internal quality control procedure was developed to assess the different steps of a proteomic analysis workflow and perform a system suitability test. The method relies on a straightforward protocol using a simple mixture of exogenous proteins, and the sequential addition of two sets of isotopically labeled peptides added to reference samples. This internal quality control procedure was applied to plasma samples to demonstrate its easy implementation, which makes it generic for most proteomics applications.

1. Introduction

Proteomics, with its ability to generate large data sets, has emphasized the necessity of comparing and integrating results across laboratories and platforms. The issue has gained acuteness as proteomics has shifted from qualitative to more quantitative studies. At present, there is a diversity of approaches and platforms that result in very heterogeneous data sets, whose integration remains very challenging. A first step toward the harmonization of proteomics results is the definition of methods and criteria to facilitate the systematic assessment of the analytical platform performance and the quality of the data generated. Furthermore, the preparation of samples using well-established procedures is necessary. These points have been widely recognized and several efforts have been undertaken in the past years toward the standardization of bottom-up proteomics LC-MS/MS analyses [1–11]. More specifically for quantitative analyses, proteomics can actually rely on the guidelines previously established in analytical and clinical chemistry [12,13]. While these recommendations relate to a single or a limited set of analytes, the general concepts outlined can be adopted in the context of proteomic quantitative LC-MS measurements. A recent workshop, focused on best practices for targeted analysis, has emphasized the necessity to define the purpose of the study (fit-for-purpose approach) [14].

In order to ensure the generation of reliable and consistent data sets, a comprehensive internal quality control procedure is required. It has to include the assessment of the sample preparation and the qualification of the instrument, which are combined in a validated analytical method. This provides a system suitability test, required prior to the analysis of actual samples [15]. The sample preparation method, which covers the sample handling, digestion, extraction and dilution, has to match the analytical question, the type of samples to be analyzed, and has to be reproducible across series of samples. The instrument and its associated operation method need to be specific and evaluated on test samples to assess the fulfillment of predefined requirements, in terms of analytical sensitivity (limits of detection and quantification), selectivity, precision (determined from replicated experiments), accuracy (based on the analysis of a reference material), and lastly robustness. Both the sample preparation and the instrument method need to be evaluated, first independently and ultimately in an integrated manner. A robust and validated protocol represents the basis for an internal quality control and its routine implementation. It allows the assessment of (i) the instrument performance, (ii) the sample preparation performance, and (iii) the system suitability.

A quantitative proteomics workflow needs to be specific, somehow addressing a well-defined analytical question. At present, most proteomics experiments are generic; nevertheless some level of systematic quality control is imperatively required. In

Abbreviations: LC, liquid chromatography; MS, mass spectrometry; SIL, stable-isotope labeled; SRM, selected reaction monitoring; PRM, parallel reaction monitoring; AUC, area under the curve; FWHM, full width at half maximum; CV, coefficient of variation; STD, standard deviation.

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an attempt to apprehend all the elements of a bottom-up LC-MS/MS proteomic workflow and monitor the different stages of the process, a simple protocol was designed. It allows the system suitability for routine proteomic analyses to be qualified while overcoming the rigidity of a full method validation. Rigorous quality controls remain required for systematic quantitative studies (e.g., preclinical), whereas relaxed constraints are applicable for initial screening experiments [14]. The protocol that was recently proposed to routinely assess the uniformity of proteomics analyses addresses this point [16]. It evaluates sample preparation and instrument performances concomitantly through the addition of isotopic variants of internal standards (peptides isotopically labeled with different motifs) at several stages of the workflow, corresponding to two peptides of each protein from the standard protein mixture. The analysis of these peptides in buffer enables to test the suitability of the LC–MS platforms, using acceptance/rejection metrics that were established based on long-term data collection. The systematic implementation of the protocol allows to monitor LC–MS performance overtime and to detect possible drifts or dysfunctions. It can also be employed to compare sensitivity levels of different platforms or analytical methods.

In an attempt to expand its applicability to clinical samples, the simple quality control procedure based on the sequential addition of multiple isotopically labeled peptides was tested on plasma samples. In this account, the standard protein mixture designed was spiked into several plasma samples, and the reproducibility of the overall workflow was assessed using control charts, which allows to define acceptance criteria. The quality control procedure is easily applicable in individual laboratories, and has shown a high level of reproducibility and robustness when applied to plasma samples, used as reference materials.

