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Evaluation of Interspecimen Trypsin Digestion Efficiency Prior to Multiple Reaction Monitoring-Based Absolute Protein Quantification with Native Protein Calibrators

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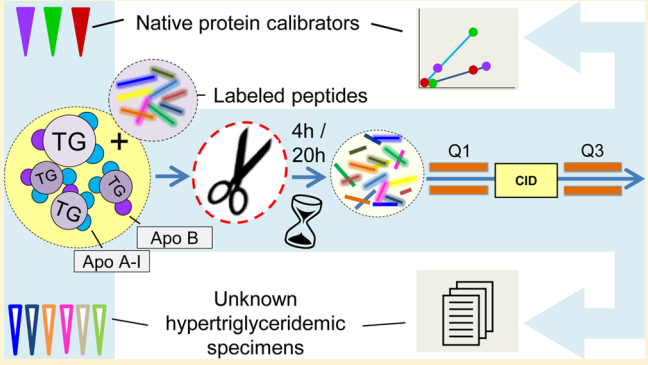
Supporting Information

ABSTRACT: Implementation of quantitative clinical chemistry proteomics (qCCP) requires targeted proteomics approaches, usually involving bottom-up multiple reaction monitoring-mass spectrometry (MRM-MS) with stable-isotope labeled standard (SIS) peptides, to move toward more accurate measurements. Two aspects of qCCP that deserve special attention are (1) proper calibration and (2) the assurance of consistent digestion. Here, we describe the evaluation of tryptic digestion efficiency by monitoring various signature peptides, missed cleavages, and modifications during proteolysis of apolipoprotein A-I and B in normo- and hypertriglyceridemic specimens. Absolute quantification of apolipoprotein A-I and B was performed by LC−MRM-MS with SIS peptide internal standards at two time points (4 and 20 h), using three native protein calibrators. Comparison with an immunoturbidimetric assay revealed recoveries of 99.4 ± 6.5% for apolipoprotein A-I and 102.6 ± 7.2% for apolipoprotein B after 4 h of trypsin digestion. Protein recoveries after 20 h trypsin incubation equaled 95.9 ± 6.9% and 106.0 ± 10.0% for apolipoproteins A-I and B, respectively. In conclusion, the use of metrologically traceable, native protein calibrators looks promising for accurate quantification of apolipoprotein A-I and B. Selection of rapidly formed peptides, that is, with no or minor missed cleavages, and the use of short trypsin incubation times for these efficiently cleaved peptides are likely to further reduce the variability introduced by trypsin digestion and to improve the traceability of test results to reach the desirable analytical performance for clinical chemistry application.



KEYWORDS: *clinical chemistry, quantitative proteomics, MRM, metrological traceability, apolipoprotein, trypsin digestion, LC−MS/MS*

1. INTRODUCTION

The application of mass spectrometry (MS) for quantification of proteins in human body fluids is rapidly expanding in different fields. Following the growing role in biomedicine[1] and biopharmaceutical industries,[2] targeted proteomics strategies have matured to such extent that implementation in clinical chemistry laboratories becomes feasible.[3] MS for absolute quantification of protein biomarkers can be a valuable alternative for clinical immunoassays that suffer from major limitations such as interfering antibodies, lack of concordance among platforms, cross-reactivity and high-dose hook effects.[4,5] Recent examples are the (candidate) LC−MS/MS reference measurement procedures for hemoglobin A1c,[6] C-peptide,[7] and urinary albumin.[8] In addition, promising MS-based methods have been reported for clinically relevant protein biomarkers, such as thyroglobulin and apolipoproteins.[9,10]

The combination of a bottom-up proteomics approach with stable-isotope labeled standard (SIS) peptides, also known as protein cleavage coupled to isotope dilution mass spectrometry (PC-IDMS), relies on the quantification of the protein content by representative signature peptides relative to the SIS peptides.[11] Hence, absolute quantification with this calibration strategy requires complete proteolytic digestion, adequate reference SIS peptides, a linear response of the endogenous peptide relative to the SIS peptides across the measurement range, and absence of any matrix effects.[9] When these requirements are not met, the trueness of the test results remains disputable. For example, a recent multiple reaction monitoring (MRM) MS method to quantify as much as 67

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putative cardiovascular disease markers demonstrated highly reproducible peptide quantification but poor concordance of absolute values with reference ranges.[12] Similarly, comparisons of targeted proteomics methods with clinical immunoassays generally show poor agreement in absolute results despite acceptable correlations.[13−15]

To enter the clinical chemistry domain, MS-based protein assays should be robust, accurate, and, preferably, automated. Moreover, routine application requires the implementation of well-known clinical chemistry concepts, such as metrological traceability, and the use of commutable reference materials.[16] Metrological traceability of test results to standards of higher order is a prerequisite for global utilization of test results, reference values, and decision limits. These high-standards for analytical performance introduce the new field of quantitative clinical chemistry proteomics (qCCP), which imposes complex proteomics workflows to meet the specific preanalytical, analytical, and postanalytical clinical chemistry quality requirements.[17,18] A major source for uncertainty in the preanalytical phase of MS-based qCCP strategies is the proteolytic digestion of the protein *measurand* into peptide *analytes* prior to MS measurement. An interlaboratory evaluation of peptide quantification by liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS), for example, showed a tremendous increase in measurement imprecision (ranging between 22 to 60%) when trypsin digestion was performed at the participating sites, which is obviously the case in clinical practice.[19,20]

Various studies have described the effects of different digestion conditions on the completeness and reproducibility of proteolytic cleavage.[21−24] In this context, it should be noted that the definition of "digestion efficiency" is used differently for identification or quantification purposes. In the case of comprehensive protein identification via a bottom-up procedure, a high *total number* of proteolytic peptides is required, preferably with suitable MS detectability. For a perfect correlation between measured peptide quantity and original protein amount, on the other hand, digestion requires completeness or, at least, precise knowledge of its extent and consistency. The reduction of uncertainty introduced by the enzymatic digestion step, therefore, requires extensive evaluation of the entire proteolytic process prior to formal validation of the complete analytical workflow.

To further compensate for digestion variability and to enhance absolute protein quantification, alternative internal standardization strategies, such as full-length labeled protein standards,[20,25] or winged SIS peptides (i.e., peptides flanked by several amino acid residues from the original protein sequence) have been reported to improve, or control, digestion efficiency.[10,26,27] In addition, calibration of MS-based proteins assays can be performed with full-length protein calibrators that follow an identical workup as the unknown specimens. Because recombinant or purified proteins do not necessarily exhibit similar digestion patterns as native human proteins,[28] native human serum standards might be required to ensure reliable calibration,[4,9] and, hence, metrological traceability of the test results. However, the use of native human serum standards requires thorough understanding of matrix effects on proteolytic digestion efficiency.

Although several studies have demonstrated the effect of protein-to-protein, peptide-to-peptide, and laboratory-to-laboratory variations on digestion efficiency, patient-to-patient variations have, so far, been poorly addressed.[9,19,23,29] This is, nonetheless, an important consideration because disease states might affect not only the protein content of the target biomarker but also processes related to the proteolysis of proteins or peptides.[30]

This work, therefore, describes the systematic evaluation of trypsin digestion efficiency for the absolute quantification of the clinically relevant biomarkers apolipoprotein A-I (Apo A-I) and B (Apo B) in various specimens. Because the ratio of Apo B/A-I is a promising indicator of cardiovascular risk,[31,32] MS-based absolute quantification of Apo A-I and Apo B is a potential alternative for direct high- and low-density lipoprotein cholesterol (HDLc and LDLc) measurements, which suffer from matrix effects in the case of dyslipidemia.[33,34] In the present study, absolute quantification of Apo A-I and Apo B in hypertriglyceridemic serum was based on calibration by three native human serum samples, which were value-assigned for the intact Apo A-I and Apo B concentration. All measurements were performed on an LC−MRM-MS platform in combination with SIS peptides for internal standardization, an approach that is ideally suited for quantitative proteomics.[35]

2. MATERIALS AND METHODS

Human Serum Collection

Three matrix-based lipid trueness verificators with different HDLc content from single donors were purchased from the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML, Nijmegen, the Netherlands) and used as native protein calibrators (NTG1, NTG2, and NTG3). These normotriglyceridemic serum samples (www.skml.nl/verificatiematerialen/lipiden-trueness-verificator; LOT 2009.0361, LOT 2009.0362, and LOT 2009.0363) were prepared in a certified production laboratory (MCA labs, Winterswijk, the Netherlands) following the CLSI C37-A guideline and thereafter value-assigned for Apo A-I and Apo B to guarantee traceability of test results to WHO-IFCC standards.[36,37] The SKML lipid trueness verificators are stable until five years after preparation when stored below −70 °C. Upon arrival, the calibration standards were thawed once and aliquoted in 50 μL portions, intended for single use only, before storage at −80 °C.

