ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

PROTEOMIC APPROACHES FOR THE IDENTIFICATION AND QUANTIFICATION OF CLINICALLY RELEVANT BIOMARKERS

Ph.D THESIS

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Department of Molecular Biology-Genetics and Biotechnology

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

KLİNİK ÖNEM TAŞIYAN BİYOBELİRTEÇLERİN BELİRLENMESİ VE MİKTAR TAYİNİ İÇİN PROTEOMİK YAKLAŞIMLAR

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To my beloved spouse and my dear mother

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October 2024

Meltem AŞICIOĞLU KÜÇÜK
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ABBREVIATIONS

AAA : Amino Acid Analyses
ACN : Acetonitrile
BSA : Bovine Serum Albumin
CAM : 2-chloroacetamide
CV : Coefficients of Variation
CRM : Certified Reference Materials
cTn : Cardiac troponin
cTnC : Cardiac troponin C
cTnI : Cardiac troponin I
cTnT : Cardiac troponin T
DDA : Data-Dependent Acquisition
DTT : Dithiothreitol
EDC : 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride
EIC : Extracted Ion Chromatography
FA : Formic Acid
FASP : Filter-Aided Sample Preparation
FDA : Food and Drug Administration
HCl : Hydrochloric acid
IAA : Iodoacetamide
ICHCLR : International consortium for harmonization of clinical laboratory
ID : Isotope Dilution
IDMS : Isotope Dilution Mass Spectrometry
ID-LC-MS/MS : Isotope Dilution Liquid Chromatography-Tandem Mass Spectrometry
IFCC WG-TNI : International Federation of Clinical Chemistry and Laboratory Medicine Working Group on Cardiac Troponin
IS : Internal Standard
ISO : International Organization for Standardization
LC : Liquid Chromatography
LC-HRMS : Liquid Chromatography combined with High-Resolution Mass Spectrometry
LC-MS : Liquid Chromatography Mass Spectrometry

LC-MSMS : Liquid Chromatography Tandem Mass Spectrometry
LOD : Limit of Detection
LOQ : Limit of Quantification
MES : 4-Morpholineethanesulfonic acid
MP : Magnetic particle
MRM : Multiple Reaction Monitoring
MS : Mass Spectrometry
NIST : National Institute of Standards and Technology
NMIJ : National Metrology Institute of Japan
NHS : N-hydroxysuccinimide
PCF : Propyl Chloroformate
PICAA : Peptide Impurity Corrected Amino Acid Analysis
PBS : Phosphate Buffered Saline
PRM : Parallel Reaction Monitoring
PSM : Peptide-Spectrum Match
RM : Reference Material
RMP : Reference Measurement Procedure
RSD : Relative Standard Deviation
QC : Quality Control
SI : International System of Units
SIL : Stable Isotope Labeled
SRM : Standard Reference Material
TCEP : Tris(2-carboxyethyl)phosphine
TIC : Total Ion Chromotography

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PROTEOMIC APPROACHES FOR THE IDENTIFICATION AND QUANTIFICATION OF CLINICALLY RELEVANT BIOMARKERS

SUMMARY

Cardiovascular diseases are a significant health issue affecting people worldwide. Early diagnosis of cardiovascular diseases is crucial for the successful treatment of conditions such as heart attacks and for preventing death. Cardiac troponin I (cTnI) is a vital biomarker for the diagnosis and risk assessment of heart attacks. In healthy individuals, cTnI levels are found below 45 nanograms per liter. During and in the hours following a heart attack, it is released into the blood stream due to heart tissuedamage, leading to an increase in its levels. cTnI can be detected in the blood in various forms, including a ternary complex (cTnC-cTnT-cTnI), a binary complex (cTnI-cTnC), and free forms. It is highly susceptible to proteolysis and enzymatic changes. Consequently, various forms of cTnI, including proteolyzed, phosphorylated, oxidized, and reduced forms, can be found in the blood. All these variables lead to differences in cTnI measurements. There are many cTnI tests available on the market. The different variations circulating in the blood can be recognized by different monoclonal antibodies specific to different epitopes of cTnI. The various versions of cTnI, along with the different antibodies used, have increased the correlations between commercial tests more than tenfold, yet standardization remains challenging. Laboratories use different clinical decision thresholds depending on the test used. Different assay cutoffs have the potential to confuse physicians, leading to the misinterpretation of cTnI results; hence, there is urgency for cTnI standardization. The standardization and/or harmonization of cTnI assays is considered a high priority by the International Consortium for Harmonization of Clinical Laboratory results (ICHCLR).

According to ISO 17511, the standardization of the measurement of a biomarker requires a metrological traceability chain. This chain begins with a primary reference measurement procedure (RMP), which assigns quantity values to a primary reference material (RM). Primary RMs are used to assign values to a secondary RM. With this secondary RM, values are assigned to working and product calibrators for routine quantification of the biomarker in patient samples. This traceability chain allows the values reported for patient care to be traced back to the International System of Units (SI). In this way, metrological traceability supports the long-term stability and comparability of routine laboratory measurement results.

The standardization or harmonization of cTnI measurement requires the development of RMs and RMPs. The traceability chain proposed by the International Federation of Clinical Chemistry and Laboratory Medicine Working Group on Standardization of Troponin I (IFCC WG-TNI) incorporates all these standardization steps. One of the tasks that the IFCC working group is focused on to establish the proposed traceability chain is the development of a higher-order RMP.

In this thesis, the focus has been on developing two different analytical methods to support the development of a RMP. Both analytical procedures involve targeted and

bottom-up proteomic approaches. In both methods, isotope dilution mass spectrometry (IDMS) has been used to determine the absolute amount of cTnI. For quantifying proteins using the IDMS method, two different strategies have been employed: the protein-based calibration strategy and the peptide-based calibration strategy. Each method has its own advantages and disadvantages.

The first developed analytical method allows for the determination of cTnI from human serum using a protein-based calibration strategy. In this context, human cardiac troponin complex material (NIST SRM 2921) has been selected for use as a calibrant. The troponin complex was purified from human heart tissue and consists of three subunits: troponin T (cTnT), troponin I (cTnI), and troponin C (cTnC). As an internal standard, isotopically labeled cTnI protein with the same sequence as cTnI has been used. To extract cTnI from a complex matrix like serum, an immunoaffinity enrichment strategy has been employed.

As the first step of immunoaffinity enrichment, two different diameters of magnetic particles were selected: micro (Dynabeads® MyOne™, 1 µm) and nano (Nanomag®-D, 130 nm). A monoclonal antibody capable of binding to cTnI was immobilized on both types of magnetic nanoparticles, and their cTnI enrichment efficiencies were compared. Magnetic nanoparticles (Nanomag®-D, 130 nm) were chosen for further experiments because the peak areas of two selected tryptic peptides of cTnI were relatively higher. Next, the maximum loading capacity of the magnetic nanoparticles was determined. It was found that when 100 µg of antibody was added to 1 mg of particles, 59.2 ± 5.7 µg/mg of antibody could be bound. Using the synthesized nanoparticle-antibody conjugate, the required amount for cTnI enrichment from 1 ml of serum was calculated, and it was determined that 10 µl of conjugate was sufficient to capture all cTnI in 1 ml of serum for analysis.

As a result of these optimizations, the isotope dilution liquid chromotograpy tandem mass spectrometry (ID-LC-MS/MS) method using the developed protein-based calibration strategy allows the measurement of cTnI in the range of 0.6 to 24 µg/L ($R > 0.996$). The limit of quantification (LOQ) was determined to be 1.8 µg/L, and the limit of detection (LOD) was 0.6 µg/L. Intermediate precision was found to be below 9.6%, and repeatability ranged from 2.0% to 8.7% for all quality control materials. The accuracy of the analyzed quality control materials was between 90% and 110%. Total measurement uncertainties (n=6) were found to be below 12.5% for all levels.

The second developed ID-LC-MS/MS method allows the determination of cTnI in human serum using a peptide-based calibration strategy. In this method, two tryptic peptides (TLLLQIAK and NITEIADLTQK) of cTnI were selected and synthesized as calibrants. Isotopically labeled versions of the selected peptides were used as internal standards. Peptide impurity correction amino acid (PICAA) analysis was performed to assign values to the synthetic peptides, thereby producing SI-traceable primary peptide standards.

Peptide-based calibration approach also employed two surrogate matrices to construct the calibration curve. The surrogate matrices were evaluated based on parameters such as linearity, accuracy, repeatability, intermediate precision, and trueness. It was observed that both matrices yielded similar results, indicating consistency in their performance.

To ensure complete cleavage of the cTnI protein and enhance proteolysis yield, optimizations such as trypsin digestion methods, enzyme-to-protein ratio, and digestion time were performed. The best trypsin cleavage yield was obtained using the

Filter-Aided Sample Preparation (FASP) method with a 1:10 enzyme-to-protein ratio and overnight digestion.

The developed analytical method using the peptide-based calibration strategy enables the quantitative determination of cTnI in the range of 0.6–21.6 µg/L. Intermediate precision RSD was less than 28.9%, and repeatability RSD was less than 10% across all concentration levels. The recovery rate ranged between 72% and 151%.

Four patient serum samples with suspected heart attack were measured using the developed method, and the results showed discrepancies of more than 50% compared to those obtained with immunoassay. Finally, the performance of the peptide-based calibration strategy was compared with the protein-based measurement strategy.

In conclusion, this thesis has developed two different ID-LC-MS/MS methods using a targeted and bottom-up proteomic approaches. Both methods were compared with each other in terms of effectiveness. This efforts aim to support the metrology community in adopting new approaches and developing SI-traceable peptide and protein primary standards and/or reference procedures tailored to specific needs.

Standardization and harmonization of cTnI across laboratories are undeniably complex tasks. However, the IFCC WG-TNI believes that cTnI measurement is standardizable. Given the critical role of cTnI in patient management, the significant effort invested is worthwhile. The proposed measurement methods will play a role in supporting the activities of the IFCC WG-TNI. These studies are necessary and logical steps towards the harmonization of results obtained from different test kits.

KLİNİK ÖNEM TAŞIYAN BİYOBELİRTEÇLERİN BELİRLENMESİ VE MİKTAR TAYİNİ İÇİN PROTEOMİK YAKLAŞIMLAR

ÖZET

Kardiyovasküler hastalıklar dünya genelinde insanları etkileyen önemli bir sağlık sorunudur. Bu hastalıkların erken teşhisi, özellikle kalp krizi gibi durumların başarılı bir şekilde tedavi edilmesi ve ölüm riskinin azaltılması açısından hayati önem taşır. Kardiyak troponin I (cTnI), kalp krizi tanısı ve risk değerlendirmesinde önemli bir biyobelirteçtir. cTnI'nın sağlıklı bireylerde kandaki seviyesi 45 nanogram/litrenin altında bulunur. Ancak kalp krizi sırasında ve sonrasındaki süreçte, kalbin hasar görmesi nedeniyle kana salınarak seviyesi artar. cTnI, kanda üçlü kompleks (cTnC-cTnT-cTnI), ikili kompleks (cTnI-cTnC) ve serbest formlar gibi farklı yapılarla tespit edilebilir. Bu biyobelirteç, proteoliz ve enzimatik değişikliklere oldukça duyarlıdır; dolayısıyla proteolize uğramış, fosforile olmuş, oksitlenmiş ve indirgenmiş cTnI formlarının çeşitli varyasyonları kanda bulunabilir. Piyasada birçok cTnI testi bulunmaktadır. Kanda dolaşan farklı varyasyonlar, cTnI'ın farklı epitoplarına özgü farklı monoklonal antikorlar tarafından tanınabilir. Bütün bu değişkenler, cTnI ölçümleri arasında farklılıklar ortaya çıkmasına sebep olur. Kardiyak troponin I'nın çeşitli versiyonları ve kullanılan farklı antikorlar, ticari testler arasındaki farklılıkları on katından fazla arttırmıştır, ancak kitlerin standardizasyonu zordur. Laboratuvarlar, kullanılan testlerin farklılığı nedeniyle farklı klinik karar eşikleri uygulamaktadır. Bu değişkenlik, hekimlerin cTnI sonuçlarını yanlış yorumlamalarına neden olabilir. Bu nedenle cTnI testlerinin standardizasyonu aciliyet taşımaktadır. Uluslararası Klinik Laboratuvar Sonuçlarının Harmonizasyonu Konsorsiyumu (ICHCLR), cTnI testlerinin standardizasyonu ve/veya harmonizasyonunu yüksek öncelikli bir konu olarak ele almaktadır.

ISO 17511'e göre, bir maddenin ölçümünün standartlaştırılması için, metrolojik izlenebilirlik zincirine gereksinim vardır. Bu zincir, birincil referans ölçüm prosedürü ile başlar, bu prosedür birincil bir referans malzemeye (RM) miktar değeri atar. Birincil RM'ler, ikincil RM'ye değer atamak için kullanılır. Bu ikincil RM'ler ile hastane örneklerinde rutin olarak ölçülen biyobelirteci ölçebilmek için, çalışma ve ürün kalibratörlerine değerler atanır. Bu izlenebilirlik zinciri, hasta için rapor edilen değerlerin uluslararası birimler sistemine (SI) kadar izlenebilmesine olanak sağlar. Bu şekilde, metrolojik izlenebilirlik, rutin ölçüm sonuçlarının uzun vadeli kararlılığını ve karşılaştırılabilirliğini destekler.

cTnI ölçümünün standartlaştırılması ya da uyumlaştırılması için, RM'lere ve referans ölçüm prosedürlerinin geliştirilmesine ihtiyaç vardır. Uluslararası Klinik Kimya ve Laboratuvar Tıbbı Federasyonu Troponin I Standartlaştırma Çalışma Grubu (IFCC WG-TNI) tarafından önerilen izlenebilirlik zinciri, bu standartlaştırma unsurlarının tamamını kapsar. IFCC çalışma grubunun odaklandığı ana görevlerden biri, üst düzey bir referans ölçüm prosedürünün geliştirilmesidir.

Bu tezde, cTnI biyobelirtecinin referans ölçüm prosedürünün geliştirilmesini desteklemek amacıyla iki farklı analitik yöntem çalışılmıştır. Her iki analitik prosedür

de hedefe yönelik ve aşağıdan-yukarıya proteomik yaklaşımlarını içermektedir. cTnI'nın mutlak miktarını belirlemek için, her iki yöntemde de izotop-seyreltme kütle spektrometrisi (IDMS) kullanılmıştır. ID-MS yöntemiyle protein miktar tayininde iki farklı strateji uygulanmıştır: protein-tabanlı kalibrasyon stratejisi ve peptit-tabanlı kalibrasyon stratejisi. Her iki yöntemin de kendine özgü avantajları ve dezavantajları bulunmaktadır.

İlk geliştirilen analitik metot, protein-tabanlı bir kalibrasyon stratejisi ile cTnI'yı insan serumundan tayin etmeyi sağlar. Bununla ilgili olarak, insan kardiyak troponin kompleks materyali (NIST SRM 2921) kalibrant olarak kullanılmak üzere seçilmiştir. İç standart olarak da, cTnI ile aynı dizilime sahip ancak izotopik olarak etiketli cTnI proteini kullanılmıştır. Serum gibi karmaşık bir matristen cTnI'yı yakalayabilmek için, immunoaffinite zenginleştirme stratejisi kullanılmıştır.

Immunoaffinite zenginleştirmenin ilk adımı olarak, mikro (Dynabeads® MyOne™ ,1 μm) ve nano (Nanomag®-D, 130 nm) olarak iki farklı boyuta sahip manyetik parçacıklar seçilmiştir. cTnI proteinine bağlanabilen bir monoklonal antikor, her iki manyetik nanoparçacığa immobilize edilerek, cTnI'yı zenginleştirme verimleri karşılaştırılmıştır. Manyetik nanoparçacık (Nanomag®-D, 130 nm) kullanılarak, cTnI'ya ait seçilen iki triptik peptit pik alanları görece daha yüksek tespit edildiği için, manyetik nanoparçacık ilerleyen deneyler için seçilmiştir. Daha sonra, manyetik nanoparçacığın maksimum yükleme kapasitesi belirlenmiştir. Buna göre, 1 mg parçacığa 100 μg antikor eklendiğinde, 59.2 ± 5.7 μg/mg antikor bağlanabildiği bulunmuştur. Sentezlenen nanoparçacık-antikor kompleksi kullanılarak, 1 ml serumdan cTnI zenginleştirmesi için gereken miktar hesaplanmış ve analiz için 1 ml serumdaki cTnI'nin tamamının eldesi için, 10 μl kompleksi kullanımının yeterli olduğu tespit edilmiştir.

Optimizasyonlar sonucunda geliştirilen protein-tabanlı kalibrasyon stratejisini kullanan izotop seyreltme kütle spektrometrisi tandem kütle spektrometrisi (ID-LC-MS/MS) metodu, cTnI'nın 0,6 ile 24 μg/L arasında ($R > 0.996$) ölçülebilmesini sağlar. Tayin sınırı (LOQ) 1,8 μg/L ve algılama sınırı (LOD) 0,6 μg/L olarak belirlenmiştir. Ara kesinlik %9,6'nın altında ve tekrarlanabilirlik tüm kalite kontrol materyalleri için %2,0-%8,7 arasında bulunmuştur. Analiz edilen kalite kontrol materyallerinin doğruluğu %90-110 arasındadır. Toplam ölçüm belirsizlikleri (n=6), tüm seviyeler için %12,5'in altında bulunmuştur.

İkinci geliştirilen ID-LC-MS/MS metodu, peptit-tabanlı bir kalibrasyon stratejisi ile cTnI'yı insan serumundan tayin etmeyi sağlar. Bu metotta kalibrant olarak, cTnI'ya ait iki triptik peptit (TLLLQIAK and NITEIADLTQK) seçilmiş ve sentetik olarak elde edilmiştir. İç standart olarak, seçilen peptitlerin izotopik versiyonları kullanılmıştır. Peptit safsızlık düzeltme amino asit (PICAA) analizi ile sentetik peptitlere değer ataması gerçekleştirilmiş ve böylece SI-izlenebilir birincil peptit standartları üretilmiştir.

cTnI proteininin tam kesimini sağlamak ve proteoliz verimini artırmak için, tripsin kesim yöntemleri, enzim-protein oranı ve kesim süresi gibi optimizasyonlar yapılmıştır. En iyi tripsin kesim verimi, Filtre Destekli Numune Hazırlama (FASP) yöntemi kullanılarak, 1:10 enzim-protein oranında ve gece boyunca kesim ile elde edilmiştir.

Peptit-tabanlı kalibrasyon yaklaşımında aynı zamanda, kalibrasyon eğrisini oluşturmak için iki vekil matris kullanılmıştır. İki vekil matris, doğrusallık, doğruluk,

tekrarlanabilirlik, ara kesinlik ve gerçeklik gibi parametrelere göre değerlendirilmiştir. Her iki matrisin de benzer sonuçlar verdiği gözlemlenmiş ve performanslarının tutarlı olduğu tespit edilmiştir.

Geliştirilen peptit-tabanlı kalibrasyon stratejisini kullanan analitik yöntem, cTnI'nin 0.6–21.6 μg/L aralığında ölçülebilmesini sağlar. Ara kesinlik %28.9'un altında, tekrarlanabilirlik tüm konsantrasyon seviyelerinde %10'un altında bulunmuştur. Geri kazanım oranı, %72 ile %151 arasında değişkenlik göstermektedir.

Kalp krizi şüphesiyle hastaneye getirilen dört hasta serumu, geliştirilen metot ile ölçülmüş, immunoassay ile alınan sonuçlar arasında %50'den fazla farklılık bulunmuştur. Son olarak, peptit bazlı kalibrasyon stratejisi ve protein bazlı ölçüm stratejisi metot performansları bakımından karşılaştırılmıştır.

Sonuç olarak, bu tezde hedefli ve aşağıdan-yukarıya proteomik yöntemleri kullanarak, iki farklı ID-LC-MS/MS metodu geliştirilmiştir. Her iki metot da birbirleriyle verimlilik açısından karşılaştırılmıştır. Bu çalışmalar, metroloji topluluğunun yeni yaklaşımları benimsemesini ve belirli ihtiyaçlara göre uyarlanmış, SI izlenebilir peptit ve protein birincil standartları ile referans prosedürlerin geliştirmesini desteklemeyi amaçlamaktadır.

Geliştirilen yöntemlerin, dış kalite değerlendirme materyalleri ve ikincil referans malzemelerde (RM) cTnI konsantrasyonunun belirlenmesinde referans ölçüm prosedürü olarak hizmet etme veya referans ölçüm prosedürünün geliştirilmesine katkıda bulunma potansiyeli bulunmaktadır. Laboratuvarlar arası cTnI standardizasyonu ve uyumlaştırılması son derece karmaşık bir süreçtir. Ancak, IFCC WG-TNI, cTnI ölçümünün standartlaştırılabilir olduğuna inanmaktadır. Kardiyak troponin I'nın hasta yönetimindeki kritik rolü göz önüne alındığında, bu alandaki büyük çaba kesinlikle değerlidir. Önerilen ölçüm yöntemleri, IFCC WG-TNI'nın faaliyetlerini desteklemekte önemli bir rol oynayacaktır.

1. INTRODUCTION

1.1 Purpose of Thesis

Cardiovascular diseases are a serious healthcare problem that affects people all over the world. According to the World Health Organization, 17.9 million people die from cardiovascular diseases each year (World Health Organisation, 2024). Cardiac biomarkers, such as cardiac troponin I (cTnI), are used for diagnosis and prognosis and facilitate successful treatment of myocardial infarction. (Apple et al., 2017).

cTnI is a quite complicated biochemical analyte. Researchers have studied cTnI for years to better understand its biochemical characteristics and post-translational processing. Although cTnI is the gold standard for the diagnosis of acute myocardial infarction, there is currently no standardization among the many different diagnostic assays. The release of cTnI into the circulation is specific for cardiac injury, and cTnI levels in the blood are too low for direct measurement with current analytical techniques. Immunoassays have great benefits in that they are quick and easy. However, routine procedures lack standardization or harmonization, and in the vast majority of situations, the target molecule of the method is not well characterized. There is currently no metrological traceability to the International System of Units (SI) for cTnI measurement due to the lack of a higher-order RMP to standardize immunoassay cTnI results.

Traceable analytical/reference methods for cTnI would significantly enhance test performance and comparability. In a recently published potential RMP, the limit of quantification (LOQ) was determined to be 2 µg/L (Schneck et al., 2018). This thesis aims to develop a complementary approach using isotope dilution mass spectrometry (IDMS) to provide traceable quantification of cTnI with two different calibration strategies. In mass spectrometry analysis, the signal of high-abundance proteins suppresses the signal of low-abundance proteins, making it extremely challenging to analyze low-abundance proteins such as cTnI (da Costa et al., 2017). It was anticipated that an effective enrichment method developed within the scope of this thesis will

address this problem. The aim of this thesis is to develop analytical measurement methods using a targeted proteomic approach for cTnI measurement, thereby contributing to the harmonization of cTnI results across clinical laboratories, assigning target values to secondary CRMs, and supporting the reliable measurement of cTnI.

1.2 Literature Review

1.2.1 Proteomics

Proteomics involves the qualitative and quantitative analysis of proteomes to understand biological processes. It allows for the identification and quantification of entire protein sets in a sample (Tyagi et al., 2010).