2. Material and method

2.1. Chemicals and reagents

Dithiothreitol, formic acid, iodoacetamide, Tris–HCl (Trizma hydrochloride), and urea were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was obtained from Promega (Madison, WI, USA). All solvents used were HPLC grade and purchased from Sigma–Aldrich.

2.1.1. Standard materials

The mixtures of three proteins and stable-isotope labeled (SIL) peptides were prepared as previously explained [16] (Supplementary Table).

2.1.2. Sample preparation

The unfolded standard protein mixture was either spiked in plasma samples or underwent the sample preparation procedure in buffer. Six plasma samples from deidentified human specimens were provided by Integrated BioBank of Luxembourg (IBBL) and treated as “not human subjects research” materials. Each plasma sample was individually mixed with the standard protein mixture (15 μL, volume corresponding to 7.5 μg of each protein) at a final concentration of 300 ng/μL. The reduction was performed with 20 mM dithiothreitol (5 mM final concentration) by incubation at 37 °C for 30 min. Then, the protein mixtures were alkylated with 75 mM iodoacetamide (15 mM final concentration) for 30 min at 25 °C in the dark before the addition of a first set of isotopically labeled peptides (H_A peptides) at a final nominal concentration of 50 fmol/μL. A solution of 25 mM Tris–HCl was used to dilute urea to 1 M and sequencing grade modified trypsin was added to a final enzyme:substrate ratio of 1:20. After an incubation of 4 h at 37 °C, peptides were cleaned on Sep-Pak tC18 cartridges (Waters, Milford, MA, USA) and eluted with 50% acetonitrile. The samples were lyophilized on a vacuum centrifuge and resolubilized in 0.1% formic acid. Prior to LC-MS/MS analysis, the mixtures were supplemented with a second set of isotopically labeled peptides (H_B peptides) at a final nominal concentration of 50 fmol/μL. In addition to the standard materials, a mixture of isotopically labeled peptides corresponding to 42 peptides from plasma was added before LC-MS/MS analysis on the quadrupole orbitrap instrument at a concentration close to that of their endogenous counterpart.

2.2. Liquid chromatography and mass spectrometry

2.2.1. LC separation

Analyses were carried out on a Ultimate 3000 RSLC nano system (Thermo Scientific). A trap column Acclaim PepMap 2 cm × 75 μm i.d., C18, 3 μm, 100 A and an analytical column Acclaim PepMap RSLC 15 cm × 75 μm i.d., C18, 2 μm, 100 A (Thermo Scientific) were used. The samples were loaded into the trap column at 5 μL/min with an aqueous solution containing 0.05% trifluoroacetic acid and 1% (v/v) HPLC grade acetonitrile. After three minutes loading, the trapping column was put on-line with the analytical column. The peptides were eluted by applying a mixture of solvent A/B. Solvent A was HPLC grade water with 0.1% (v/v) formic acid, and solvent B was HPLC grade acetonitrile with 0.1% (v/v) formic acid. Separation was performed using a linear gradient of 2–35% solvent B at 300 nL/min either over 33 min for analyses performed on the triple quadrupole instrument (SRM) or over 66 min for analyses carried out on the quadrupole orbitrap instrument (PRM). One microliter of each sample was injected.

2.2.2. Analyses on triple quadrupole instrument

Selected reaction monitoring analyses were carried out on a TSQ Vantage extended mass range triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA). A dynamic nano-electrospray source was used with uncoated SilicaTips, 12 cm length, 360 μm outer diameter, 20 μm inner diameter, 10 μm tip inner diameter. Ionization was obtained by using 1200 V of liquid junction voltage and 250 °C for the capillary temperature. The selectivity for both Q1 and Q3 was set to 0.7 Da. The collision gas pressure in Q2 was set at 1.5 mTorr argon. The time-scheduled SRM method targeted 6 triplets of isotopically labeled peptides/endogenous peptides in ± 6 min retention time windows by monitoring five transitions for each peptide within a cycle time of 2.5 s.