Six hypertriglyceridemic specimens (serum triglyceride concentration ≥1.8 mmol/L) were used for the evaluation of the effects of serum lipids on tryptic digestion and absolute quantification. Therefore, five human left-over, and deidentified, serum samples from clinical routine (HTG1 to HTG5) were selected based on their triglyceride and/or cholesterol level. In addition, left-over, and fully anonimized, serum samples from other patients, selected for normal HDLc levels, and without visible lipemia, hemolysis, or icterus, were combined to create a "normal" HDLc pool (HTGP). All clinical serum samples were collected in Vacutainer SST tubes (Becton Dickinson Diagnostics) and centrifuged at 1600*g* for 5 min at 17 °C. Within 24 h after blood collection, samples were aliquoted in 250 μL portions and stored at −80 °C until analysis.

Immunoturbidimetric Analysis

The Apo A-I and Apo B concentrations in the hypertriglyceridemic specimens were measured by singlicate immunoturbidimetric analysis (ITA) on a Cobas Integra 800 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The assay for Apo A-I has intrarun CVs of 1.0 and 0.8%, and inter-run CVs of 2.4 and 1.7% at 0.88

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Table 1. Compositions and Nomenclature of the Used Normo- and Hypertriglyceridemic Specimens

sample		concentrations						
		Apo A-I (g/L)		Apo B (g/L)				
name	type	*a*	*b*	*a*	*b*	Apo B/Apo A-I	triglyceride (mmol/L)	cholesterol (mmol/L)
Normotriglyceridemic Specimens								
NTG1	SKML trueness verificator[c], Level 1	1.15	1.11	0.76	0.75	0.66[a] 0.68[b]	1.03	3.93
NTG2	SKML trueness verificator[c], Level 2	1.60	1.55	0.80	0.79	0.50[a] 0.51[b]	0.76	4.91
NTG3	SKML trueness verificator[c], Level 3	1.60	1.55	1.29	1.26	0.81[a] 0.81[b]	1.82	6.67
Hypertriglyceridemic Specimens								
HTGP	normal HDLc pool		1.31		1.10	0.84	2.55	5.79
HTG1	left-over serum		1.53		1.37	0.90	4.11	11.55
HTG2	left-over serum		1.19		0.85	0.71	9.06	5.39
HTG3	left-over serum		1.23		2.02	1.64	11.60	10.44
HTG4	left-over serum		1.01		1.53	1.51	17.32	9.55
HTG5	left-over serum		1.49		1.06	0.71	17.76	9.04

[a]Target values traceable to WHO-IFCC standards.[36,37] [b]ITA test result: measured in duplicate for trueness verificators and in singlicate for hypertriglyceridemic specimens. [c]Prepared according to CLSI guideline C37-A (www.skml.nl/verificatiematerialen/lipiden-trueness-verificator).

Table 2. The % Bias of Apo A-I in the Native Calibrators and the % Recovery of Apo A-I in Hypertriglyceridemic Specimens

Apo A-I		$VQPYL_{121-130}$		$AKPAL_{231-239}$		$QGLLP_{240-250}$		$VSFLS_{251-262}$	
		% bias[b]							
native calibrator	target value[a] (g/L)	t4	t20	t4	t20	t4	t20	t4	t20
NTG1	1.15	0.0	−1.3	−1.9	−2.8	−4.8	−2.2	−2.4	−2.1
		0.0	1.3	1.9	2.8	4.8	2.2	2.4	2.1
NTG2	1.60	−0.1	2.2	1.1	**7.0**	−2.1	**7.2**	2.1	**12.3**
		−2.1	**−6.9**	−2.3	**−9.9**	0.3	−3.8	1.4	**−11.2**
NTG3	1.60	0.5	**9.1**	0.1	**5.4**	−1.2	4.8	0.2	**5.1**
		1.7	−4.4	1.0	−2.5	3.1	**−8.2**	−3.7	**−6.1**
		% recovery (n = 2)[d]							
sample	adjusted ITA value[c] (g/L)	t4	t20	t4	t20	t4	t20	t4	t20
HTGP	1.35	101.7	96.4	105.7	95.8	103.3	100.5	99.9	95.2
HTG1	1.58	**88.5**	**88.0**	104.9	**113.6**	93.9	102.1	94.7	98.7
HTG2	1.23	101.7	98.4	107.5	98.7	100.5	97.9	103.0	96.8
HTG3	1.27	106.0	100.0	108.8	91.3	103.7	94.7	95.3	90.0
HTG4	1.04	99.2	96.0	99.3	**89.7**	91.4	95.5	90.0	**85.9**
HTG5	1.54	104.3	100.5	99.6	94.0	94.2	**89.5**	**88.9**	92.3

[a]Protein calibrators are value-assigned and traceable to WHO-IFCC standards.[36,37] [b]Expressed as the % deviation of the measured concentration from the target value after 4 h (t4) and 20 h (t20) trypsin digestion; deviations >5% are underlined and indicated in bold. [c]On the basis of 96.8% average trueness of the ITA procedure for Apo A-I. [d]Expressed as the average recovery from duplicate samples measured by LC−MS/MS after 4 h (t4) and 20 h (t20) trypsin digestion relative to the adjusted ITA value, deviations >10% are underlined and indicated in bold.

g/L and 1.64 g/L, respectively. For Apo B, intrarun CVs of 1.2 and 1.1%, and inter-run CVs of 2.9% and 3.2% at 0.8 and 1.5 g/L, respectively, are documented. The trueness of the ITA test results was checked with duplicate analyses of SKML lipid trueness verificators in the same run. This allowed adjustment of ITA test results for calibration bias. WHO-IFCC traceable target values for Apo A-I and Apo B in the SKML lipid trueness verificators and ITA-test results for all specimens are listed in Table 1, together with their respective Apo B/Apo A-I ratios, triglyceride, and total cholesterol concentrations.

Reagents and Chemicals

All reagents and solvents were LC−MS grade quality or the highest analytic grade available. DL-Dithiothreitol (DTT), iodoacetamide (IAA), and urea were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Ammonium bicarbonate (ABC) and formic acid were from Fluka (Buchs, Switzerland). Sequencing-grade modified porcine trypsin (LOT 37507) was purchased from Promega (Madison, WI, US). Methanol absolute was supplied by Biosolve (Valkenswaard, the Netherlands).

Peptide Selection

Selection of signature peptides from Apo A-I and B was based on their Peptide Atlas Best SRM transition (PABST) ranking in the Peptide Atlas Database[38] and on published data from earlier (quantitative) MS-studies for these two proteins.[9,20,39−43] Peptides with methionine and cysteine amino acid residues were excluded, and LC−MRM-MS measurements were performed with a preselection of 10 peptides for Apo A-I and 10 peptides for Apo B. After preliminary LC−MS/MS analysis of the candidate signature peptides in serum digests, five

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peptides from Apo A-I, and four peptides from Apo B were selected based on their LC−MS/MS performance (Table 2). Synthetic peptides were prepared in-house for all analytes to serve as reference compounds for LC−MS/MS optimization.

