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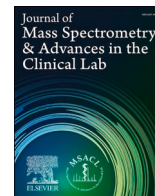
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Development of Tier 2 LC-MRM-MS protein quantification methods for liquid biopsies

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ABSTRACT

In the pursuit of personalized diagnostics and tailored treatments, quantitative protein tests contribute to a more precise definition of health and disease. The development of new quantitative protein tests should be driven by an unmet clinical need and performed in a collaborative effort that involves all stakeholders. With regard to the analytical part, mass spectrometry (MS)-based platforms are an excellent tool for quantification of specific proteins in body fluids, for example focused on cancer. The obtained readouts have great potential in determining tumor aggressiveness to facilitate treatment decisions, and can furthermore be used to monitor patient response. Internationally standardized TNM classifications of malignant tumors are beneficial for diagnosis, however treatment outcome and survival of cancer patients is poorly predicted. To this end, the importance of the tumor microenvironment has endorsed the introduction of the tumor-stroma ratio as a prognostic parameter in solid primary tumor types. Currently, the stromal content of tumor tissues is determined via routine diagnostic pathology slides. With the development of liquid chromatography (LC)-MS methods we aim at quantification of tumor-stroma specific proteins in body fluids. In this mini-review the analytical aspect of this developmental trajectory is further detailed.

Introduction

Clinical mass spectrometry (MS) is a growing and exciting field, and the number of clinical chemists that acknowledge its great potential with regard to replacing immunoassays for protein quantitation is rapidly [1] expanding. In order to develop sustainable MS-based protein assays it is important to first identify unmet clinical needs. For this purpose, both new protein markers and existing markers are considered. Furthermore, the measurement of a protein panel may be preferable, since it has become clear that disease-specific alterations are reflected in multiple proteins (signatures) rather than in a single marker. The learning objective in this mini-review is to guide the reader through the development of MS-based protein quantification methods. It is not meant to provide a comprehensive overview on quantitative protein MS. Instead it aims to exemplify the various steps in the development of MS-

based methods to address a specific clinical need. Herein, this need is exemplified with regard to the characterization of the tumor microenvironment, but the strategies followed apply to all protein quantification efforts that use a combination of proteolysis and peptide readout.

Cancer and tumor microenvironment

Cancer remains a leading cause of death worldwide, with an estimated 10 million deaths globally in 2020 [2]. Cancer patients diagnosed at an early stage demonstrate the highest likelihood of curative treatment and long-term survival, giving rise to the implementation of population screening programs for selected primary tumor types [3]. In addition to early detection as a determinant of patient survival, cancer encompasses a heterogeneous spectrum of neoplastic diseases, ranging from indolent lesions to tumors with high metastatic potential [4,5].

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This heterogeneity in tumor behavior dictates prognostic disparity and warrants the need for risk stratification tools to guide the decision-making process. Although cancer type-specific progress has been made, the current lack of robust risk stratification tools has the potential to result in the overtreatment or undertreatment of selected cancer patient subpopulations [6–8]. As such, recent research efforts have focused on the tumor microenvironment (TME) as a source for novel risk stratification tools to improve personalized medicine [9,10].

The TME comprises a dynamic ecosystem of cellular and subcellular interactions that surrounds the malignant cells and is essential to tumor progression [11]. The histologic tumor-stroma ratio (TSR) quantifies the intratumoral stromal content and was found to predict poor patient prognosis in multiple solid primary tumor types [10,12–17]. In addition to patient prognosis, the TSR was reported to predict response to neoadjuvant therapy in breast and gastrointestinal cancers [18,19,20]. Lastly, the combined assessment of the TSR and histologic tumor immune cell infiltrate was recently suggested as a novel tool in predicting response to checkpoint inhibitor therapy in colon cancer [21]. These reports support the notion of the TME as a valuable addition to current diagnostics in clinical oncology. Given the lack of TME parameters in cancer staging and grading classifications, the TSR is currently subjected to prospective validation in colon cancer in the international UNITED trial [22,23].