2.2.3. Analyses on quadrupole orbitrap instrument

Parallel reaction monitoring analyses were performed on a Q-Exactive HF mass spectrometer (Thermo Scientific, Bremen, Germany). The nano-electrospray source was identical to the one used for analyses performed on the triple quadrupole mass spectrometer. For ionization, 1500 V of liquid junction voltage was used. The acquisition method included a full scan event (mass range of 300–1500 m/z), using a resolution of 60,000 (at m/z 200), a target automatic gain control value of 1e6, and a maximum fill time of 100 ms. The second event consisted in a PRM scan event operating with a 2-Th isolation window, a resolution of 30,000 (at m/z 200), a target AGC value of 1e6, a maximum fill time of 120 ms, and a normalized collision energy set at 25. The time-scheduled method targeted the six triplets of standard peptides (two labeled and one unlabeled) derived from proteins of the standard mixture, and 42 pairs of endogenous/isotopically labeled peptides from plasma in ± 2 min chromatographic monitoring windows. Some experiments were replicated on a quadrupole orbitrap plus instrument.

3. Data processing

Data analysis was performed using Skyline (Vers. 2.6, University of Washington). The area under the curve (AUC) of each target

transition was calculated for all the isotopic variants of the peptides of the reference mixture. The AUC of each individual transition were summed together to quantify each peptide.

4. Results and discussion

4.1. Design of a simple quality control procedure

Ideally, a quality control protocol intended to assess the performance of a proteomic workflow should use isotopically labeled recombinant proteins, added at the beginning of the experiment. Such standards, corresponding to the endogenous targets in the study, yield an estimation of the digestion efficiency of the proteins of interest, provided they reflect the native proteins (e.g., modifications). Although very useful, and the ultimate choice for clinical studies, the approach remains difficult to apply generically. A quality control procedure including a mixture of exogenous proteins and the multiple-addition of internal standards, used as surrogate peptides for the standard proteins, enables to determine the recovery and monitor the reproducibility of the different stages of the process (Fig. 1A, Supplementary Table). The standard peptides added at different stages of the workflow highlight anomalies. Furthermore, for routine analysis of clinical samples, an internal standard that can be spiked into a reference sample (i.e., plasma, called reference material) representative of the sample type used in the study constitutes a generic solution for proper evaluation of the workflow. The sequential addition of isotopically labeled variants of the peptides provides a better read-out and a detailed view of data quality, indicating possible deviations.

The quality control procedure developed is simple and straightforward, and allows a routine system suitability test prior to the analysis of the actual samples. It monitors the performance of sample preparation and instrument while added in control samples. For its first iteration, to demonstrate proof-of-principle, a set of three exogenous proteins was selected (i.e., non-human, called standard protein mixture). The double addition of internal standards allows the detection of inconsistencies in the individual stages of the workflow (i.e., digestion, desalting, or LC-MS analysis) (Fig. 1A). A first set of isotopically labeled internal standards (called peptides H_A; corresponding to the peptides derived from exogenous proteins) was added prior to the digestion, and the second one (called peptides H_B; with a different isotopic incorporation) immediately before LC–MS analysis. The amounts of spiked synthetic peptides were calibrated to be equimolar, i.e., to show a triplet of signals with the same intensity when analyzed by MS or MS/MS [16]. The quality control procedure was applied to monitor the overall workflow, as depicted on the control charts, and thus represents the initial baseline used to define acceptance criteria. Regarding the sample preparation, a recovery within 85–115% range was considered as acceptable in buffer solution [17], and outliers were detected by the Grubb's test (Fig. 1B). The status of the LC-MS/MS platform was monitored through chromatography metrics related to the peak area and peak shape (full width at half maximum, FWHM), whose thresholds were set at two and three standard deviations around the mean values determined over an