SIS Peptides

SIS peptides were synthesized in-house for all selected peptide analytes using either [$^{13}C_6$, $^{15}N_1$]Val or [$^{13}C_7$, $^{15}N_1$]Leu as the labeled amino acid residue (Supplemental Table 1, Supporting Information). The purity, as determined by HPLC, was >90% for all SIS peptides except VSFLSALEEYTK (81.5%). Because protein standards are used for absolute quantification, amino acid analysis was not performed. However, for comparison of *relative* recoveries among different peptides from the same protein, SIS peptide peak areas were corrected for purity as determined by HPLC.

SIS peptide stock solutions of 30 μmol/L were prepared in deionized water with 5% (v/v) methanol and 0.05% (v/v) formic acid and stored at −80 °C. Mixtures of all nine SIS peptides with final concentrations of 5.7 and 0.29 μmol/L for the Apo A-I and Apo B-derived peptides, respectively, were prepared freshly by dilution in the same solvent. These concentrations were balanced to provide peak areas of the SIS peptides within a factor four of the peak area from the endogenous tryptic peptides (reference ranges of Apo A-I and B in serum are 30−60 and 1−3 μmol/L, respectively).

Trypsin Digestion Time Course Experiment

Duplicate samples from nine serum specimens were prepared and incubated with trypsin at 37 °C for different periods of time: 1, 2, 4, 8, 12, 16, 20, 24, and 28 h (n = 162). All solutions and buffers were prepared freshly before use. In short, serum samples were thawed at room temperature, and 5 μL of each sample was diluted to 135 μL with 50 mmol/L ABC buffer (pH 8.0). To 14 μL of this dilution (equivalent to 0.5 μL serum), 37 μL of 9 mol/L urea in 50 mmol/L ABC, 5 μL of 115 mmol/L DTT in 9 mol/L urea in 50 mmol/L ABC, and 7 μL of the SIS peptide mixture were added. After reduction and denaturation for 30 min at 56 °C, 17 μL of 101 mmol/L IAA was added, and samples were incubated for 30 min in the dark at room temperature. After alkylation, samples were diluted with 390 μL of ABC buffer to reduce the total urea concentration below 1 mol/L. Hereafter, 30 μL of trypsin solution in ABC buffer (33.3 μg/mL) was added to obtain an enzyme/protein ratio of 1:35 (w/w), and samples were digested at 37 °C. The tryptic digestion was stopped at the specified incubation time by addition of 15 μL of 10% (v/v) formic acid. After acidification, 200 μL was transferred to LC−MS vials with glass micro inserts and stored at 4 °C until LC−MS/MS analysis (≤three days). The remaining volume (∼300 μL) was stored at −80 °C in polypropylene vials.

All samples from the digestion time course experiment (n = 162) were analyzed in one batch. Samples from identical time points were analyzed subsequently with two blank samples and four standard peptide samples in-between two consecutive time series to monitor carry-over and LC−MS/MS performance.

Liquid Chromatography

An Agilent 1290 UHPLC chromatographic system was used for online SPE and subsequent peptide separation. Column temperature was maintained at 70 °C. A 20 μL volume was injected onto a Zorbax SB-C_{18} 2.1 × 15 mm column with 3.5 μm particle size (Agilent technologies, Santa Clara, CA, US)) and washed with 95% mobile phase A (5% (v/v) methanol and 0.05% (v/v) formic acid in MQ-water) at 0.5 mL/min for 2 min. Peptides were separated by gradient elution at a flow rate of 0.2 mL/min on a Zorbax SB C_{18} 2.1 × 50 mm analytical column with 1.8 μm particles (Agilent). Following valve switching, the percentage mobile phase B (95% (v/v) methanol and 0.05% formic acid (v/v) in MQ-water) increased immediately from 5 to 20% in 0.1 min, starting gradient elution from 20 to 41% mobile phase B in 7 min, then to 76% in 3.5 min and finally to 100% in 0.4 min. The column was washed with 100% mobile phase B for 2 min, before a 3 min re-equilibration with 95% mobile phase A, making a total chromatographic run time of 18 min.

MRM-MS

The UHPLC system was coupled to an Agilent 6490 triple quadrupole mass spectrometer equipped with iFunnel technology. The electrospray ionization source operated in the positive ionization mode with capillary voltage and temperature set at 3500 V and 200 °C, respectively. Peptides were measured in dynamic multiple reaction monitoring (MRM) mode that involved the scheduling of MRM transitions around the retention time of the peptide with a 0.7 min window. Doubly charged precursor ions were selected for all peptides, and one quantifier and one qualifier ion transition were measured using unit resolution for Q1 and Q3 (Supplemental Table 1, Supporting Information).

The selection of product ions was based on reference spectra obtained by direct infusion of synthetic reference peptides. Product ions with m/z values higher than the m/z value of the precursor ion were particularly selected as quantifiers to reduce potential interferences in the MRM transitions. Collision energies were optimized individually for the selected transitions.

Cycle time was set at 500 ms, providing dwell times between 9 and 48 ms, depending on the number of measured transitions.

Data Analysis

Data processing was performed with Mass Hunter Workstation software, version B.05.00 (Agilent). The integrated MS peak areas of the quantifier ions from the unlabeled peptides were divided by the MS peak areas of the equivalent MRM transitions for the SIS peptides to obtain the MS response ratio. In addition, ion transition ratios to identify potential interfering signals in the MRM measurement were obtained for each labeled and unlabeled peptide by dividing the MS peak area of the qualifier ion by the MS peak area of the quantifier ion.

The integrated MS peak areas and MS response ratios were used for quantification and construction of digestion time profiles. The digestion time profiles were fitted using nonlinear curve fitting considering two first-order formation rates and one first-order elimination rate. To allow comprehensive comparison of the digestion time profiles in the different specimens, all responses in a duplicate specimen were normalized to the maximum average response in that specimen. In addition, to visualize the differences in absolute or relative MS response of the various peptides from one protein, an intensity factor was applied based on the average maximum response of the peptide in the specimen with the highest concentration of Apo A-I (NTG3) or Apo B (HTG3). For the determination of the elimination rate of a (SIS) peptide during trypsin digestion, the natural logarithm of the (SIS) peptide MS response area was plotted against the digestion time; the slope of the linear

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regression line between t_{max} and t_{28} represents the elimination rate constant.

Calibration curves were constructed by linear regression analysis of the response ratios from duplicate measurements of three protein calibrators (n = 6), after 4 and 20 h trypsin digestion. Because the Apo A-I and Apo B target values for two out of three protein calibrators were (nearly) identical, calibration actually implied a two-point calibration curve. The relative bias for Apo A-I and Apo B in the calibration samples was expressed as the % deviation of the measured concentration from the target value. The recoveries of Apo A-I and Apo B in the hypertriglyceridemic specimens were expressed as the concentration measured by LC−MS/MS relative to the expected concentration based on the adjusted ITA values.