Regardless of the clinical relevance, assessment of the TSR requires access to histologic solid tissue biopsy or resection material obtained through invasive procedures that are reservedly applied during the course of disease [24]. Consequently, the TSR is not suitable for monitoring disease or treatment response. Considering these sample accessibility issues, recent scientific efforts have focused on liquid biopsy as a novel approach to tumor profiling. Liquid biopsy refers to the sampling of analytes from non-solid tissue specimens, such as serum or urine. Being minimally invasive, liquid biopsy offers a major advantage in sample accessibility as opposed to the conventional solid tissue biopsy, granting access to patient tumor profiling at numerous time points during the course of disease. In addition, liquid biopsy is expected to capture a comprehensive overview of the molecular tumor landscape, compensating for the loss of information when performing local solid tissue biopsy.

Despite significant advances in malignant cell-derived biomarkers, such as circulating tumor cells (CTCs) and circulating cell-free tumor DNA (ctDNA), the TME has remained surprisingly underappreciated in current liquid biopsy research [25–27]. To address TME profiling in liquid biopsy, the *Stroma Liquid Biopsy*TM (SLB) panel was developed as an experimental stroma-oriented proteomics alternative to the conventional genomic liquid biopsy biomarkers in oncology [28]. The SLB panel comprises a set of key proteins in interconnected stromal pathways (e.g. coagulation, acute phase inflammation) and is believed to capture a deranged systemic response to the presence of cancer in a plasma proteome blueprint. [28] Remarkably, we recently demonstrated that the representing genes of the SLB protein panel compose expression signatures that are expressed in malignant cells and the non-malignant cells within the TME in colon cancer. [21] Plasma protein levels of the SLB panel may, therefore, reflect histologic intratumoral stromal content and provide valuable prognostic information in liquid biopsies.

Quantitative protein mass spectrometry

Various proteins in body fluids are routinely tested in clinical laboratories for diagnostic, prognostic, and monitoring purposes. However, there is still room for improvement with regard to test performance. Quantitative protein MS is an attractive strategy compared to immunoassays. [29] MS measurements may be multiplexed and, therefore, are ideally suited for precision diagnostics in various care pathways. [30] So far, most assays have been developed are “for identification purposes only”, whereas for successful transfer of technology into useful clinical

tests, collaborations between all stakeholders are required; such as medical doctors, scientists, representatives of regulatory authorities and IVD-industry. Therefore, it is important to start with the identification of specific unmet clinical needs before setting up and evaluating a specific quantitative protein test based on a targeted bottom-up proteomics workflow with automated liquid handling and liquid chromatography (LC)-triple quadrupole-MS. Furthermore, sustainability is ascertained by introducing the concept of metrological traceability of test results through selection of suitable proteotypic peptides, optimized trypsin digestion and calibration with both internal standards and value-assigned reference calibrators [36]. To this end, it is noted that quality requirements are common practice in clinical chemistry. The earliest guidance for evaluation of LC-MS-based methods started from experience with small molecules and were based on recommendations of the US Food and Drug Administration and the European Medicines Agency [31,32]. More specific LC-MS requirements have been available since the publication of guideline C50-A from the Clinical and Laboratory Standard Institute (CLSI) in 2007 [33], which was later revised into a more systematic approach for development and validation of LC-MS methods in the CLSI-C62A guideline [34]. At the same time, the proteomics community proposed a three tiered system using a fit-for-purpose approach for the discovery of protein biomarkers and anticipated translation into a medical test [35]. Recently, a new CLSI document C64 dedicated to quantification of proteins by MS-techniques became available [36,37]. As of May 2022, in Europe, the development of MS-based tests will also have to be compliant with the new European Union In Vitro Diagnostics Regulations (EU IVDR). [38].