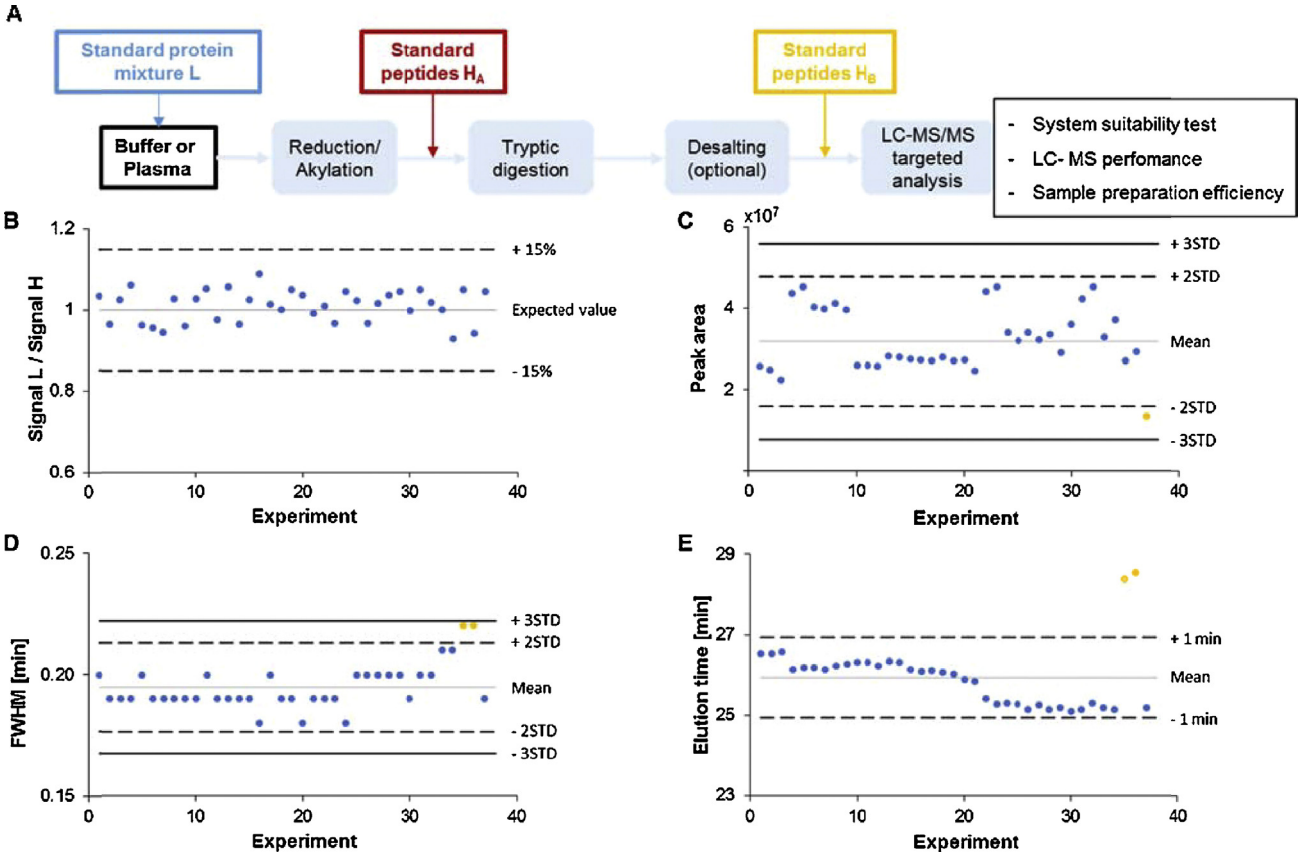

Fig. 1. Quality control procedure based on the sequential addition of isotopically labeled peptides. (A) Diagram of the analytical procedure. (B) Control chart of the sample preparation efficiency. The acceptance criteria were set at ±15% around the expected value of one. (C), (D), (E) Monitoring of the performance of the LC-MS/MS platform. (C and D) The acceptance criteria of the peak area and full width at half maximum were set at two and three standard deviations around the mean value corresponding at a warning and an action threshold, respectively. (E) The tolerance on the elution time was set at ± 1 min around the mean value. Yellow points corresponds to outliers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

extended period of time (Fig. 1C and D). Lastly, for the chromatographic elution time, an acceptance window of two minutes around the mean value of each peptide was established (Fig. 1E).

In the present study, the multiple internal standards approach was applied to clinical samples. The standard protein mixture was added to plasma samples, which were then processed with the simple protocol described above. Prior to perform LC-MS/MS analysis, the buffer solution of standard peptides was analyzed by LC-MS/MS to check the suitability of the platform, ensuring that all acceptance criteria were fulfilled. Then, plasma samples were analyzed and the standard peptides were quantified to estimate the overall efficiency of the process. Concomitantly, the standard protein mixture was processed in buffer using the same protocol, including the desalting step, to assess the efficiency of this operation. The acceptance thresholds were established based on a long-term reproducibility study of the process without the desalting step included.