Mass spectrometry is a pivotal methodology used to analyze complex protein mixtures, essential for studying both specific proteins and entire proteomes (Rappsilber, J., & Mann, M., 2002). Recently, mass spectrometry-based proteomics has been increasingly used to analyze body fluids such as blood, plasma, or serum, enhancing the investigation of cardiovascular diseases and thereby improving diagnosis, prognosis, and monitoring (Palstrøm et al., 2022). Innovative approaches are crucial for the early and accurate detection of patients, particularly in distinguishing those without the disease. It is essential for researchers to ensure thorough investigation and management not only for patients but also for those without severe illnesses. Integrating genomics, transcriptomics, proteomics, and metabolomics holds significant promise for discovering new biomarkers and developing assays (Apple et al., 2012).

There are two main strategies for proteomics research by mass spectrometry: "bottom-up," which analyzes peptides by proteolytic digestion, and "top-down," which analyzes intact proteins (Chait, 2006).

1.2.1.1 Bottom-up proteomics

Bottom-up proteomics allows for the measurement of peptides, thereby facilitating the identification and quantification of proteins (Plubell et al., 2022). This method starts with lysing and extracting proteins from cells, tissues, or body fluids. These proteins are then digested into peptides, which are more manageable for sample processing and MS. Given the large number of peptides produced, techniques like prefractionation or

enrichment are often used to simplify the sample before MS analysis. The peptides are separated via liquid chromatography (LC), and the resulting eluted peptides are analyzed by MS. The mass spectrometer measures the mass-to-charge ratios of ionized peptides in the gas phase. These measurements, combined with tandem MS (MS/MS) spectra, are used for database searches to identify peptide sequences and corresponding proteins (Altelaar et al., 2013). There are two primary approaches for peptide measurements using MS: untargeted proteomics and targeted proteomics (Palstrøm et al., 2022).

1.2.1.2 Targeted proteomics

Targeted proteomics is a robust technique for quantifying proteins using mass spectrometry in clinical applications, biomedical research, and systems biology (Shi et al., 2016). Unlike screening proteomics, which does not require prior knowledge of the proteins, targeted proteomics requires pre-existing information about the analytes to be analyzed (Borràs & Sabidó, 2017). This approach is particularly effective for analyzing and quantifying specific biomarkers. The main targeted proteomics methods include selected reaction monitoring or multiple reaction monitoring (MRM), parallel reaction monitoring (PRM), and data-independent acquisition (DIA) with targeted data extraction from MS/MS spectra. MRM and PRM fall under data-dependent acquisition (DDA) techniques (Shi et al., 2016).

MRM is a widely used targeted proteomics technique for the specific analysis and absolute quantification of biomarkers, including proteins and peptides in serum, plasma, and other biological samples. This method involves isolating the peptide precursor (parent ion) and fragmenting it to produce product ions (fragment ions), where the signal intensities of these product ions demonstrate the abundance of the peptide in the sample. The specificity of the MRM approach is due to the MRM chromatogram, which only displays signals for specific mass ions (Shi et al., 2016).

PRM is an effective method for the absolute quantification of target proteins in complex matrices. PRM offers analytical performance comparable to MRM, including sensitivity, reproducibility, accuracy, and dynamic range. Unlike MRM, which typically uses triple-quadrupole instruments, PRM employs high-resolution Orbitrap mass spectrometers. One drawback of targeted MRM analysis is the necessity of prior knowledge about the peptides of interest, specifically the transitions of the fragment

ions. PRM overcomes this limitation by enabling simultaneous monitoring of the product ions of a targeted peptide with high resolution, eliminating the need for prior knowledge of the target transitions (Palstrøm et al., 2022).

1.2.2 MS-based serum proteomics

Blood plays a crucial role in representing overall health. It is considered easily accessible and minimally invasive, providing an excellent resource for proteome analysis (Ignjatovic et al., 2019). However, serum samples present significant challenges for proteomic profiling due to its complexity and dynamic range. Serum contains approximately 15.000 protein, with concentrations ranging from 60-80 mg/mL, resulting in high complexity and dynamic range.

Plasma and serum hold great potential for disease diagnosis and biomarker discovery. However, the issue is that biomarkers are present in very low amounts in serum and plasma. Proteins such as albumin, IgG, IgA, and haptoglobin constitute almost 90% of serum, while the remaining 10% consists of moderate and low-abundance proteins. Of these, only 1% are low-abundance proteins that are used or have the potential to be used as biomarkers. In mass spectrometry analysis, the signal of high abundant proteins in serum suppresses the signal of low-abundance proteins, making the analysis of clinically important biomarkers nearly impossible in proteomics-based analysis (da Costa et al., 2017). Strategies have been developed to address these challenges.

1.2.2.1 Immunoaffinity enrichment

One approach to addressing serum complexity involves the selective capture of low-abundance proteins and peptides using functionalized magnetic particles (or beads). This strategy focuses on specific proteins or peptides. This method targets specific proteins or peptides through the conjugation of magnetic particles with antibodies. The magnetic particle-antibody complex is mixed with the complex biological matrices, where it selectively captures the target antigens. These captured antigens can then be isolated using an external magnetic force, such as a magnetic separator (Schneck et al., 2015). This enrichment process enables the specific proteins to be analyzed by mass spectrometry (Figure 1.1).

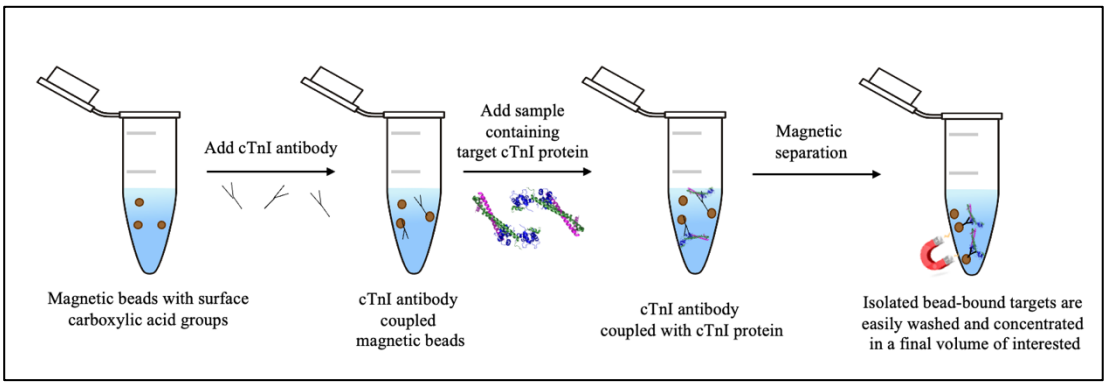

Figure 1.1 : Schematic representation of cTnI antibody immobilization on magnetic beads and the isolation of the target cTnI protein from a complex biological matrix.

The effectiveness of magnetic separation is influenced by several factors, including the physicochemical properties of antibodies or magnetic particles, the method of antibody immobilization, the surface functionality of magnetic particles, and the stability of the antibody-magnetic particle conjugates. One crucial factor affecting effectiveness is the size of the magnetic beads. Commercially available magnetic particles come in various sizes, with microbeads around 1 micron being commonly used for enriching biomarker proteins from low-abundance biological matrices. These microbeads are favored due to their low size variation and a wide range of surface functionalities that facilitate straightforward antibody coupling. However, smaller magnetic particles, less than 1 micron, can theoretically enhance capture efficiency for low-abundance proteins due to their higher surface area-to-volume ratio (Schneck et al., 2016).

As an example of peptide enrichment, Kuhn et al. developed peptide immunoaffinity enrichment coupled with stable IDMS assay for the quantification of cTnI and interleukin 33. This method can detect and quantify changes in concentration of proteins present at 1–10 µg/L in plasma (Kuhn et al., 2009).

In 2018, Schneck et al. developed an isotope dilution liquid chromotograpy tandem mass spectrometry (ID-LC-MS/MS) method for quantification of cTnI in human plasma samples using an immunoaffinity enrichment approach. In this study, researchers aimed to enrich intact cTnI protein and were able to quantify cTnI concentrations as low as 2 µg/L cTnI concentration. (Schneck et al., 2018). Palstrøm et al. recently used small molecule affinity-based probes based on agarose-immobilized p-aminobenzamidine, 8-amino-hexyl-ATP, 8-amino-hexyl-cAMP, and

O-phospho-L-tyrosine to enrich lower abundant proteins. They demonstrated the success of the immunoaffinity enrichment method for the removal of high-abundance plasma proteins and selective enrichment of low-abundance plasma proteins compared to immuno-depletion. Researchers also managed to enrich cTnI using 8-amino-hexyl-cAMP in this manner (Palstrøm et al., 2020).

1.2.2.2 Immunodepletion of serum

Due to their high abundance, proteins such as albumin, immunoglobulin, and haptoglobin have been targeted for removal from serum samples before proteomic analysis. This methodology relies on the specific interaction between antibodies and the most abundant proteins in serum. Many immunodepletion kits are commercially available for the removal of these abundant proteins. For example, Multiple Affinity Removal System (MARS; Agilent, CA, USA) columns are used to remove top six, seven or fourteen most abundant proteins; Seppro IgY14 kit (Millipore, MA, USA) is used for the removal of the fourteen most abundant proteins; Pierce™ depletion columns remove the top two or twelve most abundant proteins; and the ProteoPrep kit (Sigma, MO, USA) is used for the specific capture of two or the twenty most abundant proteins.

Considering performance, these immunodepletion kits generally have >90% efficiency and reproducibility (Lee et al., 2019). However, immunodepletion strategies are not always applicable. Some low-abundance proteins interact with high abundant proteins. Therefore, when high abundant proteins are removed from the serum, the relevant biomarker protein may also be lost. Because of this, other approaches may be better alternatives if the proteins of interest interact with high-abundance proteins (Palstrøm et al., 2022).

1.2.3 Cardiovascular biomarkers

Cardiovascular diseases are a serious healthcare problem that affects people all over the world. According to the World Health Organization, 17.9 million people die each year, making it the number one cause of death globally (World Health Organisation, 2024). Early detection of cardiovascular diseases, such as myocardial infarction, is significantly important for diagnosis, prognosis, successful treatment, and prevention of death. Biomarkers mostly indicate biochemical changes in tissue or blood. Therefore, they play a critical role in the early detection and evaluation of diseases and

in the development of drug treatments for disease states (Dhingra & Vasan, 2017). There is a long process from the initial discovery of biomarkers until they are validated and approved for daily clinical testing. Currently, official approval by the Food and Drug Administration (FDA) has only been obtained for cardiac troponin T (cTnT), cTnI, and B-type natriuretic peptide as cardiac biomarkers (Torma, 2018).