3. RESULTS AND DISCUSSION

Peptide Formation

The progress of tryptic digestion of Apo A-I and Apo B in pooled human serum samples was followed for the selected signature peptides (Figure 1). Most peptides had reached a maximum already at, or even well-before, 4 h. The peptides

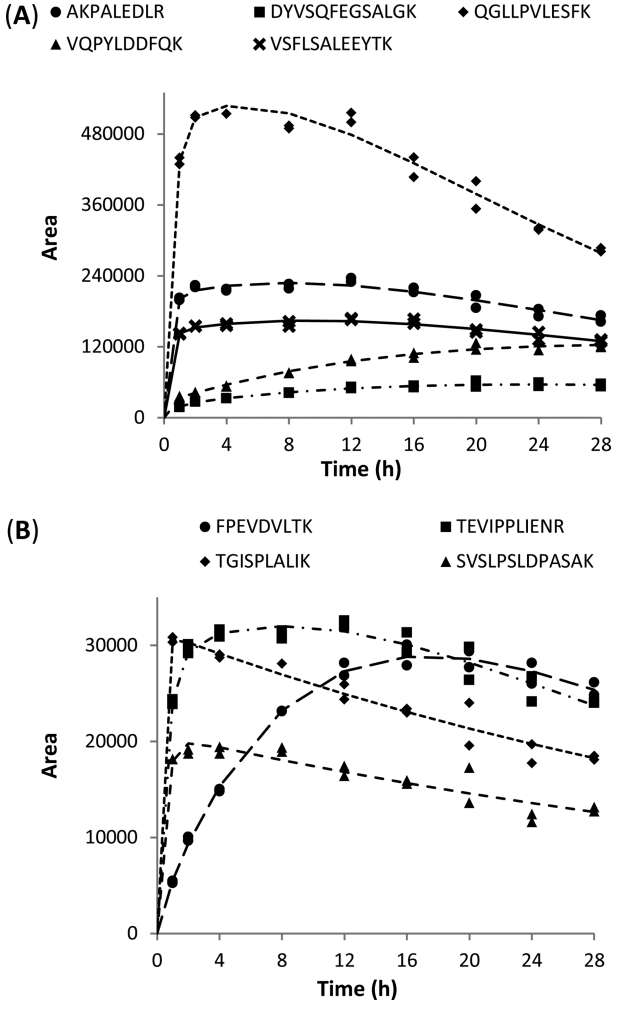

Figure 1. The absolute MS responses of of five signature peptides from Apo A-I (A) and four signature peptides of Apo B (B) during trypsin incubation in pooled human serum. Data points indicate the MS response area of duplicate samples. Curves are fitted using curve-fitting, considering rate constants for formation as well as elimination.

VQPYLDDFQK and DYVSQFEGSALGK from Apo A-I as well as FPEVDVLTK from Apo B showed a much slower formation and maximum MS responses were not reached within 28 h of incubation. This slow formation of these three peptides is in agreement with another study, despite the use of a different digestion protocol.[9]

However, the applied digestion protocol can drastically affect the formation of tryptic peptides and can, therefore, result in different (quantitative) results for the same peptide in different methods. For example, the formation rate of the Apo B peptide FPEVDVLTK, which is slowly-formed in our study, improved by the use of surfactants like sodium dodecyl sulfate (SDS) or sodium deoxycholate (DOC) instead of urea.[23] In addition, it has been reported that the use of TFE resulted in a 2−6-fold increase in peak areas of peptides from Apo A-I and B compared to the use of urea.[9] Urea was, nonetheless, used in our study because of its common application in clinical chemistry apolipoprotein measurements and standard trypsin digestion protocols. To ensure solubilization of Apo A-I and Apo B from lipoprotein particles, the urea concentration during denaturation was optimized to 6 mol/L.

The digestion time profiles also illustrate that the MS peak areas of several peptides decrease progressively, especially for QGLLPVLESFK. This is in contrast to the general assumption that proteolysis reaches completeness and yields "limit peptides".[22] However, inspection of several previously published digestion profiles of various proteins reveals that a decline in peptide response is more commonly observed, although not always interpreted as such.[9,22,23] The decreasing peptide MS response peak areas can be caused by physical or chemical instability of the peptides, that is, degradation, modification, aggregation, and/or nonspecific binding to tube materials.[9] It should be noted that the signal decrease was not observed for the peptide standards that were measured in-between consecutive LC−MS experiments, and, therefore, cannot be attributed to a possible decline of the MS signal intensity in general.

Moreover, curves fit better with our data points when a first-order elimination rate constant was considered in the nonlinear curve-fitting model based on first-order peptide formation. The nonlinear curve-fitting also revealed that for some peptides a second formation rate constant was applicable, as is especially apparent for the initially relatively rapid formation of VQPYLDDFQK (Supplemental Figure 1D, Supporting Information). This multiple step formation of VQPYLDDFQK might indicate rapid release of the tryptic peptide by trypsin, followed by a slow-formation of VQPYLDDFQK from semitryptic peptides or peptides with missed cleavages. Similar profiles were observed for the nine peptides in normo- and hypertriglyceridemic serum samples (Supplemental Figures 1 and 2, Supporting Information). Curves of AKPALEDLR and VSFLSALEEYTK showed some discrepancies at the onset of the signal reduction, but these differences are difficult to rationalize as curves are fitted through a limited number of data points. Therefore, to more explicitly investigate any difference in trypsin digestion efficiency between normo- and hypertriglyceridemic serum samples, all samples were reanalyzed to characterize and monitor any missed cleavages or modifications of the target peptides.

Screening for Proteolytic Background

For the identification of potential missed cleavages or modifications, MRM lists were constructed based on the

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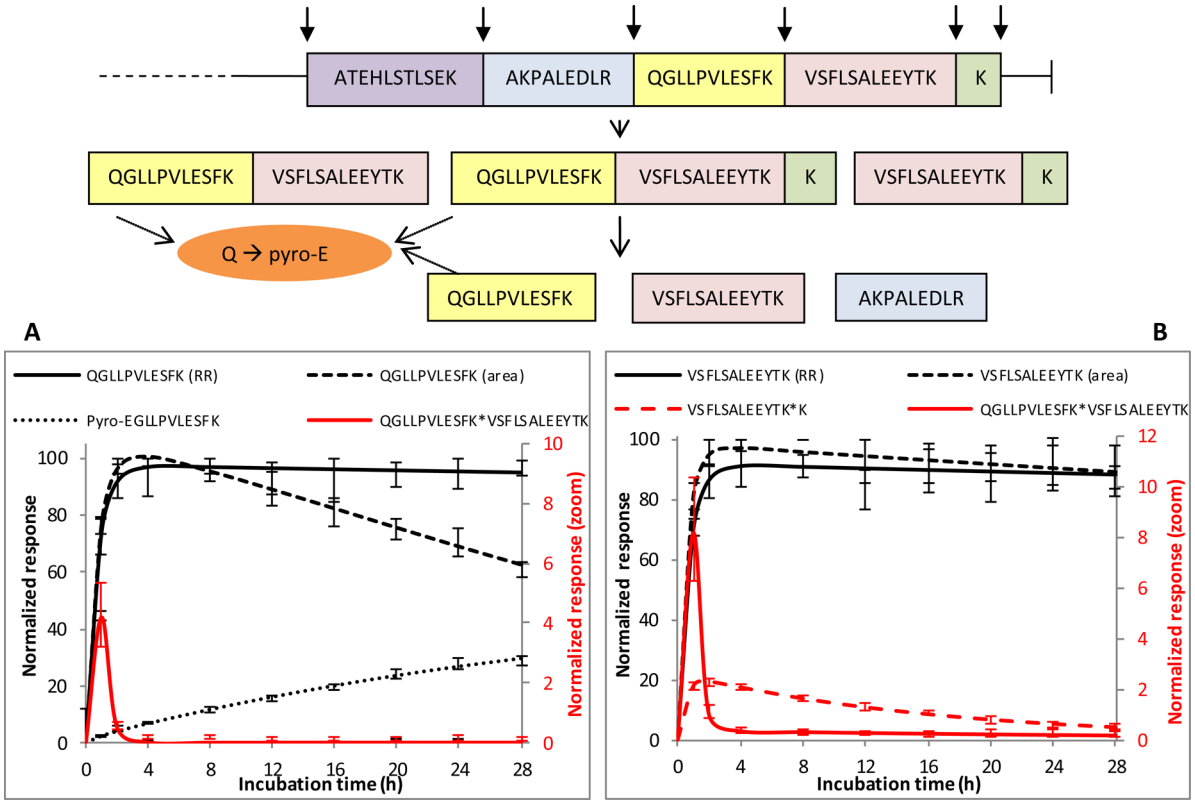