4.2. Application to plasma samples

The quality control procedure was applied to plasma samples. The experiment was replicated ten times using one representative plasma sample, in which the standard protein mixture was added at the beginning of the preparation. The digests, and spiked peptides obtained through the full analytical procedure were analyzed by LC–SRM. The SRM traces of each of the targeted standard isotopologous peptides (six triplets in total) were extracted to assess the recovery of the process. More specifically, the consistency of the protein digestion in this more complex medium was assessed by comparing the signals of the peptides derived from the standard protein mixture (L) with those of the SIL peptides added prior to digestion (H_A). As indicated in Fig. 2A, a recovery within 85–115% was systematically obtained for five peptides (CV below 11%), while it slightly, but reproducibly, dropped below the acceptance criteria (82%) for one peptide (GGYFDSIGIIR). This confirmed the robustness of the sample preparation protocol, and more specifically the digestion step, which was only marginally affected by the increase in complexity due to the plasma matrix. The estimation of the recovery of the overall process is reflected by the comparison of the signal of the L peptides with the signal of the SIL standard peptides added prior to LC–MS analysis (H_B). More specifically, the L/H_B peptides signal ratios were lower than the acceptance threshold (Fig. 2B), indicating a significantly higher abundance for peptides added prior to the LC–MS analysis as compared to the exogenous peptides derived from the spiked protein mixture. As the signal ratios of the L/H_A peptides were in line with acceptance criteria, the inconsistency of the signal ratios of the L/H_B peptides was attributed to losses occurring during the last part of the process (i.e., handling and desalting). To further investigate the reason of this deviation and identify the critical step, the procedure was applied to plasma samples and buffer solutions. The quality control performed on the sample in buffer with the desalting step (green on the charts) demonstrated higher efficiency, reflected by L/H_B ratios close to one. The desalting step in plasma samples showed one abnormality for the peptide GVLHAVK that suffered from a very low and variable recovery due to high hydrophilicity, and poor retention on reversed phase SPE cartridge. The five other standard peptides showed a reproducible recovery over the entire procedure (CV below 15%, Fig. 2B). The double addition of internal standards combined with LC-MS/MS analysis allows to assess the recovery and more importantly the consistency of the full preparation. It immediately pinpoints to discrepancies, as illustrated with the peptide GVLHAVK. However, the application of the quality control procedure in plasma demonstrated the necessity to refine the acceptance thresholds according to the matrix used in the study. The high reproducibility obtained with one plasma sample is not sufficient and an extended set of plasma samples is required to establish a baseline for a large pre-clinical study.

4.3. Quality control and robustness of the method

The robustness of the procedure was further assessed by replicating three times the experiment on five plasma samples over one month. Compared with the preliminary experiments described above, the procedure was only slightly modified to include a reversed phase SPE cartridge of higher capacity at the desalting step level. In our hand, reproducible results were obtained as illustrated in Fig. 3. The recovery determined for the digestion step (assessed by L/H_A ratio, Fig. 3A) as well as for the overall procedure (assessed by L/H_B ratio, Fig. 3B), was characterized by a low coefficient of variation, which was typically below 15% for each plasma sample analyzed by SRM on the triple quadrupole and by PRM on the quadrupole orbitrap instrument. The variation in background, inherent to samples of distinct individuals, thus did not affect the procedure efficiency. The recovery of the desalting step was moderately enhanced, benefiting from the optimization of the solid phase extraction cartridge capacity, resulting in a lower variability for the peptide GVLHAVK (CV of 15%).