cTn is a protein found in heart tissue that is essential for regulating skeletal and cardiac muscle contraction. The troponin complex in the heart consists of three subunits: cTnT, cTnI, and troponin C (cTnC), which are held together by non-covalent interactions. cTnI is composed of 209 amino acids and has a molecular weight of approximately 23,918 Da. The amino acid sequence of cTnI is shown in Figure 1.2. The structure of cTns is shown in Figure 1.3.

```
>sp|P19429|TNNI3_HUMAN Troponin I, cardiac muscle OS=Homo sapiens
MADGSSDAAREPRPAPAPIRRRSSNYRAYATEPHAKKKSKISASRKLQLKTLLLQIAKQE
LEREAEERRGEKGRALSTRCQPLELAGLGFAELQDLCRQLHARVDKVDEERYDIEAKVTK
NITEIADLTQKIFDLRGKFKRPTLRRVRISADAMMQALLGARAKESLDLRAHLKQVKKED
TEKENREVGDWRKNIDALSGMEGRKKKFES
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Figure 1.2 : Amino acid sequence of cTnI (UniProt database entry P19429).

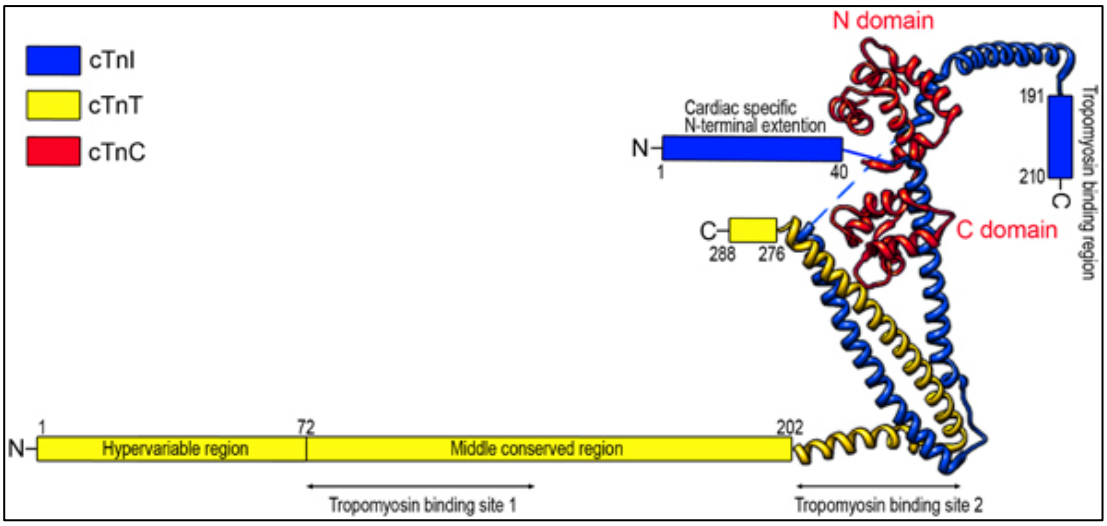

Figure 1.3 : The structure of cardiac troponins. Blue indicates cTnI; yellow represents cTnT; red shows cTnC (Sheng & Jin, 2014).

For many years, cTnI has been used clinically for the diagnosis and risk assessment of acute myocardial infarction. High levels of cTns are linked to cardiac muscle damage. The release of cTnI into the blood is specific to cardiac damage. Current procedures

in the triage of potential acute myocardial infarction patients involve evaluating the measurement of cTnI levels together with patients' symptoms, medical history, and electrocardiographic abnormalities (Bayoumy et al., 2021).

cTnI is a quite complicated biochemical analyte. Researchers have studied cTnI for years to better understand its biochemical characteristics and posttranslational processing. Although cTnI is the gold standard for the diagnosis of acute myocardial infarction, there is currently no standardization among the many different diagnostic assays. The release of cTnI into the circulation is specific for cardiac damage, and the levels of cTnI in the blood are too low for direct measurement by current analytical techniques. As the concentration is quite low (µg/L-ng/L range), current clinical measurement methods are indirect and based on immunochemical measurements, such as the ELISA method (Schneck et al., 2018). Immunoassays have great benefits in terms of being quick and easy. However, immunoassays often suffer from nonspecific antibody binding and lack of standardization. They require specific antibodies, calibrators, and detection techniques, which leads to variations among different assays (Hoofnagle et al., 2021).

1.2.3.1 Current cTnI assays, their problems and possible solutions

cTnI is a protein consisting of 210 amino acid residues and has a unique N-terminal sequence. It can undergo both C- and N-terminal proteolytic degradation and exist in various oxidized, reduced, and phosphorylated states, whether free or in complex forms. In the bloodstream, cTnT and cTnI can be found in several forms, including triple complexes (cTnC-cTnT-cTnI), binary complexes (cTnI-cTnC), and as free molecules. cTnI is highly susceptible to proteolysis and other enzymatic modifications. Various forms of cTnI have been recognized, including proteolysed, heparin-bound, phosphorylated, oxidized, and reduced forms. Degradation of cTnI can occur both in vivo and in vitro, affecting the stability of cTnI measurements. During the early phase following a myocardial infarction, the rapid release of cTnI, ongoing modifications in myocardial tissue and blood, and varied clearance mechanisms result in a wide range of cTnI isoforms. Consequently, it is hardly possible that blood samples from two different patients will show the same cTnI isoform composition (Apple, 2012).

Unlike cTnT, there are numerous cTnI tests on the market. Antibodies can detect multiple variations of cTnI in the blood, targeting specific epitopes. The use of

different versions of the cTnI antigen as standards or calibrators has significantly improved correlations between commercial assays, although achieving standardization remains challenging. An International Federation of Clinical Chemistry (IFCC) working group is investigating whether cTnI harmonization can be achieved using pooled serum-based secondary reference materials (RMs). Despite these efforts, harmonization between tests remains difficult. The primary cause of measurement differences is the epitope specificity of antibodies used in various tests. Even when using the same antibodies, various numeric concentrations are discovered. Multiple factors influence cTnI measurement, including proteolytic degradation, phosphorylation, oxidation, complexing with cTnC, heparin, heterophile or human antimouse antibodies, and cTnI-specific autoantibodies. The great majority of cTnI in blood (95%) is found as a binary cTnI–cTnC complex. All circulating cTn types should be recognized by antibodies used in tests (Apple et al., 2017). Although complete standardization may be unattainable due to the unique characteristics of cTnI, developing an SI traceable reference measurement system is a logical and necessary step toward harmonizing the results of different test kits.

1.2.3.2 Metrological traceability

As defined by the International Vocabulary of Metrology (VIM), metrological traceability or measurement traceability is a "property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty." (ISO/IEC Guide 99). Modern metrology relies on the SI units, which includes seven fundamental units: kilogram, meter, second, mole, ampere, kelvin, candela. To address the variations in measurement results for the same sample due to different measurement methods, locations, or times, a standardization initiative was started (Vesper et al., 2016; White, 2011). The establishment of reference measurement systems ensures traceability to certified reference materials (CRMs) that are directly connected to one of the seven SI units through an unbroken chain of comparisons with fully specified uncertainties (White, 2011). Value assignment of the primary standard using the primary reference method is a crucial step in developing SI traceable reference measurement systems. The International Organization for Standardization (ISO) issued guidelines for biological quantity measurement. According to ISO 17511, calibrators and control

materials used in in vitro diagnostic devices must achieve traceability to the highest order RMs available (International Organization for Standardization, 2020).
The standardization of quantitative measurements in laboratory medicine requires the consistent definition and implementation of a reference measurement system for the calibration and validation of routine methods. Such a structure is based on metrological traceability concepts and a hierarchy of analytical measurement procedures.

1.2.3.3 Reference measurement system

A metrologically correct approach to the standardization and traceability of cTnI measurements relies on the availability of a reference measurement system (Figure 1.4). This system includes the following steps: (1) a purified RMs with values assigned by mass determination, (2) a higher order reference procedure for the value assignment of secondary RMs, and (3) matrix-based (commutable) RMs, represented by a panel of carefully selected and characterized human pooled serum samples. The reference measurement system links higher-order reference methods and RMs to routine calibrators and procedures used in clinical laboratories through an unbroken, metrologically-based traceability chain. When suitable RMs are available, these materials and the manufacturer's testing procedures can be used by industry to assign values to working and product calibrators via a value transfer process. Clinical laboratories using routine procedures with validated calibrators to measure patient samples through this calibration process will acquire standardized and traceable values, with no calibration bias among the commercial procedures (Tate et al., 2010; Wu & Christenson, 2013).

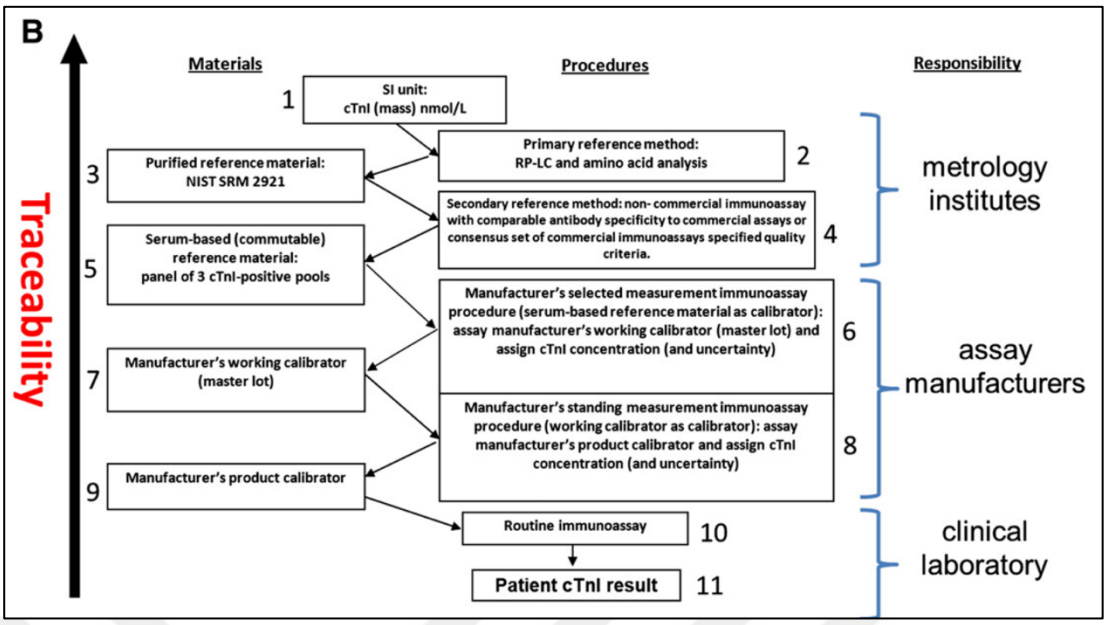

Figure 1.4 : SI traceable reference measurement system for standardization of cTnI measurement and hierarchy of analytical measurement procedure & different kinds of reference materials. SI, International System; RP-LC, reverse-pahse chromatography; NIST SRM (Tate et al., 2010; Wu & Christenson, 2013).

To provide reliable and comparable quantifications in clinical laboratories, the EU regulation 2017/746 on in vitro diagnostic medical devices indicates clearly that "the metrological traceability of values assigned to calibrators and/or control materials shall be assured through suitable reference measurement procedures and/ or suitable CRMs of a higher metrological order." (EU Commission, 2017). Morever, the standard EN ISO 17511 demands reference measurement systems including reference measurement procedures for the determination of analytes in samples of human origin (International Organization for Standardization, 2020). Biomarker such as cTn fit this definition, and, thus, the mentioned regulations and standards will apply.

1.2.4 Isotope dilution mass spectrometry

IDMS is an analytical methodology for absolute quantitative analysis. IDMS is based on two components; mass spectrometry and stable isotope labelled (SIL) internal standards (IS). IDMS involves the addition of SIL-IS to the sample, followed by MS analysis of both the analyte and the IS. The isotopically labelled standard is used to alleviate for variations in analytical performance over time and between laboratories that affect analyte levels. The combination of the sensitivity and selectivity of MS with the accuracy and precision of SIL-IS provides reliable quantitative MS data (Villanueva et al., 2014).

IDMS is also extensively used in quantitative proteomics with the help of isotope labeled protein or peptide standards. The use of an IS for method calibration improves the precision of a quantitative measurement. A constant amount of labeled standard is added to all analyzed standards and samples for internal calibration. Both the analyte and the IS signals are monitored during the analysis, with the ratio of their intensities being proportional to the analyte concentration. Under ideal conditions, the observed ratios remain unaffected by temporal variability, MS instrument response factors, matrix effects, solution volume errors, and variable analyte recovery (Villanueva et al., 2014).

In the IDMS study, the quantification of the protein of interest is performed using a calibration curve generated with the heavy (SIL-IS) and light standards (natural peptide or protein), as described in the section entitled “calibration system”.

1.2.4.1 Quantification of cTnI by ID-LC-MS/MS

The biggest obstacle to the use of cTnI in primary care is the lack of standardization in existing assays. To address this issue, a matrix-based CRMs can be use to provide cTnI measurement traceability and sample comparability.

ID-LC-MS/MS techniques are considered the ‘gold-standard’ for the absolute quantification of proteins due to their high levels of accuracy and specificity, compared to immunoassays, which offer greater sensitivity. ID-LC-MS/MS requires the use of an isotope-labeled protein internal standard analogous to the target protein, which can compensate biases associated with sample processing and instrument variability. For this reason, direct measurements based on this method are more accurate and specific. Although LC-MS/MS-based assays are not currently used in routine clinical practice, this improved technique can be employed to develop CRMs. The development of CRMs, which are fundamental to creating a traceability chain for clinical quantitative measurements, requires the highest degree of accuracy and specificity (Schneck et al., 2018).

In 2009, Keshishian et al. reported the use of strong cation-exchange chromatography in depleted patient plasma to measure cardiovascular biomarkers using a stable isotope dilution approach. In this study, low abundance proteins were measured below 50 ng/ml using MRM assay. The advantage of this technique is that it provides an antibody-free approach (Keshishian et al., 2009). In the same year, Kuhn et al. reported a novel method to quantify troponin I in plasma using peptide immunoaffinity

enrichment coupled with stable IDMS. According to this method, peptides were enriched from digested human plasma using antipeptide antibodies. This method can detect and quantify proteins at 1-10 ng/mL (Kuhn et al., 2009). In 2012, Huillet et al. studied PSAQ™ method (Protein Standard Absolute Quantification) to quantify cardiovascular biomarkers such as troponin I in human serum samples. The combination of PSAQ and selected reaction monitoring provides highly accurate biomarker quantification. Using immunoaffinity enrichment with Dynabead M-280 Streptavidin and SDS-PAGE for capturing cTnI, LOQ was detected at 5.5 ng/mL (Huillet et al., 2012).

In 2018, Schneck et al. developed a specific and sensitive ID-LC-MS/MS method for the measurement of cTnI in human plasma samples using an immunoaffinity enrichment approach. In this study, researchers were able to quantify 4.86-11.3 ng/ml cTnI concentration. For the immunoaffinity enrichment approach, researchers used amine functionalized silica coated magnetic nanoparticles they synthesized themselves, which means these nanoparticles are not commercially available (Schneck et al., 2018).

1.2.4.2 Protein-based vs. peptide-based calibration approaches

As LC-MS methodology relies on analyzing proteolytic peptides, various calibration approaches using different calibrators and internal standards have been developed. Many different calibration approaches at peptide and protein levels exist in the literature (Asicioglu et al., 2023; Huillet et al., 2012; Huynh et al., 2021; Keshishian et al., 2007; Schneck et al., 2018). The selection of calibrators and SIL-IS is crucial for ensuring the reliability and accuracy of LC-MS-based quantification (Nouri-Nigjeh et al., 2014).

The protein-based calibration method uses full-length proteins as calibrators and SIL full-length proteins as IS, which are spiked directly into samples prior to sample preparation. This approach offers high accuracy and precision by correcting for system bias and variations introduced during any sample preparation and enzymatic digestion steps, making it the “Gold Standard” for LC-MS based targeted protein quantification. From a metrological point of view, adding the calibrant prior to sample preparation ensures the traceability chain.

However, a significant drawback of this method is that producing full-length SIL-proteins with high isotope purity can be both costly and time-consuming, making it impractical for many protein classes (Nouri-Nigjeh et al., 2014).

Peptide-based calibration approach is a common technique that uses a synthesized peptide as the calibrator and a SIL analog of the synthetic peptide as IS, which is added after digestion. This method facilitates the straightforward development of quantitative assays, with both the calibrators and SIL-IS being commercially available. However, using an SIL-peptide as the IS only corrects for variations during the LC-MS analysis and does not account for variations in upstream steps like sample preparation and digestion. Additionally, this approach calculates protein concentrations based on the measured concentrations of the synthetic peptides in the digest, assuming near 100% efficiency in sample preparation and digestion (Nouri-Nigjeh et al., 2014). Another drawback of peptide-based calibration approach is the need for a correction factor due to issues like incomplete digestion, poor enrichment, and antibody selectivity. This is requires to careful monitoring over time, across different laboratories, and in various matrices to ensure its reliability. As a result, this method can introduce significant biases that may not be evident when validated with synthesized peptides spiked into matrix digests. When examined with protein-spiked quality control samples, peptide-level calibration can result in considerable quantitative bias. Furthermore, selecting two unique synthetic peptides for quantifying a single protein can yield highly discordant results for the same protein. Another drawback, from a metrological point of view, is that adding the calibrant just before the LC-MS analysis breaks the traceability chain.

Both approaches offer advantages and disadvantages in different ways. Still, both protein-based and peptide-based calibration approaches are significant for protein quantification by ID-LC-MS systems.

1.2.4.3 Peptide impurity corrected by amino acids (PICAA) analysis

PICAA analysis method is utilized to accurately determine the concentration of synthetic peptides and establish primary peptide calibrators by combining IDMS with HPLC systems. This approach involves quantifying constituent amino acids following hydrolysis through Amino Acid Analysis (AAA) and correcting for impurities. The initial step involves quantifying specific amino acids after peptide hydrolysis, which helps determine the peptide concentrations. The second step focuses on identifying and quantifying impurities that may interfere with amino acid quantification or the use of the primary standard for calibration (Josephs et al., 2019).

1.3 Objectives

This study involves the development of ID-LC-MS/MS methods for cTnI measurement using targeted proteomics approach. In the scope of the thesis: i) Using a targeted proteomics and protein-based calibration strategy, an SI-traceable ID-LC-MS/MS method was developed for quantifying cTnI in human serum. For this, a suitable immunoaffinty enrichment strategy was developed and validated. ii) An ID-LC-MS/MS-based analytical procedure was developed and validated for the quantification of cTnI in human serum using a peptide-based calibration.

1.3.1 Objective 1: Development of an ID-LC-MS/MS method using targeted proteomics for cTnI quantification by protein-based calibration

The first part of this thesis focused on developing a metrologically traceable measurement method for quantifying cTnI from serum using a targeted proteomics approach with a protein-based calibration strategy. To achieve this, an immunoaffinity enrichment strategy was employed, utilizing magnetic beads to selectively capture and isolate the cTnI protein from complex biological matrices.

Immunoaffinity enrichment strategy is widely used approach to selectively capture biomarkers of interested (Neubert et al., 2020). Magnetic particles coupled with antibodies can evenly disperse within biological matrices in the absence of an external magnetic force. During the mixing process, the antibody-coated beads specifically bind to the target protein biomarker. These complexes are then separated from the serum using a magnetic separator, allowing for the enriched biomarker to be detected via mass spectrometry. (Schneck et al., 2015).

Magnetic particles can be functionalized with various surface chemistries, such as amino, carboxyl, tosyl, or hydroxyl groups, to facilitate the attachment of antibodies. When developing enrichment protocols, selecting a magnetic particle system with high antibody coupling efficiency is crucial. Microbeads like Dynabeads® (1 µm) are commonly used for capturing protein biomarkers because they are commercially available with different surface functionalities for easy antibody attachment. However, nanoparticles (e.g., Nanomag®-D, 130 nm) offer even higher capture efficiency due to their smaller size, which provides a greater surface area-to-volume ratio, significantly enhancing protein adsorption (Schneck et al., 2016).

In this thesis, both Dynabeads® MyOne™ (1 µm) and Nanomag®-D (130 nm) were selected for the enrichment of cTnI from serum. These beads were choosen for their rapid binding chemistry, low non-specific binding, and the ability to covalently bind antibodies through COOH surface functionalities. The selection of Nanomag®-D was particularly motivated by its higher surface area-to-volume ratio, which is advantageous for capturing low-abundant proteins like cTnI.

After establishing the immunoaffinity enrichment process, the thesis moved on to the development of a metrologically traceable measurement method using a targeted proteomics approach with a HR-MS coupled with NanoLC system. This method employed a protein-based calibration strategy, which involves using a protein calibrant and SIL-IS. An advantage of this approach is that the protein internal standard, the calibrant protein, and the endogenous protein in patient samples all undergo the same sample processing steps, eliminating issues such as incomplete protein extraction or processing. This makes the method highly accurate and reliable, often considered the gold standard in IDMS. However, there are challenges associated with this approach, including the lack of metrologically traceable protein calibrants for every analyte and the difficulty and expense of obtaining SIL protein internal standards. Despite these challenges, this approach was pursued to ensure high analytical performance in the quantification of cTnI from serum.