Figure 2. Formation of peptides with modifications or missed cleavages at the C-terminus of of apolipoprotein A-I during trypsin incubation, resulting in target peptides QGLLPVLESFK (A) and VSFLSALEEYTK (B). Absolute MS response areas are normalized to the maximum response of the target peptide in each specimen (n = 9). The response ratio (RR) of the target peptide relative to the SIS peptide is additionally presented (straight black line). The range between minimum and maximum normalized MS responses in the various specimens is indicated by error bars. The normalized MS response of QGLLPVLESFK*VSFLSALEEYTK and VSFLSALEEYTK*K are plotted on a rescaled secondary y-axis (in red).

selected signature peptides with inclusion of peptides that bear one and two amino acid truncations at the C- or N-terminus, and peptides with common amino acid modifications such as N-terminal pyroglutamate (pyro-E) and carbamylation of the N-termini.[44] Peptides that bear one missed cleavage at both termini were additionally selected. For all peptides, four MRM transitions were monitored using doubly charged precursor ions, whereas triply charged precursor ions were added for peptides with missed cleavages. Collision energies and selected product ion transitions were based on the optimized settings and fragmentation pattern of the synthetic reference peptides.

From a preliminary screening with serum samples after 0.5, 1, 2, 4, 20, and 44 h of trypsin digestion, six proteolytic background peptides were identified that met the following criteria: (1) a positive signal was obtained at identical retention times for at least three MRM transitions; (2) the signal intensity changed over time; and (3) modified peptides were confirmed by a positive signal for the modified SIS peptide at an identical retention time. The six identified peptides were all derived from Apo A-I and included Pyro-EGLLPVLESFK, AK*VQPYLDDFQK, VQPYLDDFQK*K, DSGR*DY-VSQFEGSALGK, and VSFLSALEEYTK*K. No modified peptides or peptides with missed cleavages were observed for the selected peptides from Apo B. This could indicate the absence of proteolytic background and, hence, the occurrence of complete proteolysis. It should, however, be noted that the molar abundance of Apo B is lower than the molar abundance Apo A-I (~20−30×) and any low abundant proteolytic background of Apo B might simply be undetectable with the applied method. Because sufficient peptides with modifications or missed cleavages were identified for Apo A-I, these peptides were selected to specifically assess the variations in peptide formation or elimination during trypsin digestion across normo- and hypertriglyceridemic specimens.

The final MRM list consisted of the two most intense MRM transitions for the proteolytic background peptides as well as one (quantifier) ion transition ratio for the labeled and unlabeled target peptides (Supplemental Table 2, Supporting Information). All samples from the digestion time course experiment were reanalyzed with this modified LC−MRM-MS method after six months storage at −80 °C.

Formation and Stability of Rapidly Formed Peptides

For AKPALEDLR, MRM signals for the potential modification PALEDLR and carbamylated N-terminal $A_{[Cm]}$KPALEDLR were observed during the preliminary screening. However, the PALEDLR signal was low for the quantifier as well as the qualifier ion transitions, and the signal did not change over time. The absence of an unambiguous signal for PALEDLR might indicate that the well-documented, known "missed cleavage" of trypsin if a lysine or arginine residue is followed by proline, occurred consistently.[45] An MRM signal for the labeled and unlabeled $A_{[Cm]}$KPALEDLR was observed in the samples digested for more than 16 h with trypsin, but only for one MRM transition ($[M + 2H]^{2+}$ = m/z 528.3 → y_7^+ = m/z 813.4). As the MRM signal remained relatively low compared to the target peptide signal and the MS response areas for AKPALEDLR and its labeled analog decreased only slightly with time, substantial carbamylation at the N-terminus of

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AKPALEDLR was not considered. In addition, no missed-cleavage peptide of AKPALEDLR could be detected in any serum sample, which is in agreement with the rapid formation of the AKPALEDLR peptide during trypsin digestion of Apo A-I.

Screening for potential peptide modifications of the other rapidly formed peptides from Apo A-I (QGLLPVLESFK and VSFLSALEEYTK), clearly revealed an increasing signal for the monitored pyro-EGLLPVLESFK and decreasing signals for the peptides VSFLSALEEYTK*K and QGLLPVLESFK*VSFLSALEEYTK during trypsin incubation in all specimens (Figure 2). The rapid formation of pyro-glutamate from the N-terminal glutamine residue in QGLLPVLESFK explains the observed rapid decline in the QGLLPVLESFK signal during trypsin digestion. This formation is, however, not trypsin-related as it was also observed during "sample-queuing" in the autosampler at room temperature, although at much lower rate. Moreover, the relative intensity of pyro-EGLLPVLESFK compared to QGLLPVLESFK showed very little variation between the various normo- and hypertriglyceridemic serum samples at all time points and indicate similar modification rates in all specimens (Supplemental Figure 3, Supporting Information). Similar modification rates were indeed observed, and estimated, for QGLLPVLESFK ($-0.024\ h^{-1} \pm 0.0016$), and its SIS peptide ($-0.024\ h^{-1} \pm 0.0008$) in all specimens, which is illustrated for normal pooled human serum in Figure 3.

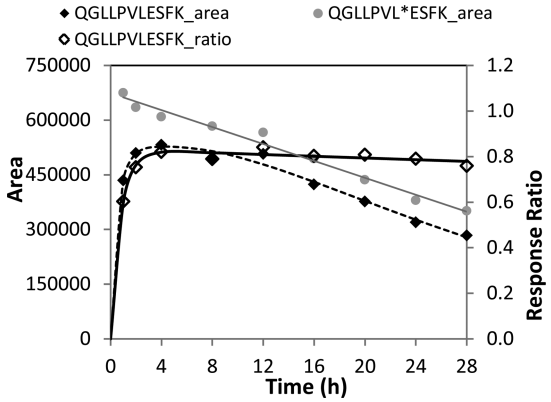

Figure 3. The average absolute (--◆--) MS peak area of QGLLPVLESFK declines during trypsin digestion in pooled human serum (n = 2). A similar decline is observed for the peak area of the SIS peptide (-●-), resulting in a stable relative response over time (-◇-).

As a consequence, the MS response ratio for QGLLPVLESFK remained stable during the digestion time course in all specimens (see section on Digestion Reproducibility). This suggests that the general recommendation to avoid selection of peptides with N-terminal glutamine residues for quantitative proteomics studies[35] is not necessarily valid when SIS peptides are added at the beginning of the workflow, prior to digestion.

For VSFLSALEEYTK, C-terminal as well as N-terminal missed cleavages were observed in all specimens (Figure 2B). Although the MS signal intensity for QGLLPVLESFK*VSFLSALEEYTK was relatively low and declined rapidly after a relatively high-abundant signal in the samples with the shortest trypsin incubation time, the peptide was observed during the entire time course. The MS signal for the C-terminal missed-cleavage peptide VSFLSALEEYTK*K, on the other hand, decreased slowly and was still present in all serum samples after 28 h trypsin digestion. It should be emphasized that the percentage normalized response does not define the percentage of the peptide with a missed cleavage or modification. In fact, as shown previously,[22] the response of peptides with missed cleavages is generally much lower than the response of the target peptides, and a low signal for a missed-cleavage peptide might actually represent a larger fraction of proteolytic background.

The presence of the neighboring basic lysine residue is known to hamper trypsin digestion[22,45] and the observed missed cleavage during the entire time course indicates that formation of VSFLSALEEYTK is not complete, despite its rapidly achieved maximum response. Nevertheless, the portion of VSFLSALEEYTK*K relative to the target peptide showed little variation between the normo- and hypertriglyceridemic serum samples at all time points and therefore suggests that the missed cleavage occurs at least consistently among various specimens. In addition, it should be stressed that the normalized MS response of VSFLSALEEYTK relative to its SIS peptide remained stable after 4 h (Figure 2B) despite the observed decrease in absolute MS response area, once more illustrating the corrective properties of the SIS peptides during trypsin digestion.