Development of a Tier 2 LC-MRM-MS assay for protein quantification

Rationalization of the requirements of all previously proposed three Tier assays has been discussed [35]. Starting at a Tier 3 level, this assay performance is suitable for exploratory studies that aim for biomarker discovery. In this mini-review the development of a Tier 2 liquid chromatography multiple reaction monitoring mass spectrometry (LC-MRM-MS) assay will be illustrated to identify and quantify proteins from the SLB panel. A Tier 2 assay requires well-defined selectivity, repeatability, sensitivity, and reproducibility and is the optimal choice for analytical performance of potential biomarkers. Once additional evidence is collected about the biomarker applicability, the assay performance is further enhanced to Tier 1, which implies protein quantification according to CLSI guidance, allowing measurement of clinical samples [35,36]. With regard to the analytical strategy, in all three Tier assays, endogenous proteins are measured after proteolysis into proteotypic peptides, commonly referred to as a bottom-up or peptide-centric approach. The analytical requirements of a Tier 2 assay mirror those of a Tier 1 assay. In both assays the endogenous peptides are quantified by LC-MRM-MS after addition of stable isotope-labeled peptide (SIL) analogues as internal calibrants, although in a Tier 2 assay this may also be done by using purified standards as calibrants [39] (Fig. 1A). The use of such calibrants allows for relative quantifications, albeit that in a Tier 1 assay accurate quantification is the goal. The role of SIL-peptides will be further discussed in a separate section (“Selection and application of SIL-peptides”), but at this stage it is important to note that conversion of thus determined peptide concentrations into true protein concentrations is not trivial. For accurate clinical chemistry quantifications protein calibrators and reference materials are required (see for more information [36]. Another key-factor for accurate quantification is detailed knowledge on the digestion kinetics of each protein of interest. Full conversion of a protein into (proteotypic) peptides is not necessarily needed for *identification* purposes; however in the case of *quantification* this step requires careful attention. Whereas for protein identification purposes a confident assignment of one, or often two peptides, is sufficient, multiple (proteotypic) peptides need to be evaluated for each single protein for quantification purposes. This evaluation starts with the determination of peptide intensities and their variations in replicate

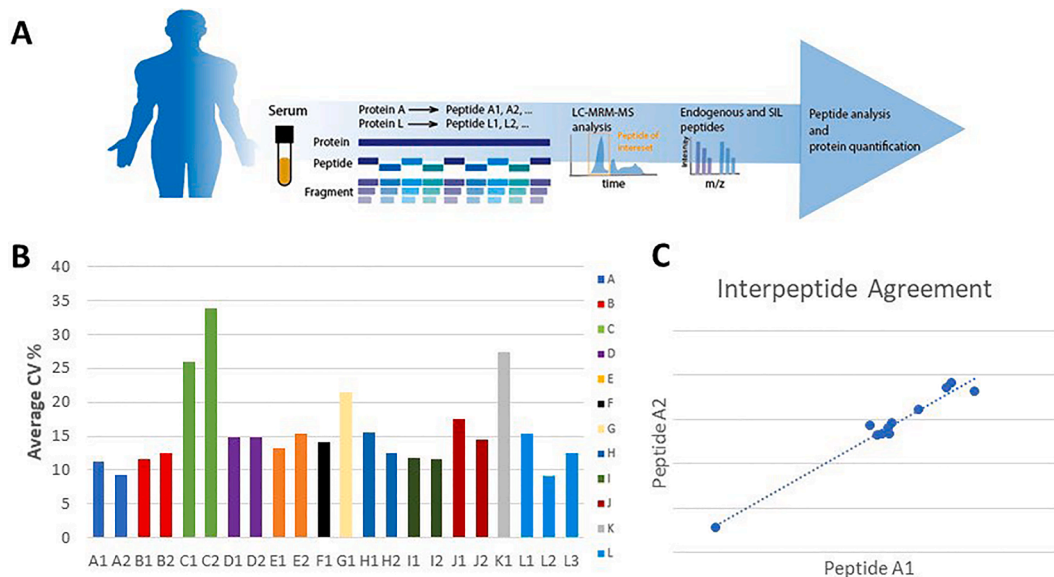


Fig. 1. Protein identification and quantification in a liquid biopsy via LC-MRM-MS analysis. (A) A liquid biopsy yields a body fluid, commonly blood via venipuncture, and differs from a tissue biopsy that is obtained via a needle or surgical intervention. Proteins in the body fluid (for instance serum) are quantified as peptides using LC-MRM-MS. The selection of optimal SIL-peptides is described in this mini-review. It is furthermore noted that initial evaluations via the scheme are performed without application of internal calibration. (B) Various peptide intensities with corresponding variations are monitored for each specific protein, with A1 and A2 as proteotypic peptides of protein A, B1 and B2 as proteotypic peptides of protein B, etcetera. (C) Correlations between various proteotypic peptides of a certain protein provide insight into the extent of protein digestion. Both axes depict arbitrary peptide signals that are normalized through SIL-calibration.

measurements without internal calibration with SIL-peptides (exemplified in Fig. 1B). The peptides that are hardly visible or have intensities that vary in different experiments are likely to fail for the final selections.