The analytical workflow begins with immobilizing anti-cTnI antibodies onto magnetic nanoparticles to form complexes. These complexes are used to isolate cTnI from serum. Next, trypsin is used to enzymatically digest the isolated cTnI. Finally, the measurement of multiple cTnI peptides is done simultaneously using ID-LC-MS/MS. Schematic diagram of the protein-based calibration strategy showed in Figure 1.5. The

method was optimized for complete cleavage and enhanced proteolysis yield, considering factors such as digestion methods, enzyme-to-protein ratio, and digestion kinetics. The final method enabled the quantification of cTnI in the range of 0.6 to 24 µg/L ($R > 0.996$), with a LOQ of 1.8 µg/L and a limit of detection (LOD) of 0.6 µg/L. The method demonstrated excellent precision and accuracy, with intermediate precision ≤ 9.6% and repeatability between 2.0% and 8.7% for all quality control materials. The accuracy of the analyzed materials was between 90% and 110%, and the total measurement uncertainties (n=6) were found to be ≤ 12.5% for all levels. The analytical method demonstrated high analytical performance in accurately quantifying cTnI levels in human serum. The proposed analytical method has the potential to facilitate the harmonization of cTnI results between clinical laboratories, assign target values to secondary CRMs and support reliable measurement of cTnI.

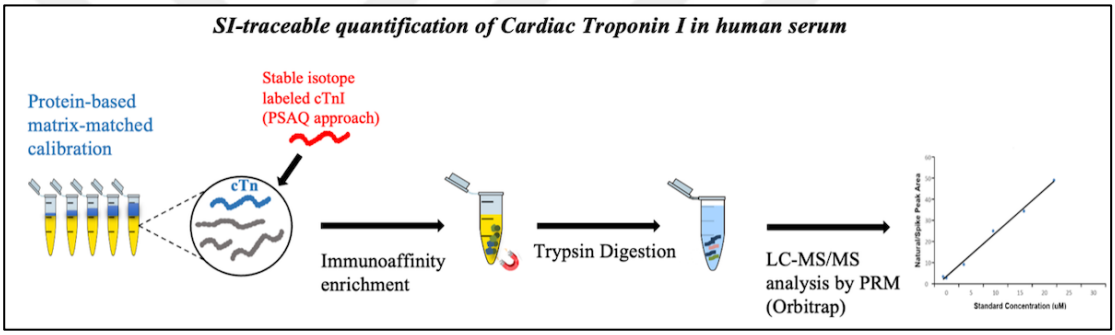

Figure 1.5 : SI traceable quantification of cTnI in human serum using protein-based calibration strategy (Asicioglu et al., 2023).

1.3.2 Objective 2: An ID-LC-MS/MS-based analytical procedure for the quantification of cTnI in human serum using a peptide-based calibration.

The second part of this thesis focused on the development and validation of an ID-LC-MS/MS-based analytical procedure for the quantification of cTnI in human serum using a peptide-based calibration strategy. This approach designed cheaaper and more accesible alternative to protein-based calibration approach for the measurement of cTnI.

This section of the research involved producing SI-traceable primary peptide calibrators to develop a quantification method. The process began with the selection and characterization of cTnI peptides (TLLLQIAK and NITEIADLTQK), followed

by the PICAA analysis to determine the absolute content of the peptide standards, ensuring traceability to SI units.

For the characterization of selected cTnI peptides (TLLLQIAK and NITEIADLTQK), the ID-LC-MS-AAA method was employed. Total AAA provided an absolute quantification of the peptide content using gas-phase hydrolysis. Intact mass LC-HRMS analysis was performed to separate impurities from the target peptides and identify them based on their exact mass. Then, LC-MS/MS analysis was carried out to confirm the peptide-related impurities and their identity through the fragmentation spectrum. Intact mass analysis showed the presence of different peptide impurities and various isoforms of the target peptide. Eight impurities were identified in TLLLQIAK, while four impurities were identified in NITEIADLTQK. The molarity value of the TLLLQIAK peptide and its corresponding expanded uncertainty in the primary stock solution was 439.51± 17.25 μM. The molarity value of the NITEIADLTQK peptide and its corresponding expanded uncertainty in the primary stock solution was 352.18±12.88 μM. The uncertainty of measurement has been calculated according to the EURACHEM/CITAC Guidelines Quantifying Uncertainty in Analytical Measurement (Josephs et al., 2019). A schematic diagram of the PICAA strategy used for preparing the primary standard for TLLLQIAK and NITEIADLTQK peptides is shown in Figure 1.6.

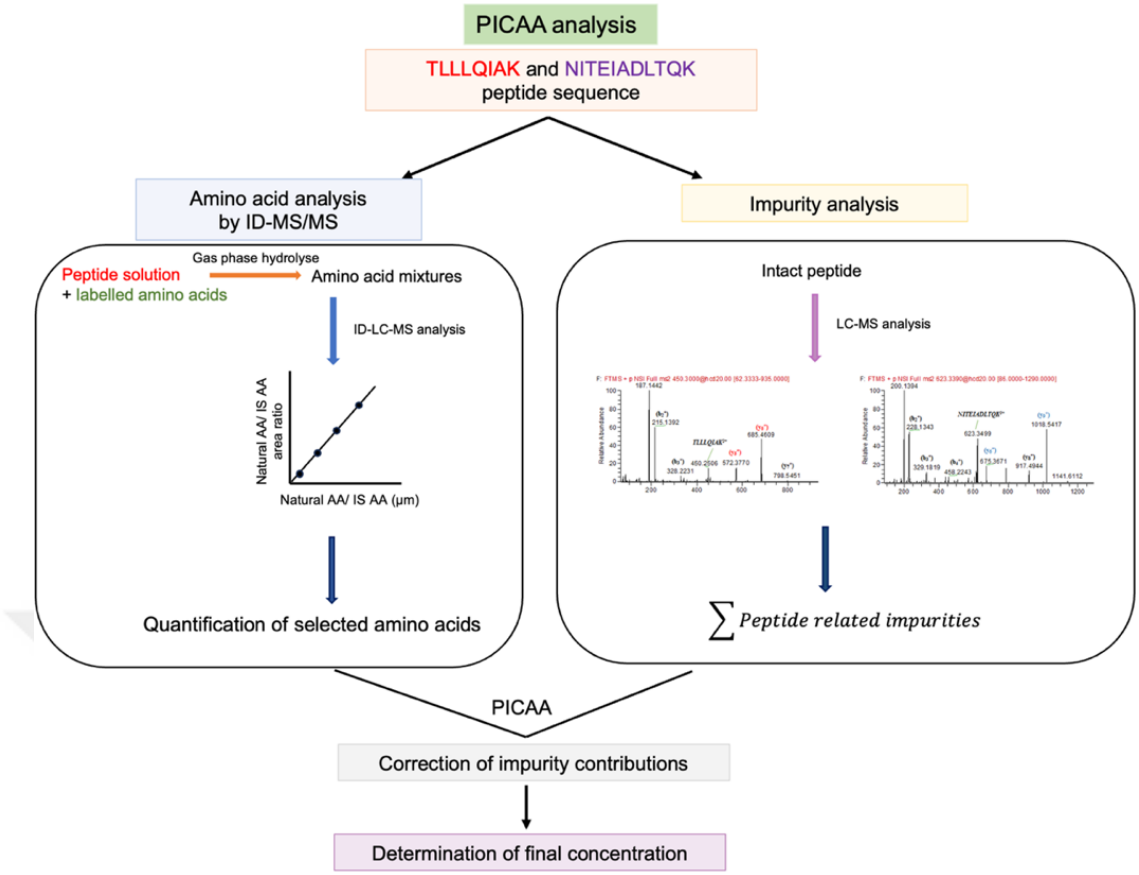

Figure 1.6 : PICAA strategy for preparation of the TLLLQIAK and NITEIADLTQK peptides primary standard (modified from Huynh, 2021; Oztug et al., 2024).

Although the use of SIL protein internal standard in ID-MS studies is considered the gold standard, it is quite expensive and difficult for every laboratory to obtian. On the other hand, a peptide-based calibration strategy has the significant advantage that synthetic peptides and their isotopically labeled versions are inexpensive to synthesize, making them more accessible. However, the disadvantage is that it does not account for variations arising from sample processing.

After procuding SI-traceable primary peptide calibrators, using these calibrator peptides and their SIL versions, second ID-LC-MS/MS method was developed. To further enhance the method's accuracy, two types of surrogate matrices were compared in the nanoLC-PRM-MS system, with parallelism between the calibration standards and the cTnI in the biological matrix being demonstrated. Schematic diagram of the peptide-based calibration strategy showed in Figure 1.7.

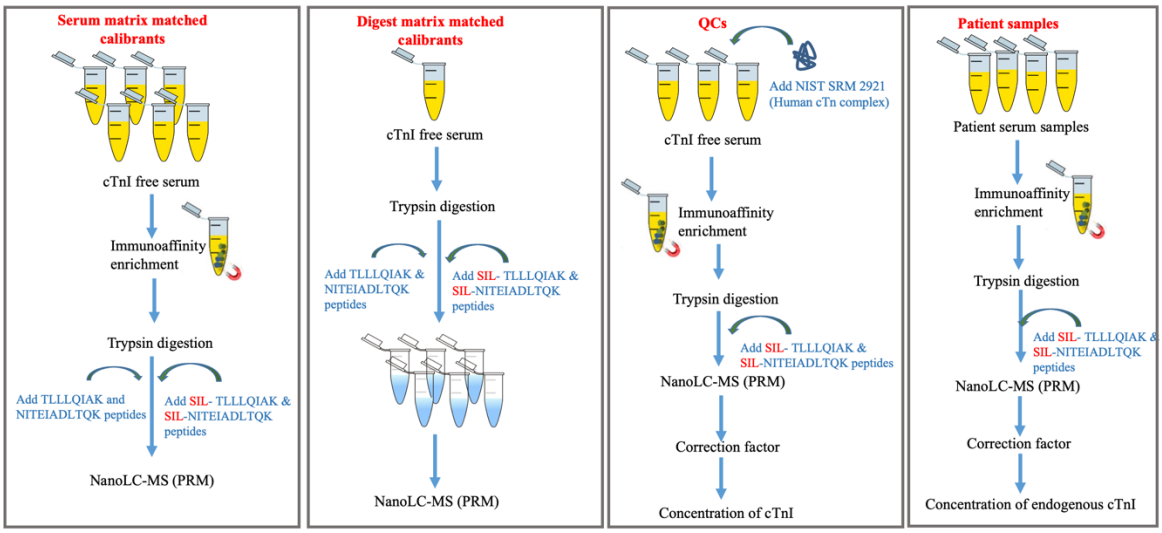

Figure 1.7 : SI-traceable quantification of cTnI in human serum using peptide-based calibration strategy (modified from Huynh et al., 2021).

The analytical method was allowed for quantification of cTnI within the range of 0.6–21.6 µg/L. Intermediate precision RSD was less than 28.9%, and repeatability RSD was less than 10% across all concentration levels. The recovery rate ranged between 72% and 151%.

Overall, this part of the thesis aimed to address the limitations of existing methods by offering an alternative approach that is more accessible to laboratories. In the final step, protein- and peptide-based calibration strategies were compared and discussed in terms of their efficiency in detecting low-abundance proteins such as cTnI.

2. MATERIALS AND METHODS

2.1 Objective 1: Development of an ID-LC-MS/MS Method Using Targeted Proteomics for cTnI Quantification by Protein-based Calibration

2.1.1 Chemicals and materials

The cTnI-free serum, a monoclonal antibody against human cTnI (19c7), human Cardiac Troponin I-T-C Complex (8T62) were purchased from Hytest Ltd. was purchased from Hytest Ltd. (Turko, Finland). Human cTn Complex Standard Reference Material (SRM) 2921 was obtained from National Institute of Standards and Technology (NIST, Gaithersburg, USA). SIL-protein was obtained from Promise Proteomics (Grenoble, France). The SIL version of the monoclonal antibody's peptide, SIL-DLPSPIER (>95% purity $^{13}C_6$, $^{15}N_4$) was purchased from New England Peptide Inc (Gardner, MA, USA) as powder. Magnetic dextran nanoparticles (nanomag®-D) with particle diameters of 130 nm and the surface functionalities COOH for the covalent binding of the antibody were purchased from Micromod Partikeltechnologie GmbH (Rostock, Germany). Dynabeads® MyOne™ Carboxylic Acid with particle size of 1 μm were obtained from Invitrogen (Carlsbad, CA, USA). 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride) (EDC), N-hydroxysuccinimide (NHS) and Pierce™ Trypsin/Lys-C Protease Mix, MS Grade, were obtained from Thermo Scientific™ (Rockford, Illinois, USA). Filter-Aided Sample Preparation (FASP) Protein Digestion Kit was obtained from Abcam (Boston, MA). Solu-Trypsin Rapid Digestion Kit (cat# MSKT0002), 2-(N-morpholino)ethanesulfonic acid (MES) (cat#4432-31-9), phosphate buffered saline (PBS) (cat #P4417), iodoacetamide (IAA) (cat #I1149), tris(2-carboxyethyl)phosphine (TCEP) (cat #75259), dithiothreitol (DTT) and 2-chloroacetamide (CAM) (cat #C0267) were purchased from Sigma-Aldrich (Darmstadt, Germany). Rapigest™ SF was obtained from Waters Corporation (Massachusetts, USA). Acetonitrile (ACN) (cas #75-05-8) was purchased from Supelco. Formic acid (FA) (cas #56302) and ammonium bicarbonate (cat #09830) was obtained from Fluka. Sodium chloride (NaCl) (cat #A2942) was purchased from

Panreac AppliChem GmbH (Ottoweg, Darmstadt). Tween©-20 (cat #A4974) was obtained from AppliChem GmbH (Ottoweg, Darmstadt).

Q-Exactive™ HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Bremen, Germany) were applied for mass detection. Easy-Spray™ ES902 C18 analytical column (75µm × 25 cm, 2 µm) were obtained from Thermo Scientific, Waltham, MA, USA. For LC, Dionex UPLC™ system and Dionex™ Ultimate 3000 system were applied (Thermo Scientific, Bremen, Germany). Savant™ SPD131DDA SpeedVac™ Concentrator coupled with Savant™ Refrigerated Vapor Traps (cat #RVT400) were used as a concentrator from Thermo Fisher Scientific Inc.

2.1.2 Comparison of magnetic particles efficiency

The monoclonal antibody clone 19C7 from Hytest in Finland was selected for this study because it demonstrated the highest binding affinity for intact cTnI among the six previously screened monoclonal cTnI antibodies (Lowenthal et al., 2011). This high binding affinity makes it the most suitable choice for capturing cTnI in immunoaffinity enrichment applications.

To optimize the immunoaffinity enrichment process, two different types of magnetic beads were utilized: carboxylic acid functionalized magnetic nanoparticles (130 nm diameter) and Dynabeads® MyOne™ Carboxylic Acid (1 µm diameter). The coupling conditions for each type of bead were as follows: The preparation of carboxylic acid functionalized magnetic nano particles (130 nm diameters) has been previously described (Asicioglu et al., 2023). Briefly, 5 mg EDC ve 5 mg NHS were mixed with 500 µl in 25 mM MES and 0.01% Tween 20 (pH:6.0). Immediately, 250 µg of magnetic nanobeads were treated with 1.25 µl NHS and 1.25 µl cold EDC to activate carboxyl groups and incubated at room temperature for 1 hour with continuous mixing. 25 mM MES and 0.01% Tween 20 (pH 6.0) were added to 25 µg of anti-cTnI antibody to make a total volume of 25 µl. This antibody solution was then added to 25 µl (250 µg) of activated beads and incubated overnight at room temperature with continuous mixing. After immobilization, the complex was treated with 10 µL of 100 mM Tris–HCl (pH 7.4), followed by washing steps. Finally, the beads were resuspended in 100 µl of 10 mM PBS, 0.01% Tween 20, and 0.05% sodium azide (pH 7.4) using probe sonication.

The preparation of Dynabeads® MyOne™ Carboxylic Acid (1 µm diameter) followed the manufacturer's instructions. Briefly, 100 µl micro particles (1 mg) were resuspended, and the supernatant was removed. Microbeads were washed with 100 µl 15 mM MES buffer (pH 6.0) three times, and Dynabeads® were resuspended with 100 µl 15 mM MES buffer (pH 6.0). The microbeads were incubated with 100 µl EDC for 30 minutes at room temperature, and the supernatant was removed. Then, 40 µg cTnI antibody diluted in 15 mM MES buffer pH 6.0 was added. The antibody and microbeads were incubated overnight at room temperature. The beads-antibody couple was washed with 100 µl PBS with 0.1% Tween-20 three times and resuspended in 400 µl PBS with 0.1% Tween®-20 and 0.1% BSA. Both couplings were done small scale to find suitable magnetic beads for enrichment.

The selected magnetic particle type was subsequently prepared in larger volume, to be used in the development of the method.

2.1.3 Optimisations of antibody-magnetic particle coupling

2.1.3.1 Determination of surface loading capacity

The ID-LC-MS/MS method presented by Schneck et al. was used to directly quantify and measure the amount of antibody bound to magnetic nanoparticles (Schneck et al., 2016). Briefly, matrix-matched external calibrants were prepared by combining the antibody and internal standard in a manner that mimicked the sample matrix. The heavy isotope labeled synthetic DLPSPIER peptides were purchased as internal standards because of their uniqueness to the constant region of the antibodies, robust MS/MS signal intensity and absence of amino acid residues prone to chemical modification, such as cysteine or methionine residues. These calibrants were used to establish a calibration curve for accurate quantification. Following antibody (clone 19C7 from Hytest) immobilization, the antibody-magnetic nanoparticle (Nanomag®-D,130 nm) were extensively washed to remove non-specifically adsorbed antibodies. The immobilized antibodies were then subjected to in situ trypsin digestion together with the nano magnetic particles. Internal standard was added to the digested samples, which were then analyzed directly by LC-MS/MS. Quantification was performed by monitoring a specific transition for DLSPIER peptides. To determine the maximum loading capacity of the magnetic nanoparticles, different antibody concentrations were used while keeping the amount of nanoparticles constant. In this study, approximately

100, 200, and 400 µg of antibody were added to the immobilization solutions containing 1 mg of nano magnetic particles.

2.1.3.2 Determination the amount of antibody-magnetic particle complex for cTnI enrichment

To calculate the amount required for cTnI enrichment from 1 ml of serum using the synthesized magnetic nano partcile-antibody conjugate, enrichment was performed using 5, 10, 20, and 30 µl of nanoparticle-antibody conjugate.

2.1.4 Preparation of solutions, calibrators and quality control samples

All solutions and standards were prepared gravimetrically using calibrated precision balances, and the concentrations are given in mg g^{-1}. SRM 2921 was purchased from the NIST and used as the calibrator stock solution and was handled according to the manufacturer's instructions. To maintain the stability of the cTn complex over time, the calibrator working solution was prepared by diluting the calibrator stock solution to approximately 1 µg/L in a protective protein, 0.1% Bovine Serum Albumin (BSA) in PBS. Final matrix-based calibrator levels ranging from 0.7-24 µg/L were prepared by diluting the calibrator working solution in 0.1% BSA in PBS. Three levels of matrix-based QC materials were prepared in a similar manner, with final concentration levels of 2.0, 5.0, and 12.0 µg/L, respectively. SIL-cTnI was dissolved in 20 mM Tris buffer (containing 0.5 mM DTT) to 200 µg/L and this solution referred to as the SIL stock solution. The SIL-DLPSPIER peptide working solution was prepared in 0.1 M HCl at a concentration of 0.5 mg/g.

2.1.5 Preparation of anti cTnI-magnetic nanoparticle complex in large volume

5mg EDC and 5 mg NHS were prepared in 500 µl 25 mM MES and 0.01% Tween 20 (pH:6.0). Magnetic nanoparticles (130 nm diameters) were treated with 12.5 µl NHS and 12.5 µl cold EDC to activate carboxyl groups and incubated at room temperature 1 hour with continuous mixing. 250 µg of anti-cTnI antibody was added to the 2.5 mg (250 µl) of activated beads and incubated overnight at room temperature with continuous mixing. After immobilization, the anti-cTnI-magnetic nanoparticle complex was treated with 100 µl 100 mM Tris-HCl (pH: 7.4) for 30 minutes with continuous mixing and placed in a magnetic separator to separate the beads. The supernatant was removed and 1 mL 10 mM PBS, 0.01% Tween 20, 0.05% Sodium

azide (pH: 7.4) was added to wash the beads. The magnetic beads were re-suspended using a sonicator and a wash step was performed three times. In the final step, 1 mL 10 mM PBS, 0.01% Tween 20, 0.05% Sodium azide (pH: 7.4) was added to the magnetic beads and resuspended by probe sonication (Asicioglu et al., 2023).

2.1.6 Sample preparation and cTnI enrichment procedure using magnetic particles

The sample preparation procedure for quantifying cTnI in serum is described in Fig 1. Briefly, six-point calibration standards were prepared by spiking cTnI calibrator working solution into 900 µL cTnI-free human serum to obtain cTnI concentrations ranging from 0.7 to 24 µg/L. A constant volume of SIL-protein cTnI working solution was spiked to obtain a concentration of 15 µg/L. Similarly, three QC materials were prepared at concentrations of 2, 5, and 12 µg/L of cTnI, each containing 15 µg/L of SIL-cTnI. Both calibrants and QCs were diluted with the same amount of PBS as the serum to reduce serum viscosity. Patient blood samples were collected in yellow capped biochemistry tubes from Kanuni Sultan Suleyman Hospital in Istanbul. Patient serum samples were obtained by spinning the blood at 2000 g for 10 minutes. The samples were obtained from individuals suspected of having a myocardial infarction. Immunoassay measurements of the samples were conducted immediately upon collection without freezing using the Siemens Atellica® Solution instrument. A reference range of cTnI with a threshold of 45 ng/L was utilized to distinguish between normal and elevated cTnI levels. Samples with cTnI concentrations exceeding 45 ng/L were categorized as "patient samples." After collection, the serum samples were promptly frozen and stored at -80°C until the day of measurement using ID-MS. This ensured the preservation of sample integrity and minimized any potential degradation. Four patient serum samples were spiked with SIL cTnI to a final concentration of 15 µg/L. For immunoaffinity enrichment of cTnI, 10 µl anti-cTnI-magnetic bead complex was added to all calibrants, quality control (QC) samples and patient serum samples and incubated overnight at +4 °C with continuous mixing. Non-specific proteins were removed by two washes with 20 mM Tris and 150 mM NaCl containing 0.05% Tween 20 followed by a single wash with ammonium bicarbonate in a magnetic separator for 30 minutes. All calibrants, QCs and patient serum samples were resuspended in 30 µl of 50 mM ammonium bicarbonate. Captured cTnI was digested using Abcam's FASP according to the manufacturer‘s instructions. Briefly, 10 mM DTT was used for 45

min at 60 °C to reduce disulfide bonds and alkylated with IAA for 20 min in the dark at room temperature. Trypsin was added and digestion was carried out overnight at 37 °C. The digests were dried using SpeedVac. All samples were reconstituted with a final volume of 20 µl of SIL-DLPSPIER (0.18 ng/µL) to determine the relative amount of anti-cTnI antibody. The digested peptide mixtures were analyzed by Nano LC-MS/MS as described in Figure 2.1.

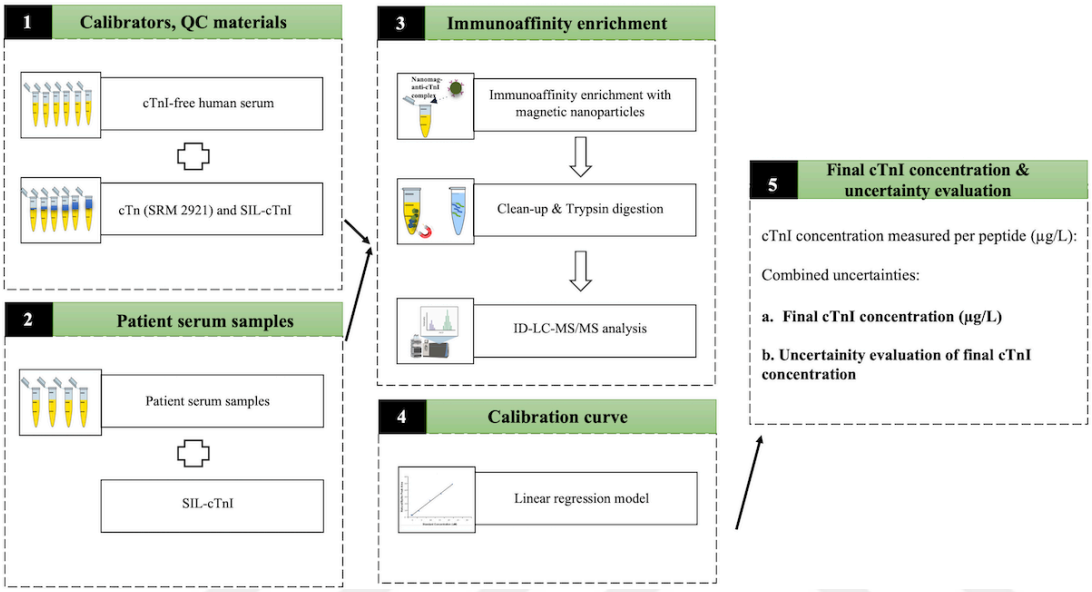

Figure 2.1 : A diagrammatic illustrating the step-by-step of the analytical workflow for the SI-traceable quantification of cTnI in human serum. Step 1: The calibration standards and QC materials were prepared using cTnI-free serum by incorporating the NIST SRM 2921 and SIL-cTnI. Step 2: Patient serum samples were prepared using cTnI positive serum samples by incorporating SIL-cTnI. Step 3: All samples were subjected immunoaffinity enrichment, followed by LC-MS/MS analysis. Step 4: Calibration curves were established for the TLLLQIAK and NITEIADLTQK peptides to determine the concentration of cTnI. Step 5: To determine the uncertainty, all sources of uncertainty from steps 1 to 4 were combined (Asicioglu et al., 2023).

2.1.7 Bottom-up detection of cTnI enriched by magnetic particles

Analyses were done in PRM mode on the high-resolution mass spectrometry Thermo Scientific™ Mass Spectrometer Q-Exactive™ HF-X (Thermo Scientific, Waltham, MA, USA) coupled with Thermo Scientific™ Dionex™ Ultimate 3000 (Thermo Scientific) liquid phase chromatography (Bourmaud et al., 2016). The digested peptides were separated on an Acclaim™ PepMap™ 100 C18 analytical column (75 × 25 µm, 2 µm) (Thermo Scientific, Waltham, MA, USA). The following MS conditions were applied: capillary temperature at 270 °C, spray voltage of 2.0 kV, S-lens RF level of 50, sheath gas flow rate and auxiliary gas heater flow rate of 0. After

optimization, 5 µL of the peptide digest was injected and the LC separation was carried out at a flow rate of 350 µL/min, a temperature of 40 °C using 100% water 0.1% of formic acid as mobile phase A and 20% acetonitrile in water, 0.1% of formic acid as mobile phase B. Peptides were eluted with the following gradient of mobile phase B: 3% for 3 min, linear from 24 to 36% in 5 min, linear from 36 to 80% in 7 min, constant in 80% for 8 min. MS2 scans were collected at a resolution of 15,000 with an automatic gain control target of 2e5, loop count of 8 and 30 microscan. Precursor ions were isolated within an isolation window a 0.6 m/z. Raw data were processed by Xcalibur Quan Browser software (Thermo Scientific). Peak areas were integrated using the Genesis algoritm. Signal extraction was applied within a mass tolerance of 15 ppm for PRM data. cTnI concentration was determined using calibration curves that were created using SRM 2921 as the calibrant. Calibration curves were plotted by correlating the peak area ratios of unlabeled to labeled cTnI peptide with the known ratios of the concentrations of unlabeled to labeled cTnI peptide.

2.1.8 Method validation

The analytical performance for quantification of cTnI in human serum using SIL-cTnI as an internal standard was validated for linear range, accuracy, trueness, precision, and the presence of carryover (Shrivastava & Gupta, 2011). The linearity of calibration curve was evaluated by regression analysis at cTnI concentrations of 0.7, 1.2, 3.5, 6, 12 and 24 µg/L. The peak area ratio of the analyte to the corresponding SIL-cTnI was plotted against the analyte concentration (µg/L). Correlation coefficients were determined for each curve. The linearity of the method was demonstrated by the recovery of the calibration samples. Recovery was reported as the percentage recovery of the measured concentration relative to the gravimetric concentration.