Furthermore, during PRM analyses on the quadrupole orbitrap instruments a series of 42 endogenous plasma peptides were concomitantly monitored with their isotopically labeled counterparts also spiked in the samples. The quantitative results of the endogenous

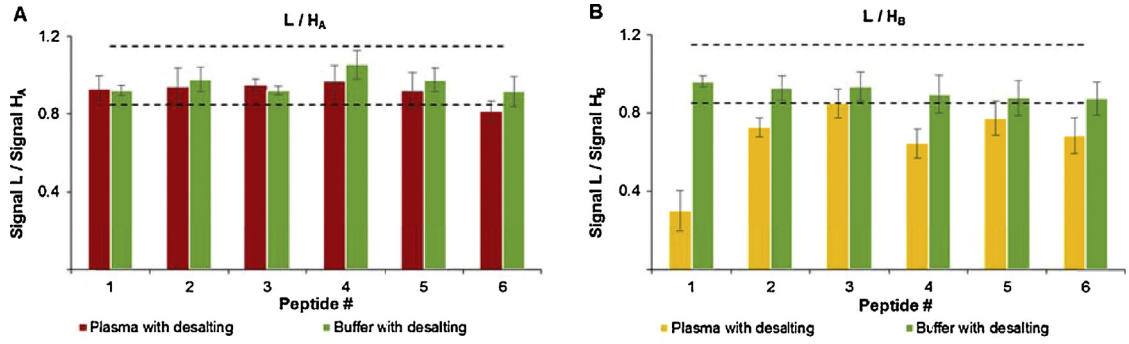

Fig. 2. Reproducibility of the double addition of internal standard in plasma sample. The sample preparation was replicated ten times over two weeks for two different conditions (in plasma samples and in buffer). The six resulting peptides were analyzed by LC-SRM on a triple quadrupole mass spectrometer. The numbers 1 to 6 correspond to peptides GVLHAVK, GVIFYESHGK, NVNDVIAPAFVK, NTVISVFGASGDLAK, VVGLSTLPEIYEK, and GGYFDSIGIIR, respectively. For each standard peptide the area ratios of the unlabeled form "L" over its isotopically labeled counterpart "H_A" (added prior to the digestion) and "H_B" (added prior to LC-MS/MS analysis) are presented in panel (A) and (B), respectively.

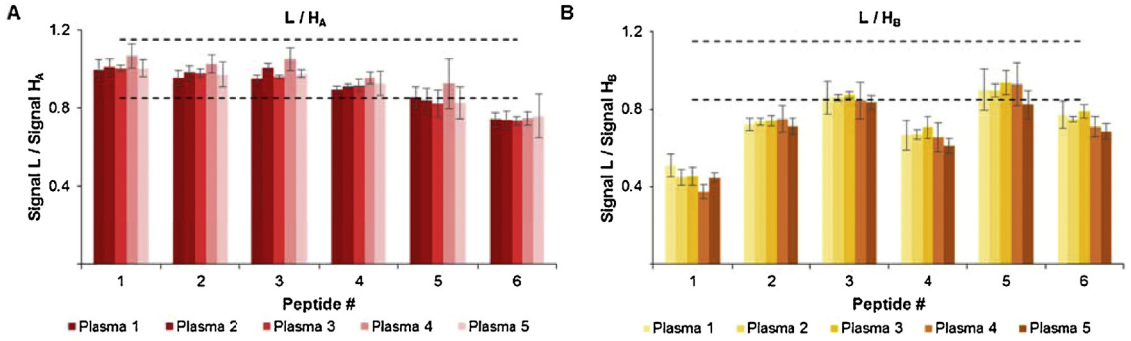

Fig. 3. Robustness of the quality control procedure applied to plasma samples. The sample preparation was performed on five distinct plasma samples and replicated three times over one month. The six resulting peptides were analyzed by LC–SRM on a triple quadrupole mass spectrometer. The numbers 1 to 6 correspond to peptides GVLHAVK, GVIFYESHGK, NVNDVIAPAFVK, NTVISVFGASGDLAK, VVGLSTLPEIYEK, and GGYFDSIGIIR, respectively. For each standard peptide the area ratios of the unlabeled form "L" over its isotopically labeled counterpart "H_A" (added prior to the digestion) and "H_B" (added prior to LC-MS/MS analysis) are presented in panel (A) and (B), respectively.