The next step in the evaluation concerns the variation in digestion. When each protein of a certain identity is fully converted into the peptide of interest, this is referred to as equimolar digestion of that specific protein, but obviously this does not always happen in practice. The evaluation of so-called interpeptide agreement at different protein concentrations provides a first clue in how efficiently a protein is digested (Fig. 1C). This topic will be further discussed in the section “Evaluation of proteolysis”. Moreover, for clinical chemistry purposes the quantification of a protein requires a pre-defined accuracy and long-term robustness. With regard to the first metric, it is not trivial to determine the *true* protein concentration, as will be explained herein. With regard to the latter aspect, the outcome of measurement should be independent of place and time to allow longitudinal analyses. Finally, it is emphasized that quantifications of clinically relevant, endogenous protein concentrations in patient samples should be accompanied by verification of identity, for which MS is also an excellent method.

From protein to peptides

Sample preparation and selection of proteotypic peptides

In order to obtain a protein digest, sample preparation should be carried out according to a standard protocol or a standard operating procedure (SOP). Commonly, proteins are denatured, disulfide bonds are cleaved by dithiothreitol (DTT) or tris(2-carboxyethyl) phosphine (TCEP), and resulting free cysteines are alkylated with iodoacetamide (carbamidomethylation) to prevent oxidation and/or refolding. The proteins are then digested using a protease, commonly trypsin in an ammonium bicarbonate (ABC) solution. Digestion is quenched by lowering the pH (commonly with acetic or formic acid) to obtain a complex mixture of peptides that are subsequently separated by reversed-phase liquid chromatography and mass analyzed and fragmented in an MS-system (commonly a triple quadrupole MS).

In the first step, proteotypic peptides are selected for each protein of interest through *in-silico* digestion. For this purpose a wide variety of software tools is online available [20,40]. Preferably, trypsin is used for protein digestion, since this protease is by far the most applied and developed enzyme for such purposes. Trypsin cleaves proteins specifically at the C-terminal side of lysines (K) and arginines (R), except when proline (P) is attached to the C-terminus of these amino acids. Furthermore, upon selection of suitable peptides, amino acids that potentially carry a post-translational modification (e.g., glycosylation, phosphorylation) or are sensitive to chemical degradation (oxidation) are avoided. The same software tools are then applied to generate theoretical collision-induced dissociation fragmentation spectra of the peptides (commonly yielding b- and y-ions) and various databases will summarize how often these peptides and their characteristic fragment b- and y-ions have been observed, and by whom, to guide the clinical (analytical) chemist in further development of MS-methods. Nevertheless, since each lab and each instrumental setup has its own characteristics, it is recommended to *in-house* evaluate (“survey”) all peptides (presence and MS/MS-fragmentation) in a protein digest of interest. This digest can be obtained from a purified (commercially available) protein, but also from an endogenous sample such as serum, plasma or a cell lysate.

LC-MS/MS analysis of proteotypic peptides

MS-based protein quantification methods are commonly based on peptide separations using LC followed by tandem MS (LC-MS/MS) analysis. In principle, proteins can also be identified and quantified by MS in their intact form (often referred to as a top-down approach), but, so far, such quantifications are limited to *relative* quantifications, which will not be discussed here. [41] For LC-MS/MS analyses of tryptic peptides, most commonly used is a triple-quadrupole (QQQ) Instrument equipped with electrospray ionization (ESI), where the system operates in a positive-ion mode. The peptides are separated based on their hydrophobicity, commonly using a reversed-phase C18 column. Variables that can be tuned to improve separation include the composition of mobile phases, the pH, the gradient, temperature and separation time. The QQQ MS is used in various manners. In order to *survey* which