In addition, the accuracy of the quantification of each calibrant was evaluated by considering the calibrants as unknown samples. The individual concentration of each peptide was determined from its respective calibration curve. The trueness and precision were evaluated using three processed replicates of QC materials at three concentration levels (about 2.0, 5.0 and 12.0 µg/L) in four independent experiments using NIST SRM 2921. The mass ratio of each sample was obtained from calibration curve that was prepared on the same day. The coefficients of variation (CV) for intra-day and inter-day variations of the assay were determined. Repeatability was

calculated by analyzing the samples at each concentration on the same day, whereas intermediate precision was determined by analyzing three samples at each concentration on four different days. The LOD and LOQ were determined by analyzing six replicate samples. For the carryover test, the signal observed at the retention time of each peptide (area below the peak) was less than 1% compared to that found in the LOD after injection of one blank sample.

The measurement uncertainty was determined according to the EURACHEM / CITAC Guide CG 4 (third edition) entitled "Quantifying Uncertainty in Analytical Measurement" (B. Magnusson and U. Örnemark (2014); Ellison Secretary et al., 2009). The uncertainty estimation considered various steps in the measurement process, including the uncertainty of the balance used for weighing, the uncertainty arising from the calibration curve, the assessment of intra-day and inter-day variances, and the evaluation of trueness. The derived overall uncertainty was multiplied by a coverage factor of k=2, corresponding to an approximate confidence level of 95% assuming a normal distribution. This multiplication resulted in the calculation of the expanded uncertainty.

2.2 Objective 2: An ID-LC-MS/MS-based Analytical Procedure for the Quantification of cTnI in Human Serum Using a Peptide-based Calibration

2.2.1 Chemicals and materials

The cTnI-free serum was purchased from Hytest Ltd. (Turko, Finland). Human cTn complex SRM 2921 was obtained from NIST (Gaithersburg, USA). Magnetic dextran nanoparticles (nanomag®-D) with particle diameters of 130 nm and the surface functionalities COOH for the covalent binding of the antibody were purchased from Micromod Partikeltechnologie GmbH (Rostock, Germany). 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride) (EDC), N-hydroxysuccinimide (NHS) and Pierce™ Trypsin/Lys-C Protease Mix, MS Grade, were obtained from Thermo Scientific™ (Rockford, Illinois, USA). FASP Protein Digestion Kit was obtained from Abcam (Boston, MA). The peptide standards TLLLQIAK and NITEIADLTQK and the stable isotope labeled standards TLLLQIAK* (> 95% purity $^{13}C_6$, $^{15}N_2$) and NITEIADLTQK* (> 95% purity $^{13}C_6$, $^{15}N_2$) were purchased from New England Peptide Inc. (Gardner, MA, USA) as powder. CRMs of amino acids L-alanine (Ala) (CRM 6011-a, 99.9±0.2%), L-leucine (Leu) (CRM 6012-a, 99.9±0.2%), L-

isoleucine (Ile) (CRM 6013-a, 99.9±0.2%) and L-proline (Pro) (CRM 6016-a, 99.9±0.2%) were obtained from the National Metrology Institute of Japan (NIMJ) (Tsukuba, Japan). The isotopically labeled amino acids Proline ($^{13}C_5$, 99%; ^{15}N, 99%), Alanine ($^{13}C_5$, 99%; ^{15}N, 99%), Leucine ($^{13}C_6$, 99%; ^{15}N, 99%) and Isoleucine ($^{13}C_9$, 99%; ^{15}N, 99%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). ACN (cas #75-05-8) was purchased from Supelco. FA (cas #56302) and ammonium bicarbonate (cat #09830) was obtained from Fluka. NaCl (cat #A2942) was purchased from Panreac AppliChem GmbH (Ottoweg, Darmstadt). Tween©-20 (cat #A4974) was obtained from AppliChem GmbH (Ottoweg, Darmstadt).

Q-Exactive™ HF-X Hybrid Quadrupole-Orbitrap MS (Thermo Scientific, Bremen, Germany) were applied for mass detection. Easy-SprayTM ES902 C18 analytical column (75µm × 25 cm, 2 µm) were obtained from Thermo Scientific, Waltham, MA, USA. EZ:faast 4 µm AAA column and Aeris 3.6 µm Peptide XB- C18 column 100 A (250 x 2.1 mm i.d.) column were obtained from Phenomenex. For LC, Dionex UPLC™ system and Dionex™ Ultimate 3000 system were applied (Thermo Scientific, Bremen, Germany). Savant™ SPD131DDA SpeedVac™ Concentrator coupled with Savant™ Refrigerated Vapor Traps (cat #RVT400) were used as a concentrator from Thermo Fisher Scientific Inc.

2.2.2 Peptide impurity corrected amino acid analysis

The proteotypic peptides TLLLQIAK and NITEIADLTQK have been previously chosen for cTnI quantification (Asicioglu et al., 2023). The amino acid sequence of cTnI and location of the selected proteotypic peptides and the schematic workflow of PICAA strategy are shown in Figure 1.2 and Figure 1.6, respectively. Certified amino acids and isotopically labeled amino acids were used for value assignment and constituted primary calibrator materials. PICAA analysis comprises two parts: (i) AAA, and (ii) Impurity analysis.

2.2.2.1 Amino acid analysis

The freeze-dried peptides were dissolved in a 0.1 mol/L HCl. This solution will be referred to as the peptide stock solution. Using peptide stock solutions, all peptides are diluted at 0.5 mg/g, gravimetrically. To prepare calibration curve points, certified amino acids (natural amino acids) from NMIJ were used. Each natural amino acid

solutions were gravimetrically prepared by 0.1 mol/L HCl. All these preparations were made fresh and independently for each hydrolysis reaction (for three days). The amount of substance content of each amino acid in the mixture ranged from approximately 30 to 1000 µmol/L. Each of the labelled amino acids was dissolved in 0.1 mol/L HCl, and the mixed labelled amino acid solution was prepared nearly the same as the concentration of the synthetic peptides. A six-point calibration curve was prepared gravimetrically for gas-phase hydrolysis method by mixing the natural and isotopically labeled amino acid mixtures. Additionally, the concentration of the natural amino acids in the calibration sample, which coincided with the midpoint of the calibration curve, was carefully aligned with the anticipated amino acid concentrations in the sample blends.

Gas-phase acid hydrolysis

Six calibration curve standards were prepared, consisting of mixture of natural and isotopically labeled amino acid solutions and two candidate peptide primary calibrators (TLLLQIAK and NITEIADLTQK) were prepared as 100 µM, containing their isotopically labeled amino acids. After weighing the solutions in the glass tubes, they were placed into Pico-Tag vacuum vial along with the calibration mixtures. A set of calibration and sample mixtures were completely dried under vacuum using Pico-Tag Workstation (Waters, Milford, MA, USA). Then, they were hydrolyzed under inert atmosphere conditions at 130 °C for 24 h in hydrolysis tubes containing 200 µl of 6 moL L^{-1} constant boiling HCl and 1% phenol. After the completion of hydrolysis, the samples were evaporated under vacuum until completely dry, and then reconstituted with 200 µl of 20 mM HCl for the derivatization process of amino acids (Oztug, Saban, et al., 2024; Oztug, Vatansever, et al., 2024).

Propyl chloroformate derivatization

After the hydrolysis was completed, individual samples were dissolved with 200 µl 0.1 mol/L HCl. The reconstituted samples were subjected to the propyl chloroformate (PCF) derivatization. The reconstituted samples were mixed with 200 µl of Propanol: Pyridine (7:1, v/v) reagent and vortexed. Then, they were mixed with 200 µl of Isooctane: PCF (5:1, v/v) and incubated for 1 min. Subsequently, 100 µl of $CHCl_3$: Isooctane: PCF (24:16:1, v/v/v) reagent was added, vortexed, and incubated for 1 min. Finally, 200 µl of 5 % HCl was added, vortexed, and centrifuged. The upper phase of

each sample was separated and transferred to clean tubes. The samples were then evaporated using the Caliper TurboVap LV evaporator under nitrogen. Lastly, each sample and calibrants in the tubes were dissolved by adding 50 µl of H2O: acetonitrile (95:5)-0.1 % FA sample solution and transferred to suitable vials for LC/MS analysis (Oztug, Saban, et al., 2024; Oztug, Vatansever, et al., 2024).

LC/MS analysis for AAA

AAA LC/MS analyses were done using a Dionex UPLC™ system (Thermo Scientific, Bremen, Germany) coupled with a Q-Exactive HF-X Hybrid Quadrupole-Orbitrap MS (Thermo Scientific, Bremen, Germany) equipped with an electrospray ion source. Derivated amino acids were separated on Phenomenex EZ: faast 4 µm AAA column. MS parameters for amino acids following PCF derivatization are presented in Table 2.1. All data were acquired in positive ion mode. After optimization, the following MS conditions were applied: resolution of 120000, capillary temperature at 250 °C, spray voltage of 3.50 kV, sheath gas flow rate of 45 kV, auxiliary gas heater flow rate of 3000 °C, AGC target of 3e6 and maximum inject time 200 ms. Then, 10 µL of the peptide extracts was injected on an Phenomonex EZ:faast 4 µm AAA column (2.0 mm × 250 mm) (Thermo Scientific, Waltham, MA, USA) at a flow rate of 250 µL/min and a temperature of 40 °C. The mobile phases consisted of 50% ACN in water, 10 mM ammonium formate (mobile phase A) and 100% methanol, 10 mM ammonium formate (mobile phase B). Peptides were eluted with the following gradient of mobile phase B: linear from 35 to 55% in 12 min, constant in 100% for 5 min. Mass data extraction and analysis were performed using QuanBrowser™ Software. Following the MS measurements, the mass fractions of peptide samples were calculated for Ala, Leu and Ile amino acids based on the integral values of signal peak areas using calibration curves. The assignment process was completed by considering the gravimetric dilution factors.

Table 2.1 : Detected m/z values for amino acids after derivatization.

Natural amino acids	PCF MS	Isotopically labeled amino acids	PCF MS	Retention time (min)
Alanine	218.000	Alanine SIL	221.980	5.34
Leucine	260.185	Leucine SIL	267.202	10.58
Isoleucine	260.18	Isoleucine SIL	261.182	11.04

2.2.2.2 Impurity analysis

In the second part of the PICAA analysis, the impurity profile of the synthetic peptides was determined by UltiMate™ 3000 UHPLC system (Thermo Scientific), and coupled with an OrbiTrap-Q Exactive High Resolution Mass Spectrometer (Thermo Scientific). Peptides were separated on an Aeris 3.6 um Peptide XB- C18 column 100 A (250 x 2.1 mm i.d.) column. Intact mass LC combined with LC-HRMS analysis was performed to separate impurities from the interested peptides and identify them based on their exact mass. Then, LC-MS/MS analysis was carried out to confirm the peptide related impurities and their identity thanks to the fragmentation spectrum. A stock solution diluted to ~0.2 mg/g in 5% ACN with 0.1% FA in water was used for analysis. Peptide related impurities were identified based on their exact mass and the fragmentation spectrum. The analysis was performed over three days, with three subsamples performed each day. Each replicate consisted of ten technical replicates of each sample. The mass fraction of the cTnI proteotypic peptides were calculated with the PICAA equation given in the equation (2.1).

$$x_p = \left(\frac{M_{\mathrm{r}}(\mathrm{P})}{Z_1}\right)\left[\frac{n_{\mathrm{AA}}}{m_{\mathrm{m}}} - \sum Y_{\mathrm{IMP}_i}\frac{X_{\mathrm{IMP}_i}}{M_{\mathrm{r}}(\mathrm{IMP}_i)}\right] \quad (2.1)$$

where,

x_p is the mass fraction of peptide in the material;

m_m is the mass of the sample analyzed;

$M_r(P)$ is the relative molecular mass of synthetic peptide;

Z_1 is the number of molecules of the amino acid of per peptide molecule;

n_{AA} is the amount of substance of amino acid of interest measured in the material;

Y_{IMPi} is the number of molecules of amino acid of interest per peptide impurity molecule (IMP_i);

X_{IMPi} is the mass fraction of peptide impurity (IMP_i);

$M_r\ (IMP_i)$ is the relative molecular mass of peptide impurity (IMP_i).

2.2.3 Comparison of trypsin digestion methods

Four trypsin digestion methods were evaluated for cTnI peptide formation yields using 0.5 µg of cTn complex from Hytest (8T62) as the substrate. These methods included the Solu-Trypsin Rapid Digestion Kit (Sigma-Aldrich) using both in-solution and FASP methodologies, the FASP Protein Digestion Kit (Abcam), and the Rapigest™ SF Surfactant (Waters).

For Solu-Trypsin Rapid Digestion, both in-solution and FASP methods were tested. Reduction and alkylation were performed using TCEP (100 mM for in-solution, 50 mM for FASP) and CAM (500 mM for in-solution, 50 mM for FASP), respectively. The trypsin-to-protein ratio and incubation time were set according to the manufacturer's instructions, with incubation at 60°C.

For FASP (Abcam) and Rapigest (Waters) methods, reduction and alkylation were done using DTT (10 mM) and IAA (15 mM for Rapigest, 0.5 M for FASP), followed by digestion with Trypsin/Lys-C Protease Mix (ThermoFisher Scientific) at 37°C. The main difference is that Trypsin/Lys-C Protease Mix allows for trypsin digestion over 2-16 hours, preferably overnight at 37°C, while Solu-Trypsin Rapid Digestion allows for trypsin digestion in one hour at 60°C which is fast. The trypsin-to-protein ratio and incubation time followed manufacturer's instructions. All digested peptides were dried using SpeedVac and reconstituted in 20 µl of 5% ACN in water with 0.1% formic acid. Three microliters of each sample were injected into the NanoLC system with a 25 cm Easy spray column. Each analysis was repeated twice for technical validation, resulting in four different digestion methods tested with two biological and two technical replicates.

2.2.4 Enzyme-to-protein amount ratio

To determine the optimal ratio, ratios of 1:100, 1:50, 1:25, and 1:10 were tested using the Pierce Trypsin/Lys-C Protease Mix with the FASP method and 0.5 µg of the cTn complex. After digestion, samples were acidified, dried, and reconstituted in 5% ACN in water with 0.1% FA. Four microliters of each sample were analyzed using PRM in the NanoLC system. To examine matrix effects, enzyme-to-protein ratios of 1:10, 1:5, and 1:2.5 were tested in the presence of antibody-magnetic nanoparticle conjugates. Serum (900 µl) diluted with PBS (900 µl) containing cTn complex (30 ng/ml) was prepared and incubated with 10 µl of antibody-nanoparticle complex overnight at 4°C.

After enrichment, denaturation, and alkylation steps, the extracts were digested with Trypsin/Lys-C for three different ratios: 1:10, 1:5, and 1:2.5. Additionally, fresh trypsin was added in the same proportions after overnight incubation, resulting in six different digestion ratios (1:10, 1:5, 1:2.5, 1:10 (x2), 1:5 (x2), 1:2.5 (x2)). The experiment was carried out using two subsamples and replicated technically two times.

2.2.5 Digestion kinetics

Digestion kinetics were investigated to find the optimal digestion time for maximal peptide yield. The study was conducted with 0.5 µg cTn complex using Pierce Trypsin/Lys-C Protease Mix at an enzyme-to-protein ratio of 1:10. Digestion times of 2h, 4h, 6h, and overnight were tested. Post-FASP, samples were acidified, dried, and reconstituted in 20 µl of 5% ACN in water with 0.1% FA. The experiment included two biological and two technical replicates.

2.2.6 Preparation of calibrator stock solutions

Stock solutions of the primary peptide calibrators TLLLQIAK, NITEIADLTQK and their isotopically labeled versions were gravimetrically prepared in 0.1 M HCl. The solutions were aliquoted and stored at -80 °C. Working stock solutions of unlabeled and labeled peptides were prepared at approximately 0.5 mg/g. in 0.1 M HCl. Secondary stock solutions of unlabeled and labeled peptides were prepared by diluting working stock solutions with 10% ACN and 0.001% Tween-20 in water to achieve concentrations of 250 nmol/L (equivalent to a cTnI concentration of 6,000 µg/L) (Molar mass of TLLLQIAK is 899.59 Da and molar mass of NITEIADLTQK is 1245.66 Da). A mixed solution of the two unlabeled peptides at 2 nmol/L (equivalent to a cTnI concentration of 48 µg/L) was prepared by mixing their secondary stock solutions in 10% ACN and 0.001% Tween-20. Similarly, a mixed solution of the two isotopically labeled peptides at 0.5 nmol/L was prepared using the secondary solutions of labeled peptides in the same solvent.

2.2.7 Preparation of QC, patient samples and immunoaffinity enrichment

The QC materials, and patient samples were processed as described previously (Asicioglu et al., 2023). Briefly, stock solution of NIST SRM 2921 Human cTn complex was prepared by diluting to approximately 1 µg/L in PBS with 0.1%BSA previously (Asicioglu et al., 2023). QC materials comprised 900 µl cTnI-free human

serum spiked with NIST SRM 2921 Human cTn complex at concentrations of 2 µg/L, 5 µg/L, and 10 µg/L, corresponding to TLLLQIAK and NITEIADLTQK peptide concentrations of 0.12 nmol/L, 0.25 nmol/L, and 0.5 nmol/L, respectively.The mixture was diluted with the same volume of PBS as the serum to reduce viscosity. For immunoaffinity enrichment of cTnI, 10 µl cTnI antibody-magnetic bead complex was added to QC and patient samples, which were then incubated overnight at 4 °C with continuous mixing. Non-specific proteins were washed and removed by two washes with a solution containing 20 mM Tris, 150 mM NaCl and 0.05% Tween 20, followed by a single wash with ammonium bicarbonate. The samples were then resuspended in 30 µL of 50 mM ammonium bicarbonate, and captured cTnI was digested using FASP Protein Digestion Kit (Abcam) according to the manufacturer's instructions. Specifically, 10 mM DTT was used for 45 min at 60 °C to reduce disulfide bonds, the samples were alkylated with IAA for 20 minutes in the dark at room temperature. Trypsin was then added, and digestion was carried out overnight at 37 °C. The digests were dried using SpeedVac. All samples were reconstituted with isotopically labeled peptides solution at 0.25 nmol/L. The digested peptide mixtures were analyzed by Nano LC–MS/MS.

2.2.8 Preparation of calibration standards

Unlike protein-based calibration, which involves identical processing steps for both calibrants and QC samples, including enrichment, peptide-based calibration differs. Peptide calibrants are directly spiked with their isotopically enriched counterparts, while QC and patient samples undergo protein enrichment, washing, and tryptic digestion before adding the internal peptide standards. Therefore, material loss and incomplete enrichment, where beads may not capture all of the analyte molecules, can occur. In addition, when preparing QC and patient samples through enrichment, nonspecific binding to beads occurs, leading to relatively high background signals in LC-MS/MS after tryptic digestion due to peptides coming from the proteins that are nonspecifically bound to the beads. This suggests that preparing calibrants in an aqueous solution might not be an appropriate matrix. Background peptides could suppress the cTnI peptides signals in QC and patient samples compared to the signals from the calibrants. Therefore, the choice of matrices for preparing the calibrants is crucial. Two types of surrogate matrices were evaluated to prepare calibrants and mimic the matrices of patient samples. (i) Serum Matrix Calibrants: This calibrant's

matrix was prepared similarly to the QC samples. 10 µL cTnI antibody-magnetic bead complex was added into cTnI-free serum and incubated overnight. The particles were then washed and digested with trypsin. After SpeedVac drying, the samples were reconstituted in a solution containing stable isotope-labeled peptides (final concentration 0.25 nmol/L) and natural peptides (final concentrations 0.03, 0.06, 0.13, 0.25, 0.50, and 1.00 nmol/L), resulting in a six-level calibration curve.

(ii) Digest Matrix Calibrants: This matrix involved digesting cTnI-free human serum with trypsin to generate a peptide pool. This peptide pool was then added in a constant amount to the calibration standards to mimic the matrix effect. Natural peptides were added in varying amounts (0.03, 0.06, 0.13, 0.25, 0.50, and 1.00 nmol/L), and a constant amount of SIL peptides (0.25 nmol/L) was added. This process resulted in the creation of a six-level calibration curve named digest matrix calibrants. When preparing the digest matrix and adding the peptide pool, two considerations were taken into account: firstly, non-specific bindings from the serum matrix were measured using a nanodrop, and a similar amount of peptide addition was made. Secondly, it was expected that the total ion chromatogram of the digest matrix would exhibit a similar total ion chromatography (TIC) profile to that of the serum matrix.

Each matrices was compared and evaluated in terms of quantify QC samples (see ESM Fig. S3). The validation included assessing the parallelism of calibration curves in the digest matrix with those in the serum matrix (slope of a curve combining 6 calibrator sets in a surrogate matrix within ±10% of slope obtained in serum; different intercepts are acceptable), linearity ($r^2 > 0.95$), repeatability, and trueness of cTnI spiked quality control samples. It could be demonstrated that the use of a surrogate matrix is a cost-effective alternative to serum matrix calibrants.

2.2.9 LC-MS/MS