Formation of Slowly Formed Peptides

For both peptides that are formed slowly during trypsin digestion of Apo A-I, namely, VQPYLDDFQK and DYVSQFEGSALGK, peptides with missed cleavages were identified in all serum samples over the entire digestion time course (Figure 4). These missed cleavages are due to either a dibasic cleavage site at the C-terminus of VQPYLDDFQK, resulting in the peptide VQPYLDDFQK*K, or the presence of an acidic amino acid residue preceding the N-terminus of DYVSQFEGSALGK, resulting in DSGR*DYVSQFEGSALGK. The proximity of positively as well as negatively charged amino acids are known to hamper trypsin cleavage.[45−47]

The presence of VQPYLDDFQK*K in all samples explains the slow formation of the target peptide VQPYLDDFQK, possibly due to very slow "cleaning" of the missed cleavage by trypsin acting as a carboxypeptidase. Similarly, the presence of DSGR*DYVSQFEGSALGK during the complete time course explains the slow formation of DYVSQFEGSALGK. The presence of these missed cleavages indicates that the formation of VQPYLDDFQK and DYVSQFEGSALGK is not complete, and their slow elimination suggest that formation is not likely to ever reach completeness for both target peptides. Moreover, the normalized MS response areas of both peptides with missed cleavages reached above 40 or 80% of the maximum response for VQPYLDDFQK and DYVSQFEGSALGK in the same sample, respectively (Figure 4). Considering the "hidden quantification danger", mentioned by Brownidge and Beynon,[22] the fraction of the peptides with missed cleavages can actually be much higher than the fraction of the target peptides measured for Apo A-I quantification. However, DSGR*DYVSQFEGSALGK and VQPYLDDFQK*K, only consist of a few additional amino acid residues compared to the target peptide, and little reduction in MS signal is, therefore, expected.

These results are indicative for the complexity of the proteolytic process. Nonetheless, the MS response areas of identified peptides with modifications or missed cleavages relative to their target peptides showed little variation among the various specimens during the complete digestion time course (Supplemental Figure 3, Supporting Information) and suggest a similar missed-cleavage pattern across the specimens.

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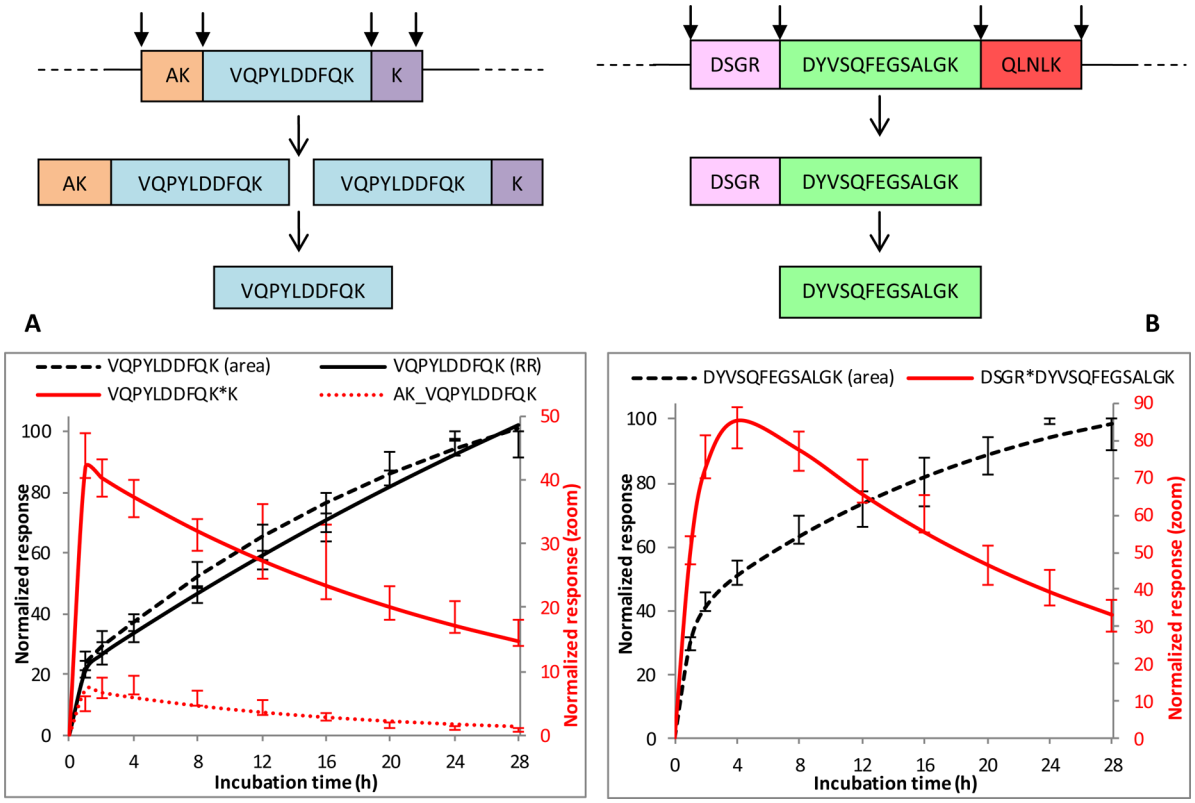

Figure 4. Formation of the slowly formed peptides VQPYLDDFQK (A) and DYVSQFEGSALGK (B) and their precursor missed cleaved peptides during trypsin incubation of Apo A-I. Absolute MS response areas are normalized to the maximum response of the target peptide in each specimen (n = 9). The response ratio (RR) of the target peptide relative to the SIS peptide is additionally presented (straight black line). The range between minimum and maximum normalized MS responses in the various specimens is indicated by error bars. Normalized MS responses of the peptides with missed cleavages are plotted on a rescaled secondary y-axis (in red).

On the other hand, the presence of peptides with missed cleavages is likely to introduce additional sources of variation contributing to the total variability of the protein digestion step, and emphasizes the importance to asses peptide cleavage during selection of the most appropriate, that is, quantotypic, signature peptides for absolute protein quantification.[29]

Digestion Reproducibility

Adequate calibration presupposes that the proteins in the calibrator samples and the unknown specimens exhibit identical, and reproducible, digestion kinetics. The MS responses of the nine peptides from Apo A-I and Apo B relative to their stable-isotope labeled counterparts during the complete digestion time course were, therefore, compared among nine normo- and hypertriglyceridemic serum specimens (Figure 5). Profiles of MS response ratios for all peptides during the complete trypsin digestion time course in all specimens are shown in Supplemental Figures 4 and 5, Supporting Information.

The results clearly illustrate that the MS response ratio remains more or less stable for the rapidly formed peptides after their maximum is reached, and demonstrate the correction of the SIS peptide for LC−MS/MS analysis and/or peptide instability. In addition, the variation in the normalized MS response ratios in all samples (n = 18) is relatively small for most peptides during the complete trypsin digestion time course (e.g., ≤ 5.0 and ≤7.6% CV at t = 4 h and t = 20 h, respectively). Only the normalized response ratio of DYVSQFEGSALGK shows wider variation between the 18 samples (e.g., 9.3 and 15.5% CV at t = 4 h and t = 20 h, respectively). If DYVSQFEGSALGK is not further considered, coefficients of variations for the normalized response ratios were between 1.9 and 6.2% and 2.1−10.6% over all time-points for the peptides from Apo A-I and Apo B, respectively, as indicated by error bars in Figure 5.