peptides can be observed in the digest, mass measurements of eluting species are continuously performed without any fragmentation of peptides. In combination with LC-separation these measurements are plotted as a total ion current (TIC) or base peak chromatogram (see Fig. 2A). Note that, in this case, peptides are observed at different charge states in the MS: common tryptic peptides have molecular masses between 800 and 2000 Da and can carry one, two or three positive charges (protons), resulting in multiple m/z -signals for each single peptide in a TIC. One mass spectrum is exemplified for a proteotypic peptides from complement C3 (CO3), namely SGSDEVQVGQQR in Fig. 2B. In order to fragment this peptide in the QQQ MS, the *product ion mode* is used: the peptide m/z is selected in the first quadrupole, the peptide of interest is subjected to CID in the second quadrupole, and a fragmentation spectrum with product ions is obtained by scanning the third quadrupole [36]. In this way specific tandem mass spectra are obtained for each

peptide that is selected. As an example, the fragmentation spectrum of peptide SGSDEVQVGQQR is plotted in Fig. 2C. Note that there is a choice of which m/z -value of a peptide is selected for further development; this decision depends on which charge state has the highest intensity in the *survey* mass spectrum (Fig. 2B), or which charge state yields the most intense fragment ions (Fig. 2C). Thus observed precursor ion-product ion pairs are referred to as transitions (m/z peptide > m/z fragment ion). Certain transitions are selected for each peptide based on the MS intensity and specificity, again exemplified for peptide SGSDEVQVGQQR in Fig. 2D. Commonly, the highest abundance transition is selected to serve as the so-called quantifier, while others are selected as so-called, qualifiers. In general, for each peptide, at least two, but preferably three, Multiple Reaction Monitoring (MRM) transitions are selected with one serving as the quantifying transition and others as qualifier transitions. All three MRM transitions should be present in the