Analyses were acquired on a Dionex™ Ultimate 3000 system (Thermo Scientific, Bremen, Germany) coupled with a Q-Exactive™ HF-X Hybrid Quadrupole-Orbitrap MS (Thermo Scientific, Bremen, Germany) equipped with an electrospray ion source. After optimization, the following MS conditions were applied: capillary temperature at 320 °C, spray voltage of 2.0 kV, S-lens RF level of 40, sheath gas flow rate of 10, and auxiliary gas heater flow rate of 0. Then, 5 µL of the peptide extracts was injected on an Easy-Spray™ (ES902) C18 analytical column (0.075mm × 25 cm, 2 µm) (Thermo Scientific, Waltham, MA, USA) at a flow rate of 250 nL/min and a

temperature of 40 °C. The mobile phases consisted of 0.1% of formic acid in water (mobile phase A) and 80% acetonitrile in water with 0.1% of formic acid (mobile phase B). Peptides were eluted with the following gradient of mobile phase B: 3% for 3 min, linear from 3 to 8% in 3 min, linear from 8 to 24% in 5 min, linear from 24 to 36% in 2 min, linear from 36 to 80% in 3 min constant in 80% for 5 min. The column was re-equilibrated at 3% B for 15 minutes before the next analysis. Analyses were done in PRM mode. Precursor ions were isolated within an isolation window of 0.6 m/z. Details of precursor ions and applied collision energy are shown in Table 2.2. MS2 scans were collected at a resolution of 30,000 with an automatic gain control target of 2e5, loop count of 6 and 50 microscan.

Table 2.2 : PRM transitions, amino acid sequence, retention time and collision energies for the proteotypic peptides.

Proteotypic peptides	Retention time (min)	Precursor ions (m/z)	Collision energy (eV)	Product ions (m/z)
TLLLQIAK	14.96	450.3	20	572.37 685.46
TLLLQIAK SIL	14.96	454.3	20	580.39 693.47
NITEIADLTQK	14.64	623.3	20	675.36 1018.54
NITEIADLTQK SIL	14.64	627.35	20	683.38 1026.56

2.2.10 Data analysis

Raw data were processed by Xcalibur Quan Browser software (Thermo Scientific, Waltham, MA, USA). Peak areas were integrated using the Genesis algorithm. Signal extraction was applied within a mass tolerance of 15 ppm for PRM data. The calibration curves were plotted by correlating the peak area ratios of unlabelled to labeled cTnI peptide with the known ratios of the concentrations of unlabelled to labelled cTnI peptide. A correction factor was established and applied to address any incompleteness in digestion and material loss prior to the internal standards spike.

2.2.11 Method validation and uncertainity evaluation for the cTnI quantification using peptide-based calibration strategy

The method validation involved evaluating the linear regression using six calibration points, corresponding to cTnI concentrations ranging from 0.6 to 21.6 µg/L. Calibration curves were created by correlating the peak area ratios of unlabeled to labeled cTnI peptides with the known ratios of their concentrations in the calibration solutions. Pearson correlation coefficients (R^2) were calculated for each curve. The method's precision, trueness, and carryover were evaluated using three QC samples (2.0, 5.0, and 10.0 µg/L) across three assays. Repeatability was obtained by analysing samples at each concentration level within the same day, while intermediate precision was determined by analysing three samples at each concentration level over three different days. The total recovery was assessed through the analysis of quality control materials spiked with cTnI. The trueness was assessed through the analysis of quality control materials spiked with cTnI and its equation given in (2.2). The correction factor was calculated with the equation given in (2.3) (Huynh et al., 2021).

$$\text{Total recovery} = \frac{\text{cTnI Concentration quantified}}{\text{cTnI concentration added}} \tag{2.2}$$

$$\text{Correction factor} = \frac{1}{\text{Total recovery}} \tag{2.3}$$

The LOD and quantification LOQ were determined by analysing six replicate samples. Carryover was assessed by ensuring the signal at each peptide's retention time was less than 1% of the LOD after injecting a blank sample. Measurement uncertainty was assessed following the EURACHEM/CITAC Guide CG 4 (third edition) titled "Quantifying Uncertainty in Analytical Measurement" (B. Magnusson and U. Örnemark (2014); Ellison Secretary et al., 2009). This assessment considered several factors in the measurement process, including balance uncertainty, calibration curve uncertainty, repeatability and intermediate precision variance assessment, and trueness evaluation. The overall uncertainty was multiplied by a coverage factor (k=2) for a 95% confidence level, resulting in the expanded uncertainty.

2.2.12 Application to patient samples

Patient blood samples were collected in yellow-capped biochemistry tubes from Kanuni Sultan Suleyman Hospital in Istanbul. Patient serum samples were obtained by spinning the blood at 2000 g for 10 minutes. The samples were obtained from individuals suspected of having a myocardial infarction. Immunoassay measurements of the samples were conducted immediately upon collection without freezing using the Siemens ADVIA Centaur® CP Immunoassay instrument. Samples with cTnI concentrations exceeding the LOQ of our method were selected for analysis. These concentrations are 12.36 µg/L, 8.34 µg/L, 10.20 µg/L, and 16.62 µg/L. After collection, the serum samples were promptly frozen and stored at -80 °C until the day of measurement using ID-MS. This ensured the preservation of sample integrity and minimized any potential degradation.

3. RESULTS AND DISCUSSION

3.1 Objective 1 : Development of an ID-LC-MS/MS Method Using Targeted Proteomics for cTnI Quantification by Protein-based Calibration

3.1.1 Selection of proteotypic peptides

The first step in the development of a an PRM-MS based analysis is the selection of a subset of peptides to be used as quantitative representatives 'Proteotypic peptides' for each candidate protein (Chen et al., 2020; Fusaro et al., 2009). The identification of optimal proteotypic peptides representing the targeted proteins is very important for the accurate quantification of the target proteins using the targeted proteomics approach. 'Proteotypic peptides' must be sequence specific, detectable and the most responsive peptides. In this study, proteotypic peptides were selected using both computational and experimental strategies. The enhanced signature peptide predictor was used to predict the high responding peptides for the cTnI protein (Chen et al., 2020). As a result of the analysis, seven peptides with the highest enhanced signature peptide predictor prediction factor were selected. These peptides are as follows, with the prediction factor from highest to lowest; NIDALSGMEGR, NITEIADLTQK, ISADAMMQALLGAR, TLLLQIAK, MADGSDAAR, EPRPAPAPIR and AYATEPHAK. The computational approach was also confirmed experimentally. Fully tryptic peptides of cTnI were screened using bottom-up proteomics. After trypsin digestion, the cTnI peptide solution was analyzed using with a Nano UPLC-Q-Exactive HF-X Orbitrap mass spectrometer with full MS/dd-MS2 (TopN) mode. Raw files were processed by Proteome Discoverer 2.5 (Thermo) using the Sequest algorithm. Advanced parameters of the Sequest algorithm were set as 10 ppm precursor mass tolerance, 0.02 Da fragment mass tolerance, static modifications of carbamidomethyl, dynamic modifications of acetyl and oxidation. Responses of tryptic cTnI peptides screened for PRM analysis are given in Table 3.1.

Table 3.1 : Responses of cTnI peptides screened for PRM analysis and assay development.

Sequence	Modifications	#PSMs	Positions	MH+[Da]	Abundance
NITEIADLTQK	-	30	[121-131]	1245.6685	6.3E+08
ISADAMMQALLGAR	2xOxidation [M6; M7]	22	[149-162]	1447.7395	5.8E+08
NIDALSGMEGR	1xOxidation [M8]	23	[194-204]	1162.5521	5.4E+08
TLLLQIAK	-	23	[51-58]	899.5924	5.0E+08
AYATEPHAK	-	27	[28-36]	987.4894	12.0E+08

Trypsin digestion hydrolyses the primary sequence of the target protein into specific peptide ending in C-terminal lysine or arginine residues. Both abundance and the number of peptide-spectrum match (PSMs) in the table aim to represent the response of the corresponding peptide, providing important insights into its relative abundance and confidence of identification. To assess potential phosphorylation sites of cTnI, the NetPhos-3.1 software was utilized, a generic phosphorylation site prediction tool for eukaryotic proteins. The results from NetPhos-3.1 indicated that threonine residues at positions 51, 124, and 129 were not predicted to be phosphorylated in cTnI. Based on this information, two signature peptides, NITEIADLTQK and TLLLQIAK, were selected for quantification. The primary selection criteria for these peptides were their high peptide-response and the fact that they have no known post-translational modifications, including phosphorylation (Blom et al., 1999; Schneck et al., 2018). ISADAMMQALLGAR and NIDALSGMEGR gave high signal responses but were not selected due to their possible post-translational modifications. NITEIADLTQK and TLLLQIAK were chosen as surrogate peptides for the quantification of cTnI using ID-LC-MS/MS.

3.1.2 Comparison of magnetic particles efficiency

Magnetic particles (or beads) are used to enrich low-abundance protein biomarkers from complex biological samples such as serum/plasma and facilitate detection with mass spectrometry (Schneck et al., 2015, 2016). One of the factors that effect of magnetic beads effectiveness is the size of magnetic beads which can be available micrometer-sized (such as Dynabeads®) or nanometer-sized. In this study, two different sizes of beads were selected for enrichment to compare their performance in capturing the target protein. To enrich cTnI from serum, Dynabeads® MyOne™ with

a size 1 µm and Nanomag®-D with a size of 130 nm were selected which are commercially available. A peptide pool was generated following FASP digestion, cTnI proteotypic peptides (TLLLQIAK, NITEIADLTQK peptides) and SIL-versions were monitored by PRM. Peak area ratios (Natural/SIL) were determined for both proteotypic peptides. The results showed a larger relative peak area using the nanobeads (Nanomag®-D, 130 nm) compared to the microbeads (Dynabeads® MyOne™, 1 µm) (Figure 3.1). This outcome aligns with theoretical expectations and is consistent with previous studies (Schneck et al., 2016), suggesting a more efficient enrichment process due to the higher surface area-to-volume ratio of the Nanomag®-D beads. Given these results, Nanomag®-D (130 nm) beads were selected for antibody coupling, and large-scale coupling processes were required for subsequent studies.

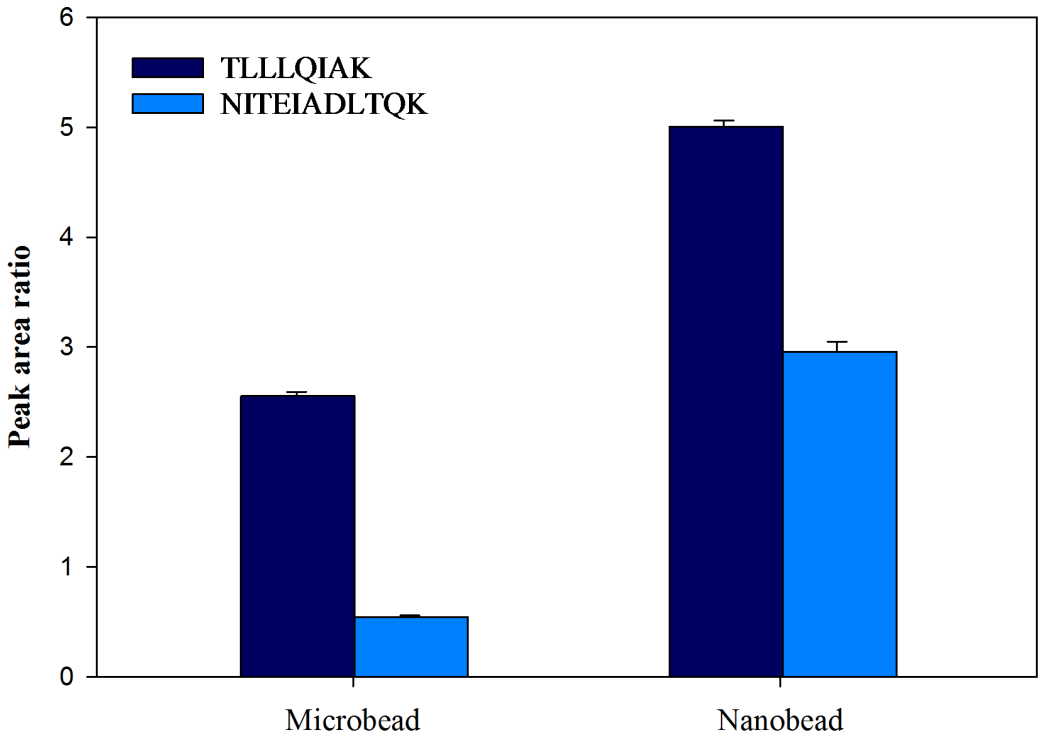

Figure 3.1 : Comparison of magnetic beads efficiency by PRM method. The experiment was conducted with four subsamples.

3.1.3 Optimizations of antibody-magnetic particles coupling

The ID-LC-MS/MS method presented by Schneck et al. was used to directly quantify and measure the amount of antibody bound to magnetic nanoparticles (Schneck et al., 2016). Figure 3.2 illustrates the methodology used to quantifying the antibody bound to magnetic particles using the ID-LC-MS/MS technique. Briefly, matrix-matched

external calibrants were prepared by combining the antibody and internal standard in a manner that mimicked the sample matrix. The SIL-DLPSPIER peptides were purchased as internal standards because of their uniqueness to the constant region of the monoclonal antibody, robust MS/MS signal intensity and absence of amino acid residues prone to chemical modification, such as cysteine or methionine residues. These calibrants were used to establish a calibration curve for accurate quantification. Following antibody immobilization, the antibody-magnetic particle conjugates were extensively washed to remove non-specifically adsorbed antibodies. The immobilized antibodies were then subjected to in situ trypsin digestion together with the magnetic particles. Internal standard was added to the digested samples, which were then analyzed directly by LC-MS/MS. Quantification was performed by monitoring a specific transition for DLSPIER peptides. To determine the maximum loading capacity of the magnetic nanoparticles, different antibody concentrations were used while keeping the amount of nanoparticles constant. In this study, approximately 100, 200, and 400 µg of antibody were added to the immobilization solutions containing 1 mg of beads. It was observed that the maximum surface loading of antibodies onto the magnetic nanoparticles was achieved at a ratio of approximately 100 µg antibody per mg of nanoparticles. In addition, further increases in antibody loading resulted in a decreased in efficiency. When immobilizing of antibodies on the surface of nanoparticles by physical adsorption, it is crucial to select the optimal antibody concentration for each specific case, which is not necessarily the highest concentration available (Byzova et al., 2017). This suggests that at high antibody concentrations, a portion of the nanoparticle surface that is not accessible for antibody binding due to steric hindrance. As shown in Figure 3.2C, the highest amount of immobilized antibody was achieved by conjugating 1 mg of nanoparticles with 100 µg of antibody (59.2 ± 5.7 µg/mg). To calculate the amount required for cTnI enrichment from 1 ml of serum using the synthesized nanoparticle-antibody conjugate, enrichment was performed using 5, 10, 20, and 30 µl of nanoparticle-antibody. As shown in Figure 3.2, the amount of peptide measured reaches saturation after the use of 10 µl of nanoparticle-antibody in serum cTnI enrichment. Therefore, the use of 10 µl of conjugate appears to be sufficient to capture of the cTnI in 1 ml of serum for the analysis. The remaining part of the study was performed using these optimized parameters.

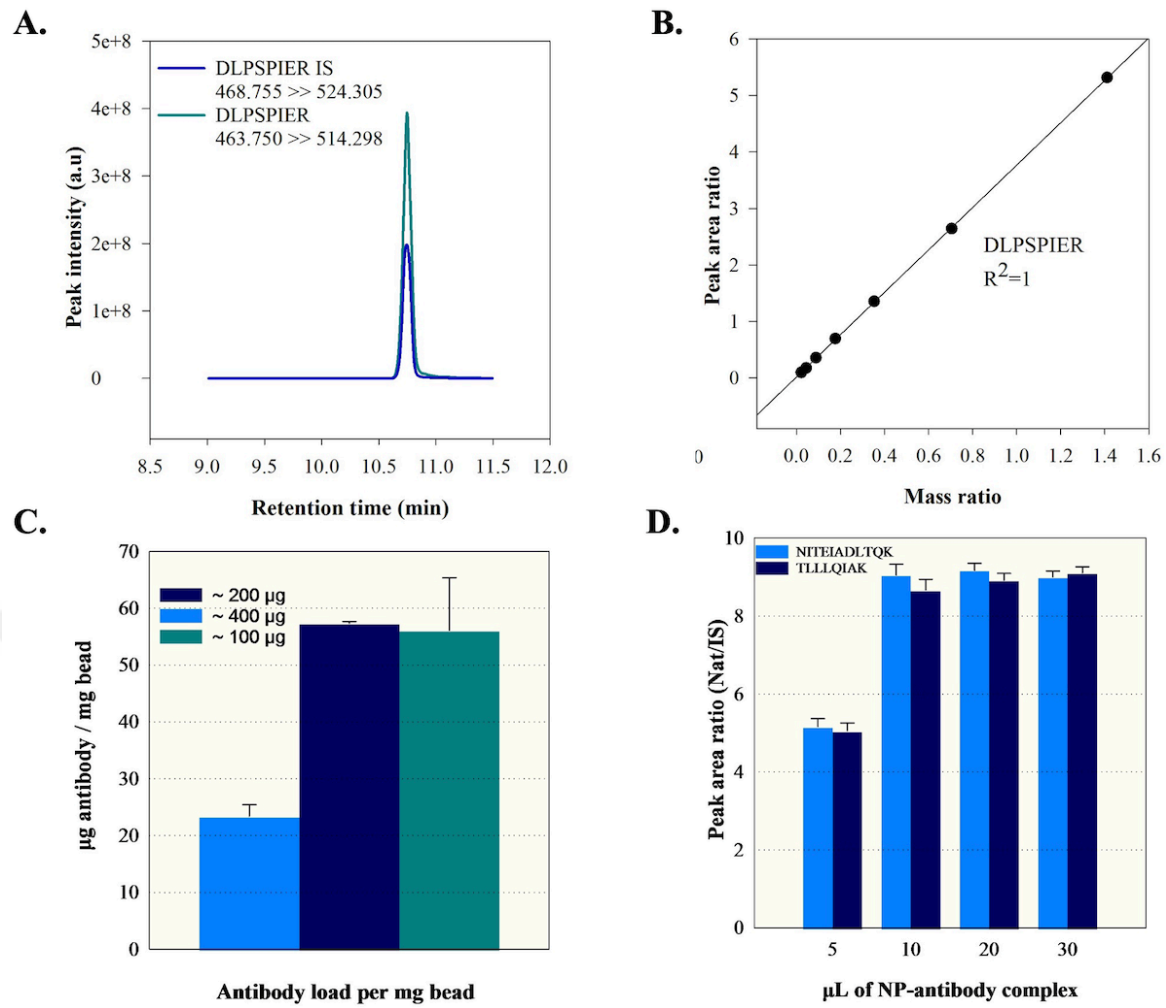

Figure 3.2 : (A) Extracted ion chromatograms. (EIC) for both DLSPIER and IS-DLSPIER peptides. (B) The calibration curve. (C) Bar graph depicting the amount of immobilized anti-cTnI antibody per milligram of nanoparticles. The standard deviation error bars indicate the variability between duplicate preparations of the conjugates. (D) Bar graph depicting the amount of nanoparticles-antibody conjugate required for 1 ml serum enrichment (Asicioglu et al., 2023).

3.1.4 Quantification of cTnI in human serum

Monitoring more than one proteotypic peptide as a quantifier increases confidence in the accuracy of protein analysis in matrix-based assays. Considering the wide range of cTnI modifications and the variability of cTnI isoforms among different patients' plasma, the measurement of multiple peptides provides greater confidence and accuracy (Labugger et al., 2000). Therefore, the proteotypic peptides NITEIADLTQK and TLLLQIAK were selected for cTnI quantification and simultaneously monitored by PRM. The collision energies for each peptide were optimized experimentally optimized by PRM monitoring. The optimized parameters were utilized to observe the

fragmentation transitions of the two cTnI peptides (NITEIADLTQK and TLLLQIAK) and a single antibody peptide (DLPSPIER) in both labeled and unlabeled forms. Table 3.2 summarizes the PRM transitions of selected peptides for cTnI measurement by the nanoparticle enrichment method using the intact protein calibrant (NIST SRM 2921).

Table 3.2 : PRM transitions, amino acid sequence and retention time for the detection of the peptides.

Proteotypic peptides	Retention time (min)	Precursor ions (m/z)	Collision energy (eV)	Product ions (m/z)
TLLLQIAK	13.89	450.30	20	572.377; 685.460
TLLLQIAK (U-N15)	13.89	455.28	20	579.356; 693.437
NITEIADLTQK	11.28	623.35	20	675.367; 1018.542
NITEIADLTQK (U-N15)	11.28	630.31	20	683.311; 1029.506
DLPSPIER	9.73	463.75	25	514.298; 698.383
DLPSPIER IS	9.73	468.755	25	524.305; 708.390

The PRM MS/MS spectrum of the selected cTnI proteolytic peptides and the corresponding extracted ion chromatograms are shown in Figure 3.3. The peak areas of the selected proteotypic peptides were used to establish a calibration curve based on IDMS and quantitative analysis.

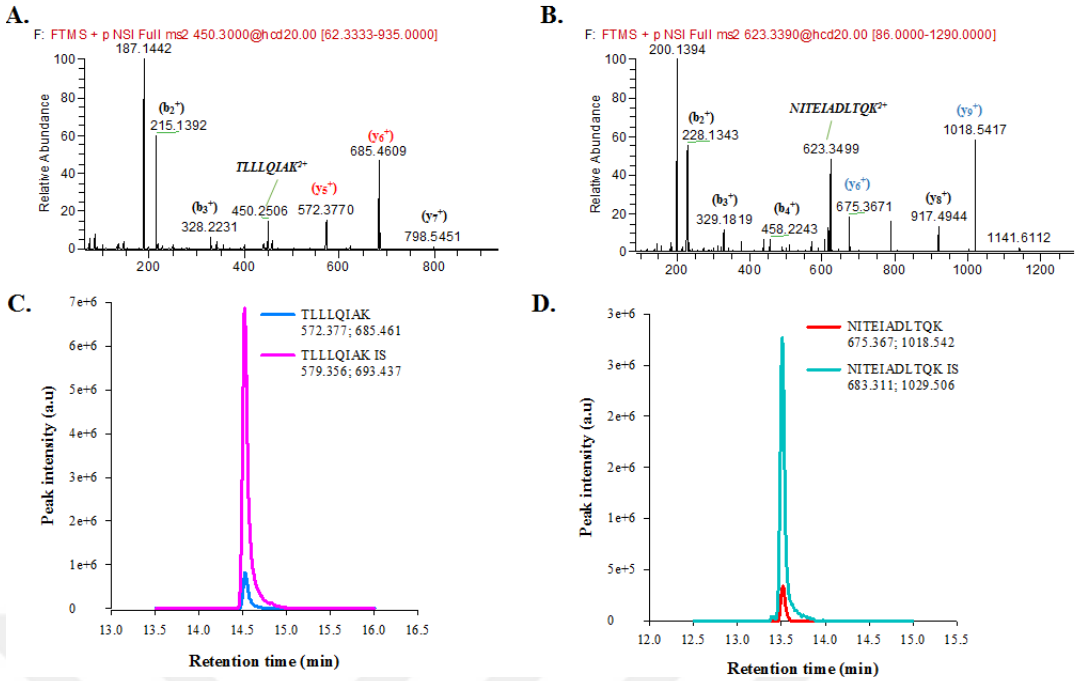

Figure 3.3 : PRM analysis of tryptic digests of cTnI spiked serum. (A) MS/MS spectrum of targeted precursor ion TLLLQIAK^{2+} (selected product ions y_5^+ and y_6^+ for quantification in red). (B) MS/MS spectrum of targeted precursor ion NITEIADLTQK^{2+} (selected product ions y_6^+ and y_9^+ for quantification in blue). (C) EICs obtained when quantifying cTnI-free human serum spiked with a cTnI at LOQ level (1.8 µg/L) and SIL-cTnI at 15 µg/L showing coelution of the TLLLQIAK peptide and its isotopically labeled counterpart. (D) EICs obtained when quantifying cTnI-free human serum spiked with a cTnI at LOQ level (1.8 µg/L) and SIL-cTnI at 15 µg/L showing coelution of the NITEIADLTQK peptide and its isotopically labeled counterpart. Precursor ions were isolated using an isolation window of 0.6 m/z (Asicioglu et al., 2023).

This study utilized NIST SRM 2921, a protein calibrant, along with its labeled internal standard, to establish a calibration curve. Endogenous cTnI from patient serum samples and SIL-cTnI were isolated by immunoaffinity enrichment strategy and subsequently analyzed by ID-MS. There are different forms of cTnI in blood such as triple complex (cTnC-cTnT-cTnI), binary complex (cTnI-cTnC), free form, proteolyzed form, heparin bound form, phosphorylated form, oxidized and reduced forms. Furthermore, cTnI can be degraded both in vivo and in vitro (Apple, 2012). Due to this variability and heterogeneity, there is no perfect matrix-matched protein calibrant for LC-MS quantification strategy. Nevertheless, SRM 2921 has been purified from human heart tissue and because of its heterogeneity is currently the best choice for purpose (Bunk & Welch, 2006).

3.1.5 Method validation

3.1.5.1 Linear range

The method was validated using the NIST SRM 2921 as calibrant. To assess the linearity of the method response, the integrated peak area ratios of cTnI peptide transitions (unlabeled to labeled) were plotted against the mass ratios of SRM 2921. The quantities used for the plot ranged from 0.7 to 24 µg/L, with specific concentrations of 0.7, 1.2, 3.5, 6.0, 12.0 and 24.0 µg/L. The peak areas were automatically integrated by computer using the Quan Browser software program (Thermo Scientific). The method response was found to be linear for between 0.7 µg/L and 23.3 µg/L of cTnI with a regression coefficient of 0.996 and 0.992 for the peptides TLLLQIAK and NITEIADLTQK respectively according to the equations presented in Table 3.3.

Table 3.3 : Linear range of method response by surrogate peptides TLLLQIAK and NITEIADLTQK.

Parameters	TLLLQIAK	NITEIADLTQK
Linear range for cTnI (µg/L)	0.6-23.3	0.7-23.3
Regression equation	$Y_{\text{ratio peak area}} = 2.6219C_{cTnI}-2.3795$	$Y_{\text{ratio peak area}} = 1.7415C_{cTnI}-1.2778$
Correlation coefficient	0.996	0.992
LOQ of cTnI (µg/L)	1.8	4.8
LOD of cTnI (µg/L)	0.6	1.6

The LOD was calculated as defined by ICH (Shrivastava & Gupta, 2011) using the formula LOD = 3 × Sa/b, where b is the slope of the calibration curve and Sa is the standard deviation of the intercept. The LOD values were found to be 0.6 ng and 1.6 ng for cTnI using the peptides TLLLQIAK and NITEIADLTQK for quantification, respectively. The LOQ was also determined according to the defined criteria, LOQ = 10 × Sa/b (Shrivastava & Gupta, 2011), and was found to be 1.8 ng and 4.8 ng for cTnI using the peptides TLLLQIAK and NITEIADLTQK for quantification, respectively.

3.1.5.2 Accuracy

The accuracy is defined under precision and trueness of the test results. For precision of the measurement system, the repeatability and intermediate precision of the method were evaluated by intra-day (analysis of QC solutions in replicates of three in the same day) and inter-day (in replicates of three on four different days) assay variance, respectively. At the QC concentration levels evaluated, the repeatability (intra-run) and intermediate precision (inter-run) of the acceptance standards should be less than 10% (percent RSD). $RSD_{Repeatability}$ (3.1) and $RSD_{intermediate\ precision}$ (3.2) were calculated using the formulas given below. For QCs, the values obtained for repeatability and intermediate precision were less than 10% RSD (Table 3.4). These values met the acceptance requirements, indicating that the current method has sufficient precision.

$$RSD_{Repeatability} = \frac{\sqrt{MS_{within}}}{W_{mean}} x\ 100 \quad (3.1)$$

$$RSD_{IntermediatePrecision} = \frac{\sqrt{MS_{between} - MS_{within}}}{W_{mean}} x\ 100 \quad (3.2)$$

Table 3.4 : The precision results of different cTnI concentrations.

	TLLLQIAK (µg/L)			NITEIADLTQK (µg/L)		
Repeatability (n=3)	**2**	**5**	**12**	**2**	**5**	**12**
Mean ± SD	2.1 ± 0.1	4.7 ± 0.8	11.9 ± 0.1	2.0 ± 0.1	4.9 ± 0.3	13.3 ± 0.3
$RSD_{Repaatability}$	4.9	6.8	2.2	8.7	4.4	2.0
Intermediate precision (n=12)						
Mean ± SD	2.1 ± 0.1	4.9 ± 0.4	11.4 ± 0.5	2.1 ± 0.2	5.0 ± 0.5	12.7 ± 0.7
$RSD_{Int.precision}$	6.4	9.6	5.2	8.7	4.4	7.6

The trueness assessment was performed with the recovery evaluated using NIST SRM 2921. Three QC levels 2.0 (low), 5.0 (medium), and 12.0 (high) µg/L were assessed for each peptide. The obtained recovery data for TLLLQIAK was 104.2 %, 97.3 %

and 94.8 % for 2, 5 and 10 µg/L cTnI levels respectively. The obtained recovery data for NITEIADLTQK was 104.5 %, 99.9 % and 106.1 % for 2, 5 and 10 µg/L cTnI levels respectively. Accordingly, % recovery values were calculated and presented in Table 3.5. Those values met the recovery acceptability requirements, indicating that the current approach was adequate in terms of trueness.