Interspecimen Peptide Correlations

Stable MS response ratios and similar profiles in serum specimens are essential to guarantee digestion reproducibility and, hence, accurate quantification. Therefore, interspecimen correlations in the normo- and hypertriglyceridemic specimens were evaluated between different peptides from the same protein. Correlations (R^2) between the MS response ratios of all peptides from Apo A-I in the nine specimens were between 0.729 and 0.939 for all peptide combinations, except DYVSQFEGSALGK, during the entire trypsin digestion time course. The interspecimen correlations between the different Apo A-I peptides are illustrated for time points 4 and 20 h in Figure 6. This figure furthermore illustrates little sample-to-sample variation between hypertriglyceridemic sera (open symbols) and normotriglyceridemic sera (dark symbols).

The poorest interspecimen correlation was observed between the peptides VQPYLDDFQK and VSFLSALEEYTK, which can be partly explained by the different digestions kinetics of the slowly formed peptide VQPYLDDFQK, compared to the, rapidly formed, peptide VSFLSALEEYTK. As a consequence, the intersample correlation between these two peptides increases with increased digestion yield of VQPYLDDFQK, as illustrated for time points 4 and 20 h in Figure 6. Moreover, comparison of the absolute MS-responses of two slowly formed

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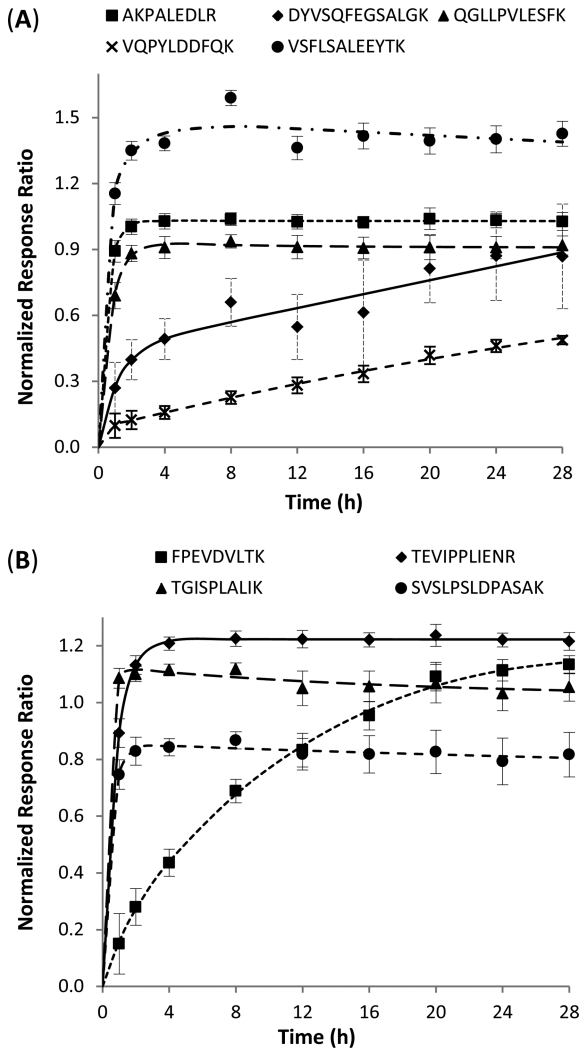

Figure 5. The normalized relative MS responses of five signature peptides from Apo A-I (A) and four signature peptides of Apo B (B) during trypsin digestion. SIS peptides were added prior to digestion. The MS response ratios in each specimen were normalized to their maximum and then corrected by the intensity factor of each peptide (defined by the maximum response ratio in the sample with the highest concentration Apo A-I or Apo B). Data points indicate the average normalized response ratio of duplicate samples in the nine specimens with standard deviations (n = 18) indicated by error bars. Curves are fitted using curve-fitting, considering rate constants for formation as well as elimination.

peptides, that is, VQPYLDDFQK and DYVSQFEGSALGK, showed excellent correlation (R^2 = 0.95). A similar correlation for these two peptides was observed in an earlier study (R^2 = 0.96).[9] This excellent correlation in absolute MS response suggests that the poor reproducibility of the response ratio for DYVSQFEGSALGK is due to variations in the LC–MS/MS response of the SIS peptide rather than inconsistencies during trypsin digestion. Possible reasons could be interference in the used MRM transition, or, unnoticed, irregularities in peak integration. Because the variations in MS response ratio of DYVSQFEGSALGK complicated further assessment of trypsin digestion efficiency in normo- and hypertriglyceridemic sera, and showed no relation with sample type or digestion time, the response ratio of DYVSQFEGSALGK was not taken into further consideration during this study.

Comparison of MS response ratios of the different Apo B-derived peptides revealed excellent correlation between the different peptides in all specimens at all time-points (R^2 > 0.95 for all peptide combinations from t = 1 to t = 28 h), as illustrated for t = 4 h and t = 20 h in Figure 7. These excellent correlations suggest that all four signature peptides from Apo B are generated (and eliminated) consistently during the incubation with trypsin in normo- as well as hypertriglyceridemic sera. Consistency in peptide formation does, nonetheless, not necessarily indicate completeness of digestion, nor does it indicate absolute recovery.

The lower correlations between the Apo A-I-derived peptides compared to the Apo B-derived peptides can be explained by a more complicated proteolysis of the Apo A-I derived peptides, as was demonstrated by monitoring the proteolytic background during trypsin digestion. However, the observed proteolytic background of Apo A-I was relatively consistent in the various serum specimens (Supplemental Figure 3, Supporting Information), and any differences in digestion time profiles between the serum specimens are, therefore, likely a summation of small variations in cleavage efficiency as well as small variations in LC–MS/MS performance.

Digestion Completeness and Peptide Recovery

The best approach to assess the digestion efficiency and elucidate potential matrix effects is to calculate the absolute peptide recovery in the various normo- and hypertriglyceridemic serum samples. Moreover, when SIS peptides are added in identical molar quantities, the obtained maximum MS response ratio for all peptides from the same protein is expected to be identical. In Figure 5, the normalized response ratios are corrected by an intensity factor to illustrate the differences in maximally achieved MS response ratios of different peptides from the same protein. Although the MS responses were corrected for purity of the SIS peptides as determined by HPLC, the exact molar peptide content is not known and differences in the maximally achieved relative MS responses can only provide an indication of peptide recovery.

For Apo A-I, it is obvious that the maximum MS response ratio of the slowly formed peptide VQPYLDDFQK is still relatively low after 28 h of trypsin digestion, indicating incomplete digestion. The relative MS response ratio of VSFLSALEEYTK, on the other hand, appears relatively high and might either reflect optimal recovery of this peptide or underestimation of the peptide content. The latter is not unthinkable as the peptide purity of labeled VSFLSALEEYTK was lower (81.5%) than the purities of the other peptides. For Apo B, the differences in maximally achieved MS response ratios of the four peptides are smaller (between 0.8 and 1.2) but remain difficult to interpret as long as the exact SIS peptide content is unknown.