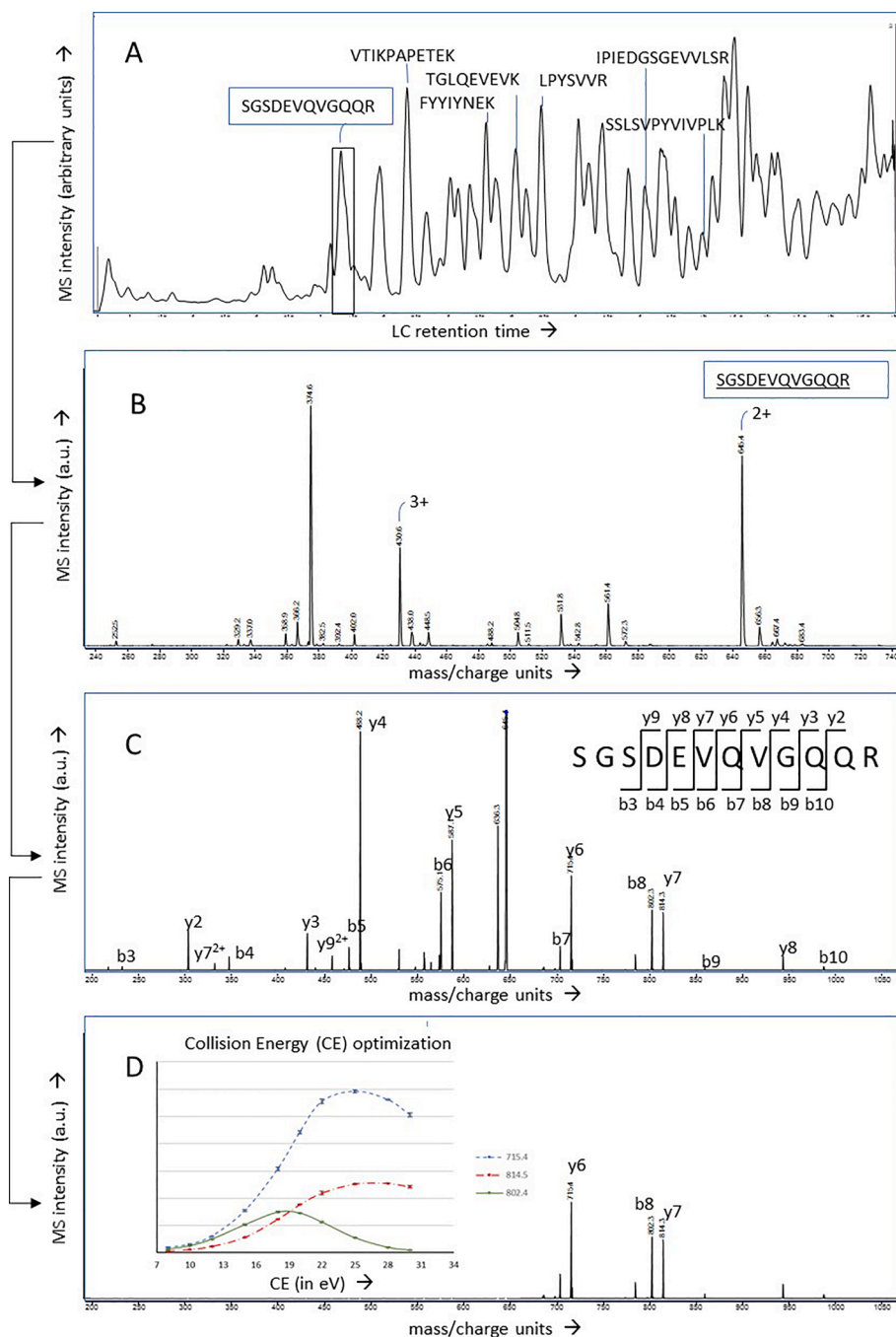


Fig. 2. Workflow for detailed analysis of proteotypic peptides from endogenous protein CO3. (A) Total Ion Chromatogram of a peptide digest obtained from purified CO3. As an example, eight proteotypic peptides are assigned based on peptide mass, namely FYYIYNEK, SSSLVPPYVIVPLK, TGLQEVEVK, LPYSVVR, VTIKPAPETEK, IPIEDGSGEVVLSR, SGSDEVQVGQQR and VHQYFNVELIQPGAVK. All eight are further evaluated whereas results are exemplified for SGSDEVQVGQQR. (B) Summed mass spectrum at retention time 4.3 min containing SGSDEVQVGQQR peptide with m/z values of charge states 2+ and 3+. (C) MS/MS spectrum of SGSDEVQVGQQR with theoretical fragmentation and corresponding b- or y-ions assigned to corresponding peaks. (D) Fragment spectrum of SGSDEVQVGQQR acquired from the MRM method with corresponding b- and y-ions assigned to the peaks. The inset exemplifies the optimization of collision energies for each transition.

resulting data and the ratios between these three intensities (often referred to as ion ratios) should be relatively stable.

Subsequently, the collision energy (CE) of each transition is optimized and later included in the MRM method. Upon qualification and quantification of the proteins of interest, optimal fragmentation of the corresponding peptides is achieved by optimization of the collision energies in the QQQ MS. Different transitions (m/z peptide > m/z ions) are selected from product ion scans (or tandem mass spectra) and measurements to focus on finding an optimum by means of intensity typically by varying from 8 eV to 30 eV, exemplified for peptide SGSEDEVQVGQQR in Fig. 2D. Once the optimal peptides of the proteins of interest are determined, and the MRM methods, including optimized collision energies, are finalized, the peptide separation part can be further optimized with regard to retention times and LC-peak shapes. The total analysis time is minimized to allow for the high-throughput requirements for routine applications in medical laboratories.

Evaluation of proteolysis

Protein digestion and corresponding digestion curves

An important, although often overlooked, aspect in MS-based protein quantification concerns digestion kinetics. The efficiency of proteolysis requires careful attention and needs to be assessed using increasing time points of protein digestion at 37 °C, for example 10 min, 30 min, 1 h, 3 h, 5 h and 22 h. For this purpose, digestions are quenched through acidification and results are normalized to $t = 22$ h. Peptides representing a fast tryptic digestion followed by a plateau are preferred for use as a so-called quantifier peptide, although it should be taken into account that a subsequent decrease in signal may occur, thus limiting suitability for quantitative assays. All peptides that have slow digestion kinetics that do not reach a plateau within 22 h are excluded. Based on the results of the digestion kinetics experiment, proteotypic peptides that are suitable for quantification of the endogenous protein of interest can be selected. Typically, a second peptide is selected as a “qualifier peptide”. In Fig. 3, the digestion curves are shown for four proteotypic peptides, namely