Table 3.5 : The trueness results of different cTnI concentrations.

	TLLLQIAK (µg/L)			NITEIADLTQK (µg/L)		
% Trueness (n=3)	**2**	**5**	**12**	**2**	**5**	**12**
Mean, Day 1	106.9	94.1	99.4	101.7	98.2	110.8
Mean, Day 2	96.6	101.4	92.5	100.7	103.1	108.2
Mean, Day 3	107.2	89.6	96.3	111.2	100.8	107.8
Mean, Day 4	105.9	104.0	91.0	104.4	97.6	97.5
Mean ± SD	104.2 ± 4.4	97.3 ± 5.7	94.8 ± 3.3	104.5 ± 4.1	99.9 ± 2.2	106.1 ± 5.1

3.1.5.3 Carryover

In the present method, a solution of acetonitrile/water (80/20; v/v) with 0.1% formic acid was used to rinse the syringe and injection port. This wash procedure was performed several times before and after each injection. Under these washing conditions, the signal observed at the retention time of each peptide (area below the peak) was less than 1% compared to that found in the LOD after injection of a blank sample.

3.1.6 Evaluation of measurement uncertainty

The uncertainty of the method was evaluated according to EURACHEM / CITAC Guide CG 4 (third edition) entitled “Quantifying Uncertainty in Analytical Measurement” (B. Magnusson and U. Örnemark (2014); S L R Ellison and A Williams (2012). Uncertainty sources of arising from operations steps such as pipetting, dilution, balances and volumetric equipment are covered by the reproducibility, intermediate precision and recovery uncertainties. The sources of uncertainty identified for the present method are the following defined parameters: balance, the repeatability

standard deviation s_r (3.3), the contribution of the grouping factor to the total variation ($s_{between}$) (3.4) the uncertainty of the calibration curve (3.5). The formula of the standard combined uncertainty (3.6) given below. The expanded uncertainty has been calculated considering a coverage factor of 2 for a confidence of approximately 95%. The breakdown of the uncertainty budget is presented in Table 3.6.

$$s_r = \sqrt{MS_{withingroup}} \tag{3.3}$$

$$s_{between} = \sqrt{\frac{MS_{within} - MS_{between}}{n_{repeat}}} \tag{3.4}$$

$$u_{cal} = \frac{s}{m} x \sqrt{\frac{1}{n_{rep}} + \frac{1}{n_{cal}} + \frac{(x_{pred} - x_{mean})^2}{\sum(x_i - x_{mean})^2}} \tag{3.5}$$

$$u_{combined} = k \sqrt{\frac{u_{rep}{}^2}{n_{rep}} + \frac{u_{intPre}{}^2}{n_{days}} + u_{calibration}{}^2 + u_{balance}{}^2} \tag{3.6}$$

Table 3.6 : Breakdown of the uncertainty budget.

	TLLLQIAK (µg/L)			NITEIADLTQK (µg/L)		
	2	5	12	2	5	12
Mean	2.1	4.9	11.4	2.1	5.0	12.7
Uncertainty components						
s_r	0.1	0.2	0.3	0.2	0.2	0.5
$s_{between}$	0.1	0.3	0.2	0.2	0.2	0.3
u_{cal}	0.2	0.2	0.2	0.2	0.2	0.2
Balance	0.0	0.0	0.0	0.0	0.0	0.0
k	2	2	2	2	2	2
Uncertainty, u_c	0.3	0.5	0.5	0.3	0.4	0.6
Expanded Uncertainty, U	0.6	0.9	0.9	0.7	0.7	1.2

3.1.7 Application to clinical samples

To evaluate the results of the developed reference method, the candidate reference method was applied to patient serum samples. Although it was almost mimicked the patient sample by adding the human cTn complex to human cTnI free serum, it is still important to quantify the developed method on clinical samples considering variety of cTnI modification and unique cTn profile of individuals. Unfortunately, in all samples collected (n=25), the levels of cTnI were found to be below the LOQ of our method. From all the collected patient samples, the four samples which cTnI values are above the detection limit were selected and analyzed by the developed ID-LC-MS/MS method.

Table 3.7 presents the results obtained from both the ID-LC-MS/MS method and the Siemens Atellica® Solution immunoassay. The recovery and uncertainty was not calculated because the cTnI values of the selected samples, as detected by the immunoassay, were below the quantification limit of the developed ID-MS method. Based on the results of the validation study, these samples could be detected but might not provide fully accurate quantitative results. The experimental results confirmed our prediction. However, the fact that the developed methodology performed similarly on the patient samples demonstrates the reliability of the method. This is further supported by the high recovery values observed for the QC samples prepared at concentrations of 2, 5, and 12 µg/L, as shown in Table 3.5.

Table 3.7 : Application to clinical samples.

	cTnI concentration from immunoassay measurements (µg/L)	ID-LC-MS/MS	
		TLLLQIAK (µg/L)	NITEIADLTQK (µg/L)
Patient 1	0.6	0.4	0.5
Patient 2	1.6	0.8	0.8
Patient 3	0.7	0.6	0.5
Patient 4	0.6	0.3	0.4

In future developments, it is important to improve the developed method by achieving a lower LOQ. Additionally, a larger number of samples should be utilized to assess the applicability of the method to clinical sample.

3.2 Objective 2 : An ID-LC-MS/MS-based Analytical Procedure for the Quantification of cTnI in Human Serum Using a Peptide-based Calibration

3.2.1 PICAA analysis

3.2.2 Amino acid analysis

AAA provided an absolute quantification of the amino acid content in the primary stock solution. Three amino acids (Ala, Leu, and Ile) were selected for quantification, and analysis was performed in triplicate over three days. The AAA determined that the TLLLQIAK peptide has an 85% mass fraction, while the NITEIADLTQK peptide has a 65% mass fraction. Characterizing peptide-related impurities is essential because AAA can overestimate the mass fraction values, as these impurities also contain the amino acids used for the analysis.

3.2.3 Impurity analysis

HR-LC-MS/MS was used to identify peptide-related impurities. Intact mass analysis revealed the presence of different peptide impurities and various isoforms of the TLLLQIAK peptide, identifying eight impurities. Table 3.8 includes the identity, mass fraction, and uncertainty for each characterized impurity. The identified impurities are mostly truncated forms of the TLLLQIAK peptide (LLLQIAK), forms with the loss of an amino acid (TLLLIAK, TLLLQIK), forms with the incorporation of an additional amino acid (TLLLIKT, ALLLQIAK, TLLLQIAAT), or forms with different post-translational modifications (TLLLQ(deaminated)IAK, Formyl-LLLQAK). Similarly, intact mass analysis revealed various peptide impurities and isoforms of the NITEIADLTQK peptide, identifying four impurities detailed in Table 3.9. These impurities are mostly forms with the loss and addition of an amino acid (NITEIADLK, NITEILTQK, NITEIADLTK, NIIEIADLTQK), which are commonly found in synthetic peptides. Contributions from peptide-related impurities were subtracted from the AAA results. The molar concentration of the TLLLQIAK peptide in the primary stock solution, including its expanded uncertainty, was (439.51 ± 17.25) µmol/L (Table 3.10). The molar concentration of the NITEIADLTQK peptide in the primary stock solution, including its expanded uncertainty, was (352.18 ± 12.88) µmol/L (Table 3.11). The assigned values and corresponding combined uncertainties are summarized in Table 3.12.

Table 3.8 : Purity analysis of TLLLQIAK with identification and quantification of peptide-based related impurities present in the primary stock solution as confirmed by LC-HRMS/MS and quantified by LC-HRMS.

Code	Standard peptide and its impurities	RT (min)	m/z	Mass	Mass fraction (mg/g)	Standard uncertainty ($u_{standard}$) (mg/g)	Coverage factor (k)	Expanded uncertainty ($U_{expanded}$) for about 95% confidence interval (mg/g)
	TLLLQIAK	13.44	2+	899.5911	-	-	-	-
I1	LLLQIAK	12.68	2+	798.5439	4.34	0.13	2	0.26
I2	TLLLIAK	13.05	2+	771.5325	0.61	0.11	2	0.21
I3	TLLLIKT	12.86	2+	801.5446	19.14	0.32	2	0.65
I4	TLLLQIK	13.48	2+	828.5541	1.03	0.10	2	0.21
I5	TLLLQ(Deamidated)IAK	14.22	2+	900.5755	20.40	0.33	2	0.65
I6	ALLLQIAK	14.54	2+	869.5818	24.98	0.53	2	1.06
I7	TLLLQIAAT	13.37	2+	943.5821	0.38	0.11	2	0.21
I8	Formyl-LLLQAK	15.19	2+	713.4559	0.76	0.11	2	0.21

(*)Impurity represented as I.

Table 3.9 : Purity analysis of NITEIADLTQK with identification and quantification of peptide-based related impurities present in the primary stock solution as confirmed by LC-HRMS/MS and quantified by LC-HRMS.

Code	Standard peptide and its impurities	RT (min)	m/z	Mass	Mass fraction (mg/g)	Standard uncertainty ($u_{standard}$) (mg/g)	Coverage factor (k)	Expanded uncertainty ($U_{expanded}$) for about 95% confidence interval (mg/g)
	NITEIADLTQK	12.59	2+	1245.6682	-	-	-	-
I1	NITEIADLTK	12.18	2+	1016.563	19.37	0.17	2	0.34
I2	NIIEIADLTQK	12.79	2+	1117.6095	0.75	0.07	2	0.14
I3	NITEILTQK	12.51	2+	1059.6047	4.34	0.04	2	0.08
I4	NITEIADLK	14.68	2+	1257.6677	0.58	0.07	2	0.14

(*)Impurity represented as I.

Table 3.10 : The total amino acid concentration of TLLLQIAK and the associated relative uncertainties.

TLLLQIAK	Ala	Leu	Ile	Relative contribution of uncertainty %		
				Ala	Leu	Ile
Concentration (μM)	439.93	445.28	433.32			
Uncertanity Components						
IntPre (μM)	6.82	9.51	3.79	41.07	55.50	27.29
Variance Between Days (AA) (μM)	5.07	8.96	0.00			
Variance Between Hydrolysis (AA) (μM)	4.56	3.18	3.79			
Uncertanity of Intact Analyisis (μM)	2.34	2.34	2.34	14.12	13.69	16.87
Measurement Precision	3.46	3.46	3.46	20.82	20.18	24.88
Calibration Curve	3.10	0.93	3.43	18.69	5.43	24.70
Purity of Amino Acid std	0.88	0.89	0.87	5.30	5.20	6.25
The accuracy of Balance	0.00	0.00	0.00	0.00	0.00	0.00
Combined Standard Uncertanity (μM)	8.62	10.47	6.66	16.61	17.13	13.90
Expanded Standard Uncertanity (k=2)	17.25	20.93	13.32			
Relative Expanded Standard Uncertainty (%)	3.92	4.70	3.07	100.00	100.00	100.00

Table 3.11 : The total amino acid concentration of NITEIADLTQK and the associated relative uncertainties.

NITEIADLTQK	Ala	Leu	Ile	Relative contribution of uncertainty %		
				Ala	Leu	Ile
Concentration (μM)	344.27	337.78	374.49			
Uncertanity Components						
IntPre (μM)	3.70	6.54	9.58	29.96	51.82	57.43
Variance Between Days (AA) (μM)	2.73	6.19	2.86			
Variance Between Hydrolysis (AA) (μM)	2.49	2.12	9.14			
Uncertanity of Intact Analyisis (μM)	0.67	0.67	0.67	5.46	5.34	4.04
Measurement Precision	3.24	3.24	3.24	26.27	25.70	19.45
Calibration Curve	4.04	1.49	2.43	32.73	11.79	14.58
Purity of Amino Acid std	0.69	0.68	0.75	5.58	5.36	4.50
The accuracy of Balance	0.00	0.00	0.00	0.00	0.00	0.00
Combined Standard Uncertanity (μM)	6.44	7.51	10.45	12.35	12.63	16.69
Expanded Standard Uncertanity (k=2)	12.88	15.03	20.91			
Relative Expanded Standard Uncertainty (%)	3.74	4.45	5.58	100.00	100.00	100.00

Table 3.12 : Assigned peptide concentrations and summary of relative uncertainties.

Peptides	Quantitative values (µM)	Combined relative uncertainty (µmol/L)	Combined relative uncertainty (%)
TLLLQIAK	439.51	17.25	3.92
NITEIADLTQK	352.18	12.88	3.74

3.2.4 Trypsin digestion optimizations

Quantifying a protein at low-abundance levels within a complex matrix such as serum poses an analytical challenge. Particularly for protein measurement using a peptide-based calibration strategy, it is essential to provide complete digestion (Huynh et al., 2021). To achieve this, parameters of the digestion method such as enzyme-to-protein ratio, and digestion kinetics have to be optimized. This optimization aims to increase protein isolation and proteolysis yield, while minimizing interferences and potential material loss during sample preparation.

Initially, four different digestion protocols were evaluated testing two different methods, different enzyme-to-ratios and different digestion times. Peptides were analyzed through PRM, monitoring two cTnI proteotypic peptides (TLLLQIAK and NITEIADLTQK). The largest peak areas for both peptides were observed using the FASP method with Pierce Trypsin/Lys-C Protease Mix. The largest peak areas for both peptides were observed using the FASP method with Pierce Trypsin/Lys-C Protease Mix (Figure 3.4A). Based on these findings, FASP emerged as the most effective digestion method for measuring cTnI and was selected for further studies.

Next, the enzyme-to-protein ratio was optimized by testing ratios of 1:10, 1:25, 1:50, and 1:100 using the FASP method. PRM analysis showed that while both peptides appeared to reach saturation at a 1:25 ratio, the decision was made to use a 1:10 ratio to ensure complete digestion (Figure 3.4B).

Following this, a digestion kinetics study was conducted with incubation times of 2 hours, 4 hours, 6 hours, and overnight. Signal intensity was maximal for both the TLLLQIAK and NITEIADLTQK peptides after overnight digestion, making it the most effective digestion time for measuring cTnI Figure 3.4C.

In the final stage of optimizations, digestion was conducted within the matrix and after enrichment to mimic real experimental conditions. This was done to account for the

complexities introduced by antibody-magnetic nanoparticle conjugates and the unique challenges of serum-based samples. Further enzyme-to-protein ratios of 1:5 and 1:2.5 were tested. Additionally, fresh trypsin was added after overnight digestion for the 1:10, 1:5, and 1:2.5 ratios to assess if extended digestion would improve results, as porcine trypsin loses most of its activity after 24 hours at 37°C. The samples were then incubated for an additional six hours, bringing the total digestion time to 24 hours. However, no significant improvement was observed with the addition of extra trypsin Figure 3.4D. Thus, it was concluded that a 1:10 enzyme-to-protein ratio with overnight FASP digestion at 37 °C is the optimal condition where no further digestion occurs.

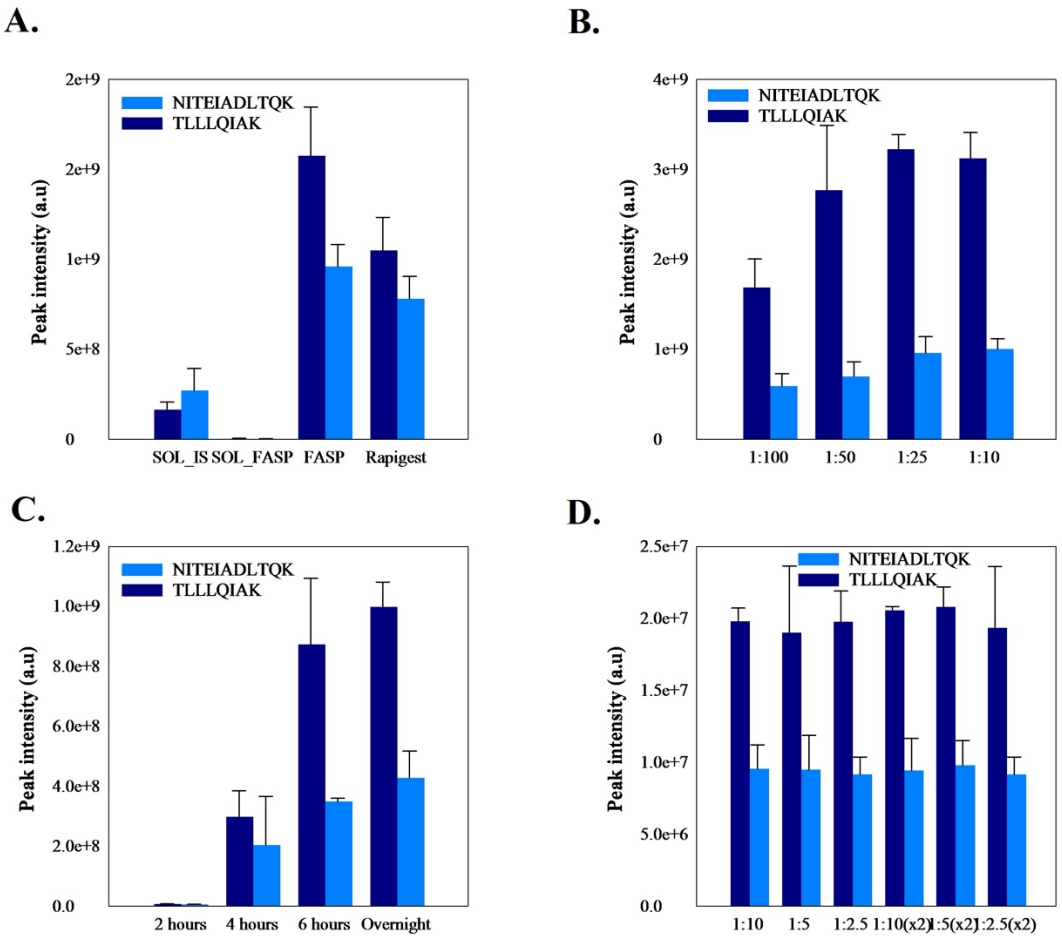

Figure 3.4 : The average areas of chromatographic peaks for two cTnI specific peptides. (A) Using various digestion methods (SOL_IS, SOL_FASP, Rapigest, FASP). (B) FASP-digestion at different enzyme-to-protein ratios (1:10, 1:25, 1:50, 1:100). (C) At different digestion times (2h, 4h, 6h, overnight). (D) Peak intensities were compared for digestion products using enzyme-to-protein ratios of 1:10, 1:5, and 1:2.5 after overnight and 24-hour digestion. Fresh trypsin was added to the samples undergoing 24-hour digestion after the initial overnight digestion. Each experiment was conducted with two subsamples and two technical replicates.

3.2.5 Method validation

The cTnI proteotypic peptides, TLLLQIAK and NITEIADLTQK, were quantified using ID-LC-MS/MS with the PRM method. The optimized LC gradient minimized ionization suppression and improved peptide separation. The EICs for calibration standards at LOQ levels are shown in Figure 3.5. The MS/MS spectra of the two selected peptides and their isotopically labeled standard peptide are given in Figure 3.6. By using these optimized conditions, robust and accurate quantification of low-abundance cTnI in human serum was achieved.

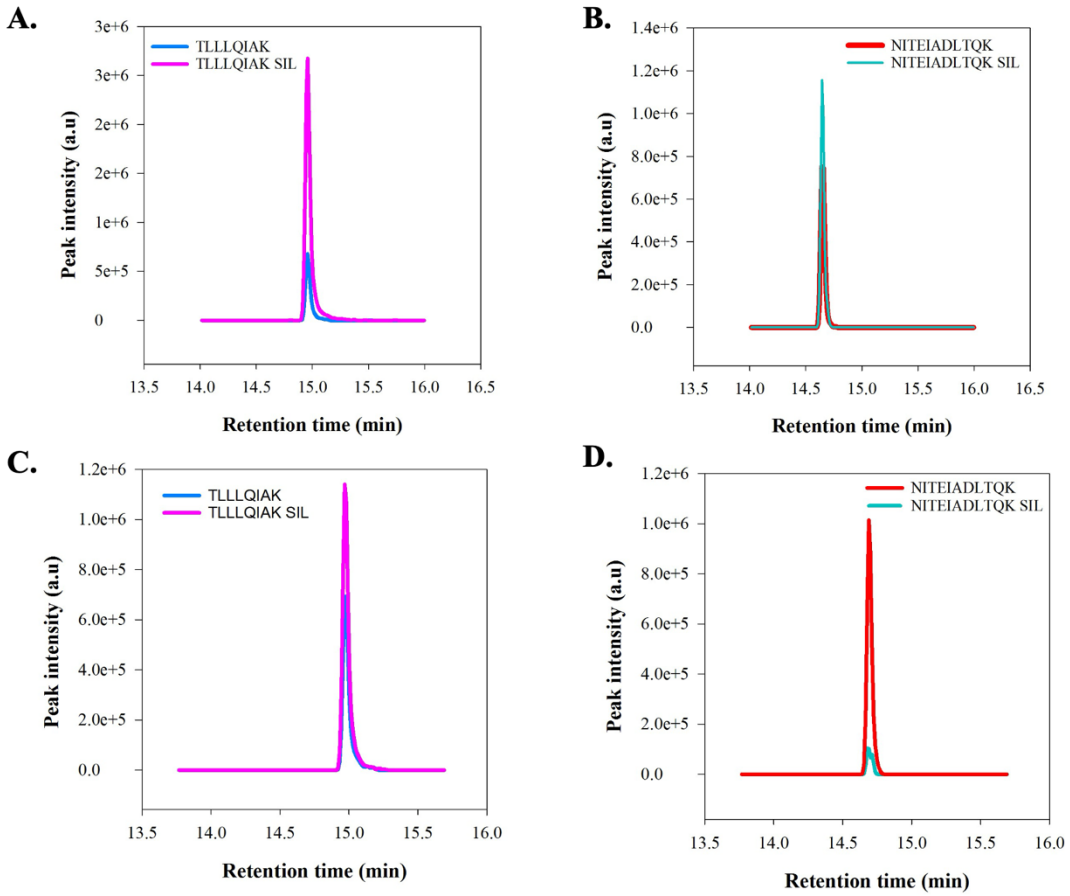

Figure 3.5 : (A) EIC of TLLLQIAK from serum matrix calibrants at LOQ (~2.5 µg/L). (B) EIC of NITEIADLTQK from serum matrix calibrants at LOQ (~2.5 µg/L). (C) EIC of TLLLQIAK from digest matrix calibrants at LOQ (~2.5 µg/L). (D) EIC of NITEIADLTQK from digest matrix calibrants at LOQ (~2.5 µg/L). Precursor ions were isolated within an isolation window of 0.6.

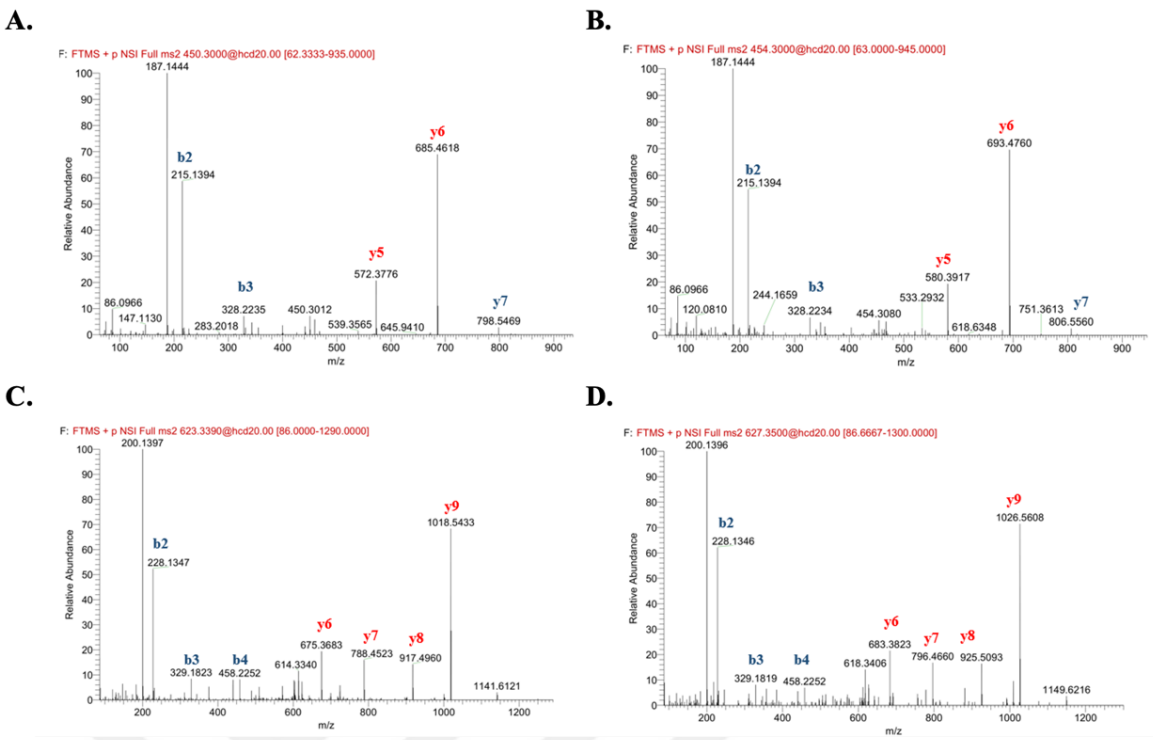

Figure 3.6 : The MS/MS spectra of the selected two cTnI proteotypic peptides and their isotopically labeled versions. (A) MS/MS spectrums of the TLLLQIAK peptide at m/z 450.3 (z=2). (B) MS/MS spectrums of the isotopically labeled TLLLQIAK peptide at m/z 454.28 (z=2). (C) MS/MS spectrums of the NITEIADLTQK peptide at m/z 623.33 (z=2). (D) MS/MS spectrums of the isotopically labeled NITEIADLTQK peptide at m/z 627.35 (z=2). The fragmentation profile was obtained from calibration solutions.

3.2.5.1 Linear range

The linearity of the six-point digest matrix calibration curve for cTnI concentrations ranging from 0.6 to 21.6 µg/L showed regression coefficients of 0.998 and 0.992 for the peptides TLLLQIAK and NITEIADLTQK, respectively (Table 3.13). Similarly, the linearity of the six-point serum matrix calibration curve demonstrated regression coefficients of 0.995 and 0.998 for TLLLQIAK and NITEIADLTQK, respectively (Table 3.13). The slopes for the serum matrix curves were within ±10% of those in the digest matrix, indicating good correlation between the two calibration methods (Table 3.14). The LOD was calculated as defined by ICH (Shrivastava & Gupta, 2011) using the formula LOD=3×Sa/b, where b is the slope of the calibration curve and Sa is the standard deviation of the intercept. Using the digest matrix calibration, the LOD values were found to be 0.8 ng and 1.5 ng for cTnI using the peptides TLLLQIAK and NITEIADLTQK for quantification, respectively (Table 3.13). The LOQ was also

determined according to the defined criteria, LOQ=10×Sa/b (Shrivastava & Gupta, 2011), and was found to be 2.5 ng and 4.3 ng for cTnI using the peptides TLLLQIAK and NITEIADLTQK for quantification, respectively. Using the serum matrix calibration, the LOD values were found to be 0.9 ng and 0.9 ng for cTnI using the peptides TLLLQIAK and NITEIADLTQK for quantification, respectively (Table 3.13. The LOQ was found to be 2.8 ng and 2.7 ng for cTnI using the peptides TLLLQIAK and NITEIADLTQK for quantification, respectively.

Table 3.13 : Calibration curves parameters (n=6 per level).

Parameters	TLLLQIAK		NITEIADLTQK	
	Serum Matrix	Digest Matrix	Serum Matrix	Digest Matrix
Optimum range for cTnI (µg/L)	0.9-22.0	0.6-21.6	1.5-28.3	0.6-22.1
Regression equation	$Y_{ratio\ peak\ area}$ = 0.562CcTnI +0.0215	$Y_{ratio\ peak\ area}$ = 0.586CcTnI +0.0083	$Y_{ratio\ peak\ area}$ = 1.382CcTnI -0.0564	$Y_{ratio\ peak\ area}$ = 2.115CcTnI -0.0126
Correlation coefficient	0.995	0.998	0.998	0.992
LOQ of cTnI (µg/L)	2.8	2.5	2.7	4.3
LOD of cTnI (µg/L)	0.9	0.8	0.9	1.5

3.2.5.2 Precision and trueness

The trueness and intermediate precision assessment were performed as described in section 2.2.8. The repeatability and intermediate precision results from both calibration curve is shown in Table 3.14. The $RSD_{Repeatability}$ values for both peptides (TLLLQIAK and NITEIADLTQK) across different concentrations are less than 10% for both matrices, indicating good repeatability. The $RSD_{Intermediate\ precision}$ values are higher and range between 9.5% and 28.9% indicating that the method may need further optimization to reduce variability. Overall, while the method demonstrates acceptable precision for many conditions, attention should be paid to improving intermediate precision for better reliability across different days.

QC samples prepared using NIST SRM 2921 at three different levels (2, 5, and 10 µg/L) were measured with the developed method, and it was found that the measured values for all levels were 18.3 times lower than the certified values (n=4 for each level measurement) for serum matrix calibrant and 20.5 times lower than the certified values

(n=4 for each level measurement) for digest matrix calibrant. As mentioned before, peptide-based calibration differs from protein-based calibration in that peptide calibrants are directly spiked, while QC and patient samples undergo protein enrichment, washing, and tryptic digestion before adding the internal peptide standard. This process can lead to material loss and incomplete enrichment, where beads may not capture all the analyte. To address these variations, a correction factor was calculated using the equation provided by Huynh et al., published in 2021 (Huynh et al., 2021) resulting in a correction factor 18.3 for serum matrix calibrant and 20.5 for digest matrix calibrant. The trueness calculations presented above were calculated after the application of the correction factor.

Trueness was evaluated with the recovery of three QC samples for each peptide. Using the serum matrix calibration curve, the recovery data (mean of three days) for 2, 5, and 10 µg/L TLLLQIAK were 97.4%, 93.7%, and 94.1%, respectively. For NITEIADLTQK, the recovery data were 124.0%, 100.7%, and 102.6% for 2, 5, and 10 µg/L cTnI levels, respectively. Using the digest matrix calibration curve, the recovery data (mean of three days) for 2, 5, and 10 µg/L TLLLQIAK were 114.5%, 106.8%, and 106.8%, respectively. For NITEIADLTQK, the recovery data were 98.1%, 80.2%, and 105.2% for 2, 5, and 10 µg/L cTnI levels, respectively. Detailed data are available in (Table 3.15).

The trueness of the method for TLLLQIAK is satisfactory in both the digest and serum matrices. Minor deviations are observed but are within acceptable ranges for most applications. The trueness for NITEIADLTQK shows more variability. Upon examining Table 3.15, a significant variation in the recoveries was observed for both peptides across the two matrices. This large spread was attributed to the peptide-based calibration strategy employed. The reason is that such a wide variation was not observed in the trueness assessment of the protein-based calibration strategy (Asicioglu et al., 2023).

Table 3.14 : Precision of cTnI spiked quality control samples in cTnI-free serum calculated calibration standards prepared in serum matrix and digest matrix.

	cTnI concentration (µg/L)	Serum Matrix				Digest Matrix			
		Repeatability		Intermediate precision		Repeatability		Intermediate precision	
		Mean ± SD	$RSD_{Repeatability}$	Mean ± SD	RSD_{IntPre}	Mean ± SD	$RSD_{Repeatability}$	Mean ± SD	RSD_{IntPre}
TLLLQIAK	2	1.9 ± 0.1	3.2	2.0 ± 0.1	9.9	2.0 ± 0.1	4.3	2.1 ± 0.1	12.5
	5	5.5 ± 0.3	3.0	5.1 ± 0.7	28.9	5.5 ± 0.3	3	5.2 ± 0.6	26.4
	10	10.8 ± 5.0	5.8	10.0 ± 1.1	22.9	10.8 ± 5.0	5.7	10.2 ± 1.0	19.5