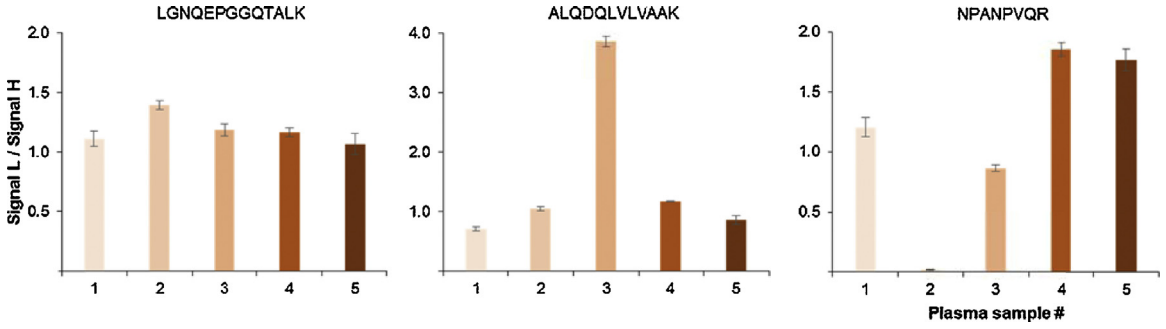

Fig. 4. Reproducibility of the measurement of endogenous peptides from plasma samples mesured concomitantly with the internal standard peptides. The sample preparation was performed on five distinct plasma samples and replicated three times over one month. The resulting peptides were analyzed by LC–PRM on quadrupole orbitrap instruments. The area ratios of three endogenous peptides over their isotopically labeled counterparts added prior to LC-MS/MS analysis are presented on each chart.

peptides are presented in Supplementary material. They exhibited high reproducibility within each plasma sample while reflecting the variable inter-sample abundance. This is illustrated by the results obtained for three selected peptides presented in Fig. 4. All these peptides were measured with a low CV (typically below 10%) in replicated analyses of each plasma sample while two of them exhibited a variable abundance across the samples. Indeed, peptide LGNQEPGGQTALK (corresponding to alpha-2-antiplasmin) was reproducibly and consistently measured in a similar amount in all plasma digests. However, peptides ALQDQLVLVAAK (corresponding to angiotensinogen) and NPANPVQR (corresponding to haptoglobin) were present in different amounts in the various plasma samples. For instance, peptide ALQDQLVLVAAK was three to five times more concentrated in plasma #3 compared to the other samples whereas peptide NPANPVQR was present in a very low concentration in the digest from plasma #2 i.e., 40–100 times less concentrated than in the other samples.

These results demonstrate the pertinence of the quality control when applied to plasma samples, as it is able to highlight abnormalities in the level of the endogenous peptides of interest. The standards spiked in biological samples allow the reproducibility and the performance of both sample preparation and analyses on LC–MS platforms to be monitored, as required to ensure reliability of the results.

5. Conclusion

The routine use of LC–MS based proteomics methods in biomedical studies and the necessity to generate comparable results have emphasized the need of standardization of the analytical process. A simple quality control procedure was proposed to address this requirement; it is based on the combination of a straightforward protocol and the sequential addition of two sets of six standard peptides with characteristic isotopic patterns. The amino acid sequence of these peptides is identical to surrogates peptides generated from the exogenous proteins added into the sample. The aim of this procedure is to perform a system suitability test, and to monitor the performance of both the sample handling and the LC–MS platforms involved in the analytical process. In proteomics experiments, in which the samples processed range from fairly to highly complex, a quality control obtained by spiking an internal standard into the sample at the beginning of the process is required. The exogenous protein mixture was spiked in highly complex samples and underwent the sample preparation and analyses using standard LC–MS methods. The procedure applied to plasma reference material demonstrated high reproducibility. Furthermore, in addition to the standard peptides monitored, the analysis included endogenous peptides from plasma digest.

In a pre-validation study performed on test samples, the boundaries of the assays were assessed, and were used to define metrics and estimate acceptance criteria. They represent a basis for large studies, performed to validate the acceptance/rejection thresholds. According to common practice, the mean and standard deviation (STD) of a set of values collected over longer periods allow the definition of the warning (2STD) and the action thresholds (3STD), respectively. Furthermore, the procedure guides system suitability checks; according to previous recommendations [18] an internal quality control is required every twenty samples for routine analyses, and every five or two samples for more complex processes. Considering the complexity of clinical

proteomics samples, the latest scenario is the most likely to happen.

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