FYIYNEK, SLSVPIVIVPLK, SGSEDEVQVGQQR and VHQYFNVE-LIQGAVK. Digestion curves can be classified into four types, namely “fast and plateau” (A), “fast and decrease” (B), “fast and limited decrease” (C), and “slow and no plateau” (D). Only those that follow “fast and plateau” are suitable as quantifier peptides. One may argue that other types also qualify as long as these are perfectly reproducible (“protein X always converts into 20 % of the proteotypic peptide”); however, in practice we have never encountered such peptides. In our opinion, digestion curve types (B), (C) and (D) point toward proteotypic peptides that are not suited for accurate protein quantification. It is noted that, at this stage of the development process, no SIL-peptides have been applied for internal calibration purposes yet. Therefore, decreases in peptide signal after fast formation, such as in curves (B) and (C) could (partially) be explained by ion suppression effects due to increasing complexity of the protein digest. Changing (extending) the LC-gradient could be applied to study this effect, but it is emphasized that peptides that follow digestion curve (A) remain the ones that are preferred for further development.

Selection and application of SIL-peptides

By following the here-described steps, the analytical chemist has gathered extensive information on proteotypic peptides that correspond to the protein of interest. Ideally, these data should be universal so that labs can learn from each other and transfer knowledge and expertise. However, it is noted that, in practice, the pre-analytical phase of clinical samples significantly varies over different sites (not discussed here in detail), instrumental setups differ (MS vendor type, LC-column) and sample preparation protocols are often lab-specific. It is, therefore, recommended to perform each evaluation *in-house* and not to take shortcuts. Such an evaluation may initially appear tedious, but in the end, it will increase the quality of protein quantification data. Based on these evaluations the most optimal peptides can be selected for internal normalization, *i.e.* the SIL-peptides. SIL-peptides are synthesized peptides with structural formulas that are identical to the endogenous peptides of interest, but differ in mass due to the inclusion of specific

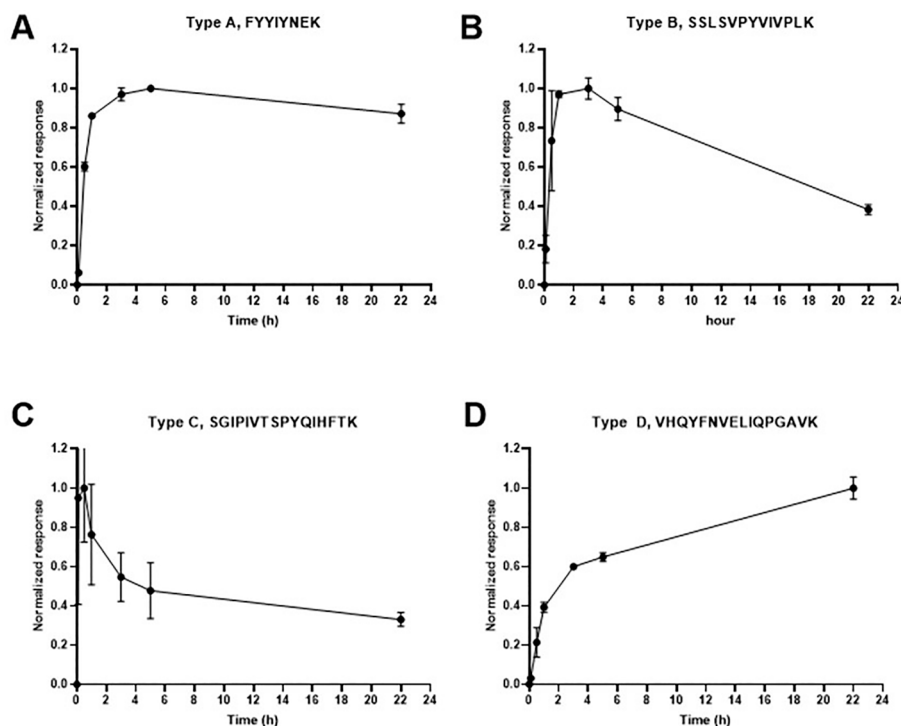


Fig. 3. Examples of digestion curves of four proteotypic peptides from complement C3. Digestion curves can be classified into four types, namely “fast and plateau” (A), “fast and decrease” (B), “fast and limited decrease” (C), and “slow and no plateau” (D).

amino acids that contain ^{13}C and ^{15}N , *i.e.* heavy amino acids. A SIL-peptide is spiked into the samples of interest as early as possible during the sample preparation process in order to correct for losses of the corresponding target peptide. It is expected that modifications to the endogenous peptide during all processing steps will occur in an identical manner for the SIL-peptide and, consequently, the relative ratio between endogenous and SIL peptide remains constant.