NITEIADLTQK	2	2.7 ± 0.3	4.8	2.6 ± 0.2	9.5	1.9 ± 0.0	1.8	1.8 ± 0.1	11.8
	5	5.7 ± 0.7	4.4	5.5 ± 0.5	18.8	3.9 ± 0.2	6.6	3.9 ± 0.4	19.7
	10	11.2 ± 1.0	2.3	10.9 ± 1.1	22.6	10.7 ± 1.1	3.3	10.0 ± 1.0	22.7

Table 3.15 : Trueness of cTnI spiked quality control samples in cTnI-free serum calculated calibration standards prepared in serum matrix and digest matrix.

	cTnI concentration (µg/L)	Trueness (%)							
		Serum Matrix				Digest Matrix			
		Mean, Day1	Mean, Day2	Mean, Day3	Mean ± SD	Mean, Day1	Mean, Day2	Mean, Day3	Mean ± SD
TLLLQIAK	2	84.3	120.8	87.1	97.4 ± 16.6	97.8	145.0	100.8	114.5 ± 21.6
	5	104.9	78.5	97.6	93.7 ± 11.2	118.8	91.1	110.4	106.8 ± 11.6
	10	103.3	82.9	96.0	94.1 ± 8.4	116.1	96.2	108.0	106.8 ± 8.2
NITEIADLTQK	2	118.5	151.8	101.6	124.0 ± 20.8	94.7	110.2	89.2	98.1 ± 8.9
	5	109.5	90.1	102.4	100.7 ± 8.0	84.1	72.1	84.4	80.2 ± 5.7
	10	107.0	92.0	108.7	102.6 ± 7.5	114.4	93.2	108.0	105.2 ± 8.9

3.2.5.3 Carryover

In this method, a solution consisting of acetonitrile and water in a ratio of 50:50 (v/v) with 0.1% formic acid was utilized to rinse both the syringe and the injection port. This washing protocol was repeated multiple times before and after each injection. With these washing parameters, the signal detected at the retention time of each peptide (area under the peak) was below 1% compared to the signal observed at the LOD following the injection of a blank sample.

3.2.6 Uncertainty evaluation

The measurement uncertainty was performed according to EURACHEM/CITAC Guide CG 4 (third edition) entitled "Quantifying Uncertainty in Analytical Measurement" (Ellison Secretary et al., 2009; Eurachem Guides, 2020). The uncertainties arising from operational procedures include factors such as volumetric equipment, dilution, pipetting, balances, intermediate precision, and recovery uncertainties. The uncertainties identified for the developed method encompass specific parameters including balance, the standard deviation of repeatability (s_r), the contribution of the grouping factor to the total variation ($s_{between}$), and the uncertainty associated with the calibration curve (u_{cal}). The formula for standard combined uncertainty, along with previously provided uncertainty components, has been outlined (Asicioglu et al., 2023). The expanded uncertainty has been calculated with a coverage factor of 2 to achieve a confidence level of approximately 95%. The breakdown of the uncertainty budget using digest matrix calibration curve is shown in Table 3.16, while serum matrix calibration curve is shown in Table 3.17. The relative expanded uncertainties for the digest matrix from 22% to 33% for TLLQIAK and 24% to 29% for NITEIADLTQK, whereas for the serum matrix, they range from 17% to 35% for TLLQIAK and 26% to 28% for NITEIADLTQK.

Table 3.16 : The breakdown of the uncertainty budget of digest matrix calibration curve.

	TLLLQIAK (µg/L)			NITEIADLTQK (µg/L)		
	2	5	10	2	5	10
Mean	2.1	5.2	10.2	1.8	3.9	10.0
Uncertainty components						
s_r	0.2	0.8	1.1	0.1	0.4	1.3
$s_{between}$	0.1	0.2	0.6	0.0	0.3	0.3
u_{cal}	0.0	0.0	0.0	0.0	0.0	0.1
Balance	0.0	0.0	0.0	0.0	0.0	0.0
NIST SRM 2921	4.5	4.5	4.5	4.5	4.5	4.5
Uncertainty, u_c	0.2	0.8	1.4	0.2	0.6	1.4
Expanded Uncertainty, U	0.5	1.7	2.7	0.4	1.1	2.9
Relative Expanded Uncertainty, U (%)	21.8	32.4	26.9	23.8	28.4	28.6

*k: 2 (confidence level of approximately 95%)
**NIST SRM 2921: Uncertainty of SRM

Table 3.17 : The breakdown of the uncertainty budget of serum matrix calibration curve.

	TLLLQIAK (µg/L)			NITEIADLTQK (µg/L)		
	2	5	10	2	5	10
Mean	2.0	5.1	10.0	2.6	5.5	10.9
Uncertainty components						
s_r	0.1	0.8	1.3	0.1	0.6	1.4
$s_{between}$	0.1	0.2	0.6	0.1	0.2	0.2
u_{cal}	0.0	0.0	0.0	0.0	0.2	0.3
Balance	0.0	0.0	0.0	0.0	0.0	0.0
NIST SRM 2921	4.5	4.5	4.5	4.5	4.5	4.5
Uncertainty, u_c	0.2	0.9	1.5	0.3	0.7	1.5
Expanded Uncertainty, U	0.3	1.8	3.0	0.7	1.4	3.1
Relative Expanded Uncertainty, U (%)	16.8	35.2	30.2	25.7	25.9	28.2

*k: 2 (confidence level of approximately 95%)
**NIST SRM 2921: Uncertainty of SRM

3.2.7 Application to patient samples

The developed ID-LC-MS/MS method, along with two surrogate calibration matrices, was applied to patient serum samples to assess its performance. Four patient samples were analyzed using this method. Table 3.18 and Table 3.19 reveal that the results obtained by the commercially available Siemens ADVIA Centaur immunoassay were consistently higher compared to those obtained by ID-LC-MS/MS. The analysis of the same patient with two different calibration curves yielded highly similar results, with comparable differences observed when compared to the immunoassay results. This observed discrepancy may stem from differences in calibration systems between the IDMS and immunoassay. Notably, a significant portion of cTnI in blood exists as a binary cTnI-cTnC complex, potentially contributing to these variations (Apple et al., 2017). Overall, the measurement results, precision, and the relative difference between ID-LC-MS/MS and the immunoassay were consistent across both matrices of the calibrants.

Table 3.18 : Comparison between commercially available immunoassays and the ID-LC-MS/MS method for four patient serum samples using serum matrix calibrant as a surrogate matrix.

	cTnI concentration from immunoassay measurements (µg/L)	Serum matrix calibration curve			
		TLLLQIAK (µg/L)	NITEIADLTQK (µg/L)	Average of two peptide (µg/L)	Relative difference between ID-LC-MS/MS and IA (%)
Patient 1	12.36	7.26	5.81	6.53	-0.47
Patient 2	8.34	3.07	1.53	2.30	-0.72
Patient 3	10.20	2.88	3.18	3.03	-0.70
Patient 4	16.62	8.64	8.49	8.57	-0.48

*Relative difference (LC-MS/MS-Immunoassay)/Immunoassay

Table 3.19 : Comparison between commercially available immunoassays and the ID-LC-MS/MS method for four patient serum samples using digest matrix calibrant as a surrogate matrix.

	cTnI concentration from immunoassay measurements (µg/L)	Digest matrix calibration curve			
		TLLLQIAK (µg/L)	NITEIADLTQK (µg/L)	Average of two peptide (µg/L)	Relative difference between ID-LC-MS/MS and IA (%)
Patient 1	12.36	6.48	3.22	4.85	-0.61
Patient 2	8.34	2.78	1.01	1.90	-0.77
Patient 3	10.20	2.65	2.01	2.33	-0.77
Patient 4	16.62	6.94	8.12	7.53	-0.55

*Relative difference (LC-MS/MS-Immunoassay)/Immunoassay

4. CONCLUSIONS AND RECOMMENDATIONS

In the first part of the thesis, an ID-LC-MS/MS method was developed to quantify cTnI in human serum using protein-based calibration approach. This method consisted of two different ID-LC-MS/MS methods. The first method was used to quantify anti-cTnI antibody bound to magnetic beads, while the second method was used to quantify cTnI in human serum. These two methods allowed for the specific and accurate measurement of both the antibody and the target protein in their respective matrices.

ID-LC-MS/MS offers advantages such as the ability to differentiate interfering substances, independence from specific reagents, and direct measurement of targeted peptides, making it a valuable method for quantifying antibodies immobilized to magnetic particles and assessing the quality of the enrichment process. The efficiency of the enrichment is undoubtedly influenced by a number of factors, including the physiochemical characteristics of the antibody/ magnetic particles, the method used to immobilize the antibody, and the surface properties of the magnetic particle. Together, these factors contribute to the overall performance and effectiveness of the enrichment process. An essential factor for successful protein capture is the presence of an adequate amount of antibody bound to the surface of the magnetic particles. In this study, magnetic nanoparticles were chosen for protein enrichment because of their superior ability to bind antibodies compared to microparticles. In a previous study, four types of magnetic particles were used to conjugate anti-cTnI antibody. These particles included both microparticles and nanoparticles, which were coated with epoxy and amine groups on their surfaces (Schneck et al., 2016). In this study, to enrich cTnI from serum, Dynabeads® MyOne™ with a size 1 µm and Nanomag®-D with a size of 130 nm, were compared which both of are commercially available. For both cTnI proteotypic peptides, Nanomag®-D,130 nm, COOH as found to be more effective in enriching cTnI (Figure 3.1). The observed results align with theoretical expectations and are consistent with a previous study (Schneck et al., 2016). This outcome is attributed to the higher surface area-to-volume ratio associated with Nanomag®-D, suggesting a more efficient enrichment process for the target proteins.

In order to achieve complete cleavage and enhance proteolysis yield of the subsequent tryptic peptides of interest, method optimization such as digestion methods, enzyme-to-protein ratio, and digestion kinetics was performed. The best proteotypic yield was obtained using the FASP method, with a 1:10 enzyme-to-protein ratio and overnight digestion (Figure 3.2).

Schneck et al (2016), observed that the nanoparticles coated with glutaraldehyde groups yielded the highest concentration of immobilized antibody. Specifically, by adding approximately 300 µg of antibody, they were able to achieve an antibody concentration of 44 µg/mg of magnetic particles. This finding suggests that the nanoparticles with glutaraldehyde groups were the most effective in facilitating the conjugation and immobilization of the antibody onto the magnetic particles (Schneck et al., 2016). In this study, experiments were conducted using commercially available magnetic nanoparticles, coated with COOH groups and capable of covalently binding to antibodies, to further investigate the impact of various characteristics of magnetic particles on their enrichment performance. The results showed a significant improvement in the immobilization of anti-cTnI, with more than two and a half times better immobilization at a rate of 59.2 ± 5.7 µg/mg (Figure 3.2C). Interestingly, this improved immobilization was achieved by using a lower amount of antibody, specifically 100 µg. Consequently, A higher amount of antibodies was immobilized onto the magnetic particles, while the amount of antibody required was minimized. These observations highlighted the importance of optimizing the antibody-to-nanoparticle ratio to achieve the desired immobilization efficiency. By determining the optimal ratio, researchers can ensure the maximum utilization of both the nanoparticles and the antibodies, leading to improved performance in various applications that rely on antibody immobilization, such as biosensors, immunoassays, or magnetic separation techniques.

As noted in the previous section, Schneck et al. have previously developed an ID-LC-MS/MS method for the quantification of cTnI in plasma. The reported cTnI values ranged from 4.9 to 11.3 µg/L (Schneck et al., 2018). Although the study provides validation parameters, it lacks information on the LOD, LOQ, and uncertainty budget specifically for the plasma or serum samples. The LOD and LOQ values reported in the study, 163 pg and 325 pg respectively, are given for measurements in buffer rather than plasma or serum. The LOD and LOQ values in buffer may not accurately reflect the limit of detection and quantification achievable in plasma or serum due to potential

matrix effects and interferences. Furthermore, uncertainty estimation is crucial for assessing the reliability and accuracy of measurement results, taking into account various sources of uncertainty in the analytical process.

The method developed and validated in our study for the quantification of cTnI in human serum has LOQ of 0.6 µg/L and LOD of 1.8 µg/L. These values indicate the lowest concentration of cTnI that can be reliably quantified in human serum samples using an ID-LC-MS/MS method. Furthermore, in this study, a recovery rate of approximately 95% was achieved using only 10 µl of antibody-nanoparticle conjugates for the enrichment of cTnI from human serum. This indicates that the method efficiently captures and recovers the target analyte from the serum samples, demonstrating its effectiveness in sample preparation and enrichment for cTnI analysis.

Furthermore, in this study, a recovery rate of approximately 95% was achieved using only 10 µl of antibody-nanoparticle conjugates for the enrichment of cTnI from human serum. This indicates that the method effectively captures and recovers the target analyte from the serum samples, demonstrating its efficiency in sample preparation and enrichment for cTnI analysis.

Previous studies have reported that approximately 30% of cTnI was recovered using small amounts of SRM 2921 were used from human plasma (Schneck et al., 2018; Schneck & Lee, 2016). Furthermore, Schneck et al. reported a recovery of cTnI with 93% efficiency using 30 µl of antibody-nanoparticle conjugates, but it is important to note that this recovery was observed in a buffer solution (specifically, 0.2% BSA in PBS, 1.8 (Schneck & Lee, 2016) human serum (Schneck & Lee, 2016). The higher recovery rate observed in this study with human serum samples using a smaller volume of antibody-nanoparticle conjugates suggests the effectiveness of the developed method for cTnI enrichment in serum samples.

In contrast to the commonly used quantitative MS-based assays, the method developed in this study was integrated into a Nano LC system. The aim was to establish a reliable and reproducible method for measurement of cTnI in human serum. After processing, the protein was recovered within a concentration range of 0.6-24.0 µg/L. It is noteworthy that the LOQ and LOD values obtained in this study were lower compared to previous studies that measured cTnI using MS-based methods. This suggests that the method developed in this study has improved sensitivity in detecting lower concentrations of cTnI in human serum samples (Huillet et al., 2012; Keshishian et al.,

2009; Kuhn et al., 2009; Schneck et al., 2018). Furthermore, the correlation coefficient of the calibration curve was found to be greater than 0.996. This high correlation coefficient indicates a strong and reliable relationship between the concentration of cTnI in the serum samples and the corresponding signal response obtained from the peptides used for detection. The high correlation coefficient of the calibration curve confirms the accuracy and precision of the developed method for the quantification of cTnI in serum.

In the second part of the thesis, a method based on peptide calibration approach using immunoaffinity enrichment was proposed to quantify cTnI at microgram per liter level in human serum by nanoLC-MS. In the first part of the thesis, an ID-LC-MS/MS method for cTnI based on immunoaffinity enrichment of intact cTnI by a monoclonal cTnI antibody immobilized on magnetic nanoparticle was developed. In our previous method, SIL recombinant cTnI is added as an internal standard prior to the immunoaffinity enrichment step, and certified reference material NIST SRM 2921 is used for calibration. After several washing steps of the magnetic nanoparticle and trypsin digestion of the magnetic nanoparticle-bound antibody-cTn complex, cTnI peptides were detected (Asicioglu et al., 2023). Using an internal cTnI protein standard helps compensate any potential losses in the enrichment of endogenous cTnI, washing and handling procedures. In the previous ID-LC-MS/MS method, the achieved limits of quantification for cTnI were 1.8 µg/L from peptide TLLLQIAK, 4.8 µg/L from peptide NITEIADLTQK. In this study, it was achieved approximately same level at 2.5 µg/L from peptide TLLLQIAK, 4.3 µg/L from peptide NITEIADLTQK (Asicioglu et al., 2023). Table 4.1 displays the achieved method parameters for both protein-based and peptide-based calibration strategies for cTnI quantification. When compared to current routine assays, unfortunately, neither of our ID-LC-MS/MS methods meets the necessary sensitivity requirements to cover the full range of diagnostically significant cTnI concentrations which begin at 47 ng/L. Nevertheless, when compared in terms of quantification range, linearity, LOQ, and LOD parameters, both methods have yielded quite similar results. While the peptide-based calibration strategy appears to be more advantageous in terms of cost and time, the presented method encounters several drawbacks and constraints: (i) One of the major drawbacks of peptide-based calibration strategy is the challenge in constituting QC samples. The proposed method involves quantifying cTnI in human serum using a peptide-based calibration. However, the need for a correction factor to accurately quantify cTnI in serum samples

is evidenced by the quantification of QC materials (cTnI protein spiked in serum). Despite the reproducibility of protein enrichment recovery and tryptic digestion efficiency, variations in the correction factor could occur, particularly in cases where digestion was not performed as expected in crucial steps of sample preparation. Therefore, it is crucial to monitor the validity of the correction factor over time and across different laboratories to ensure its reliability. Considering the perspective of establishing a reference method for cTnI, the use of such a correction factor raises significant concerns, and a protein-based calibration should be favored over a peptide-based calibration. (ii) Using isotope-labeled peptides for standardization can introduce a systematic error if there is incomplete cTnI digestion. Additionally, the immunoaffinity enrichment step's capture and enrichment of intact cTnI cannot be compensated by adding a peptide internal standard. Addressing these limitations is crucial to develop a reliable measurement method. However, given that calibrants and internal standards are inexpensive and accessible, developing a peptide-based measurement method logical and appropriate to use. On the other hand, the protein-based calibration strategy, despite being potentially disadvantaged by the expensive isotopically labeled protein production, can be considered a relatively better approach as it does not require a correction factor and eliminates variations arising from sample processing. Although very successful ID-LC-MS/MS methods based on a peptide-based calibration strategy were developed, we believe that peptide-based strategies involving preliminary sample preparation steps like immunoaffinity enrichment are not suitable for low-abundant proteins such as cTnI. For low-abundant proteins, a protein-based calibration strategy is much more effective because the sample preparation steps are consistent for both calibrants and QCs, which helps compensate for potential losses.

Moreover, the proposed method also evaluated two calibration curve models with two surrogate matrices (i) quantification in a serum matrix calibration solutions, and (ii) quantification in a digest matrix calibration solutions. In terms of method performance criteria, there is no significant difference between two approaches. Our results show that synthetic peptide within a solution containing 10% ACN, 0.1% FA, along with a human serum digest peptide pool, can be used as surrogate matrix to generate calibration curve for quantification of cTnI using peptide-based calibration approach. For the two peptide and their SIL version tested in this evaluation, serum matrix and digest matrix calibration solutions showed similar performance in terms of parallelism

of curves, performance of quality control samples, based on the linearity, repeatability, intermediate precision and trueness in both matrices. Additionally, they showed very similar results on patient outcome measures. When evaluated in terms of comparison of two different matrices, using digest matrix as a calibrant solutions would make sense both in terms of cost reduction and easier applicability.

From a metrological perspective, ensuring accurate and comparable measurement results for cTnI is essential for this clinically significant protein biomarker. Although the suggested method enables the establishment of metrological traceability of results to SI units, it has significant limitations that hinder its utility as a reference method. In the future, further developments, such as improvements in the detection sensitivities of mass spectrometers, will allow lower LOQ values to be measured and perhaps to better cover clinical decision ranges (Cobbaert et al., 2021; Smit et al., 2021). The development of metrologically traceable LC-MS methods for biomarkers such cTnI is crucial step in improving measurement standardization and laboratory-to-laboratory harmonization. This study may serve as traceable analytical method for detecting cTnI in serum samples.

Table 4.1 : Comparison of ID-LC-MS/MS method parameters in both protein-based and peptide-based calibration strategies.

	Protein-based calibration		Peptide-based calibration			
	TLLLQIAK	NITEIADLTQK	TLLLQIAK		NITEIADLTQK	
Surrogate matrix	Serum-matrix matched		Serum-matrix	Digest matrix	Serum-matrix	Digest matrix
Linear range for cTnI (µg/L)	0.7-23.3	0.7-23.3	0.9-22.0	0.6-21.6	1.5-28.3	0.6-22.1
Correlation coefficient	0.996	0.992	0.995	0.998	0.998	0.992
LOQ of cTnI (µg/L)	1.8	4.8	2.8	2.5	2.7	4.3
LOD of cTnI (µg/L)	0.6	1.6	0.9	0.8	0.9	1.5
Correction factor	N/A		18.3	20.5	18.3	20.5

N/A= Not available.

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