Preferably, the ratio between endogenous and SIL peptide lies between 0.1 and 10, reflecting a dynamic intensity range that is easily covered by all analytical instruments. Obviously, the purity of SIL-peptide requires careful attention, especially when complex samples are analyzed (such as liquid biopsies) and various proteins are targeted. During synthesis (minor) side-products may end up in the final product, such as peptides lacking the *N*-terminal amino acid or peptides that contain protective groups, which can interfere with MRM-selections. Secondly, it is noted that a synthetic peptide product may contain remaining salts or (crystal)water and that weighing a certain amount does always reflect the true molarity. However, this latter phenomenon does not hamper the quantification process, since the relative ratios of endogenous peptide and SIL-peptide do not reflect *true* protein concentrations. All Tier assays allow comparison of protein quantities between different samples, but for accurate clinical chemistry quantifications, protein calibrators and reference materials are required (see for more information [36]). Note that, in the context of quantification purposes, the terms “accurate” and “absolute” are not the same, despite the fact that often absolute quantifications are (incorrectly) interpreted as results that reflect trueness. For this purpose, a full length recombinant SIL-protein is beneficial, although this protein may still not be identical to the endogenous one in native human matrix. A recombinant protein may differ with regard to secondary, tertiary or quaternary structure from the endogenous protein, or differ in post-translational modifications (or proteoform profile), thereby introducing a digestion bias.

Concluding remarks

In this mini-review the technical aspect of LC-MRM-MS assay development has been detailed. In our laboratory, we are using these assays for the quantification of tumor-stroma specific proteins in body fluids in order to characterize the TME, but the methodologies described herein can similarly be applied to other quantitative MS-protein determinations. A Tier 2 assay corresponds to quality and performance that is sufficient to measure changes in the expression levels of proteins as a result of biological changes in the human body, such as a disease. The TME is a valuable resource for clinically relevant information in cancer diagnostics and therapeutics. Recent scientific efforts have focused on minimally-invasive liquid biopsy as a novel approach to tumor profiling. As an alternative, or complementary to the conventional genomic liquid biopsy biomarkers, robust detection and quantification of TME-associated proteins in patient sera can provide prognostic information that may ultimately guide the treatment decision-making process in clinical oncology.

The degree of analytical validation and the precision of an MS-based Tier 2 assay is moderate-to-high compared to a Tier 1 assay, whereas both assays require high reproducibility. With such assays, relative changes of the selected proteotypic peptide levels are determined, without reporting true protein concentrations. When later aiming for a lab-developed test, which even extends beyond the Tier 1 level, the clinical LC-MS/MS-based assays should be validated according to the CLSI guidelines. Only then, is further biomarker translation, including clinical evaluation, feasible. Following specific guidelines will improve the overall quality of methods, ease reproduction, and, furthermore, is actually required in the new EU IVDR. We encourage the bottom-up proteomics community to develop LC-MS assays according to the provided guidance while keeping clinical needs in mind. [42].

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Informed consent statement

Not applicable.

CRediT authorship contribution statement

Nina Diederiks: Conceptualization, Methodology, Writing – review & editing. **Cor J. Ravensbergen:** Writing – review & editing. **Maxim Treep:** Methodology. **Madelein van Wezel:** Methodology, Writing – review & editing. **Matt Kuruc:** . **L. Renee Ruhaak:** Methodology, Writing – review & editing. **Rob A.E.M. Tollenaar:** Conceptualization, Supervision. **Christa M. Cobbaert:** Conceptualization, Supervision. **Yuri E.M. van der Burgt:** Conceptualization, Methodology, Writing – review & editing. **Wilma E. Mesker:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: M.K. is the vice president of the issuing organization of the patent-pending Stroma Liquid Biopsy™ product, the Biotech Support Group (BSG). All remaining authors have declared no conflict of interest.

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