Protein Recovery

The digestion time profiles of the MS peak areas of the various peptides as well as the observed presence of peptides with modifications or missed cleavages indicate the difficulty to obtain complete recovery of multiple peptides from the same protein. Especially in targeted proteomics studies where quantification is based solely on SIS peptides, generally added *after* digestion, incomplete digestion and peptide instability seriously affect the accuracy of quantification and can explain the observed discrepancies in quantitative outcomes with the use of different peptides.[14,48,49] Our results demonstrate that the addition of SIS peptides *prior* to digestion is essential to

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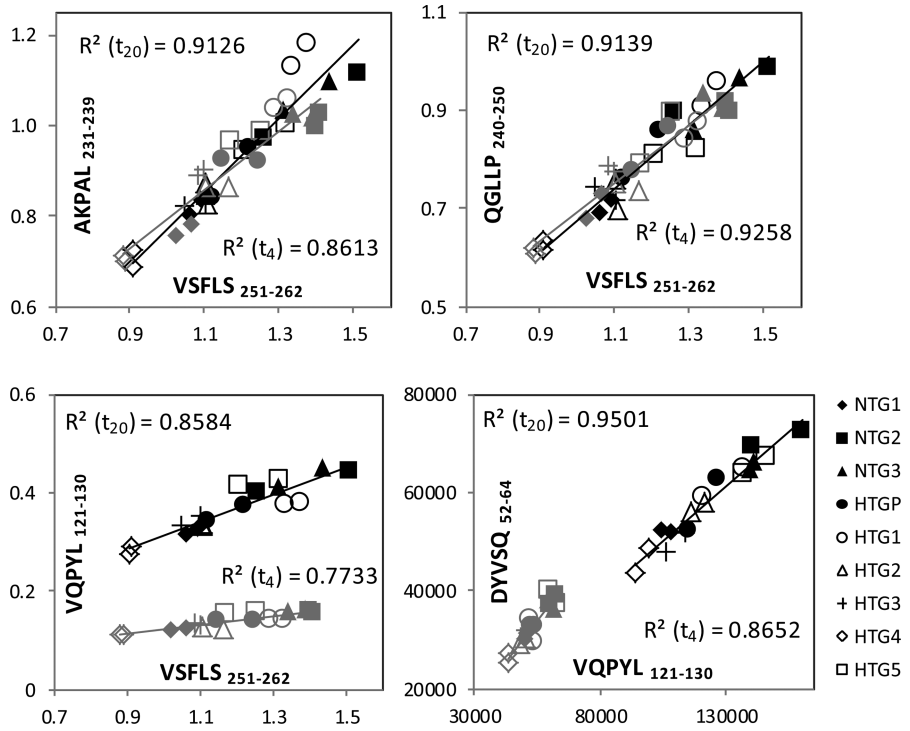

Figure 6. The correlation between interspecimen MS-response ratios of VSFLSALEEYTK (*x*-axis) and AKPALEDR, VQPYLDDFQK, and QGLLPVLESFK (*y*-axes) after 4 h (gray) and 20 h (black) trypsin digestion. For DYVSQFEGSALK, the MS response ratios could not be used due to large variations in the MS response of the SIS peptide. Alternatively, the absolute MS response area is correlated to the absolute MS response area of VQPYLDDFQK. The legend shows the different symbols for the different specimens.

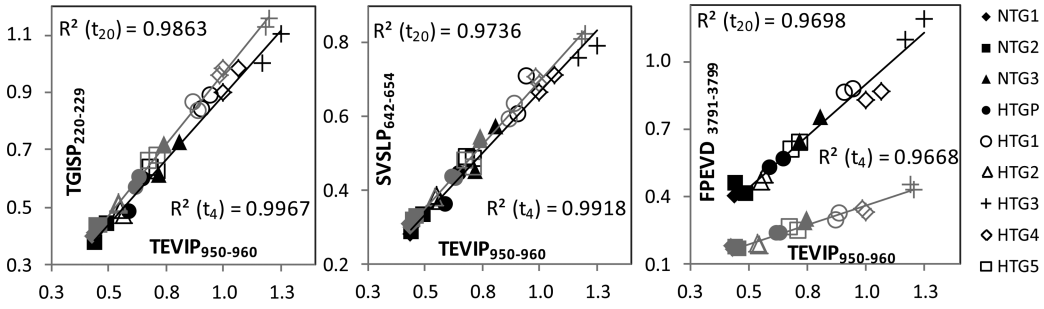

Figure 7. Comparison of MS response ratios in different specimens between the four Apo B-derived peptides at t = 4 h (gray) and t = 20 h (black). The legend shows the different symbols for the different specimens.

correct for instability during trypsin digestion, as is clearly illustrated for the rapidly degrading peptide QGLLPVLESFK in Figure 3.

In addition, the use of protein calibrators can further enhance the accuracy of the measurement and additionally eliminate the specific requirement for digestion completeness. The use of value-assigned native protein calibrators is of particular importance to anchor test results to the metrological traceability chain. The % deviation of the measured Apo A-I and Apo B concentrations in the protein calibrators from the target values after 4 and 20 h trypsin digestion are expressed as the relative bias in Tables 2 and 3. In addition, protein recoveries of Apo A-I and Apo B in the hypertriglyceridemic serum samples were calculated based on the LC–MS/MS measurement result for each peptide individually relative to the expected concentration based on the adjusted ITA value for Apo A-I (Table 2) and Apo B (Table 3). The recoveries of Apo A-I and Apo B in all specimens based on the average values of four signature peptides are shown in Supplemental Figure 6, Supporting Information and revealed recoveries of 99.4 ± 6.5% for Apo A-I and 102.6 ± 7.2% for Apo B after 4 h of trypsin digestion. Protein recoveries after 20 h trypsin incubation equaled 95.9 ± 6.9% and 106.0 ± 10.0% for Apo A-I and Apo B, respectively.

Although no correlation between measurement inaccuracy and serum triglyceride concentration was observed, the results clearly demonstrated larger deviations from the value-assigned concentrations of the three native protein calibrators at t = 20 h compared to t = 4 h for Apo A-I as well as Apo B (Tables 2 and 3). The use of relatively short or even accelerated digestions[21,24,50] has previously been reported to reduce the uncertainty introduced by the digestion step and might be beneficial to minimize the measurement bias observed in this study. The larger deviations after 20 h trypsin digestion were, however, less obvious for the unknown specimens, although the calculated recoveries for Apo B showed an increased tendency for positive bias (≥10%) after longer trypsin digestion (Table 3). The results, on the other hand, support the usefulness of the

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Table 3. The % Bias of Apo B in the Native Calibrators and the % Recovery of Apo B in Hypertriglyceridemic Specimens

Apo B		$TGISP_{220-229}$		$SVSLP_{642-654}$		$TEVIP_{950-960}$		$FPEVD_{3791-3799}$	
		% bias[b]							
native calibrator	target value[a] (g/L)	t4	t20	t4	t20	t4	t20	t4	t20
NTG1	0.76	−2.1	2.4	0.8	−2.7	−0.7	−1.2	**6.6**	−1.9
		1.4	3.1	−1.3	2.2	0.2	0.2	0.2	0.7
NTG2	0.80	0.2	5.0	1.9	**7.2**	1.9	**5.7**	**−5.1**	−4.5
		0.6	**−10.7**	−1.3	**−6.6**	−1.4	−4.7	−1.9	**5.7**
NTG3	1.39	0.5	**8.9**	0.7	**11.1**	−0.3	**5.6**	−2.7	**7.3**
		−0.5	**−8.7**	−0.7	**−11.2**	0.3	**−5.7**	3.0	**-7.4**
		% Recovery (n = 2)[d]							
sample	adjusted ITA value[c] (g/L)	t4	t20	t4	t20	t4	t20	t4	t20
HTGP	1.12	94.9	92.3	93.4	92.1	98.3	94.7	92.2	**89.6**
HTG1	1.39	108.7	**120.4**	104.0	**117.8**	109.4	**111.8**	97.3	**114.1**
HTG2	0.86	109.1	107.7	108.4	**113.2**	109.1	109.7	98.9	103.7
HTG3	2.05	98.0	99.5	92.7	93.7	100.3	98.6	92.0	101.4
HTG4	1.56	**110.7**	**117.8**	105.4	**110.4**	**110.1**	**111.4**	95.0	99.7
HTG5	1.08	**112.0**	**112.8**	105.4	**114.4**	**111.8**	109.8	106.1	106.4

[a]Protein calibrators are value-assigned and traceable to WHO-IFCC standards.[36,37] [b]Expressed as the % deviation of the measured concentration from the target value after 4 h (t4) and 20 h (t20) trypsin digestion; deviations >5% are underlined and indicated in bold. [c]On the basis of 98.4% average trueness of the ITA procedure for Apo A-I. [d]Expressed as the average recovery from duplicate samples measured by LC−MS/MS after 4 h (t4) and 20 h (t20) trypsin digestion relative to the adjusted ITA value, deviations >10% are underlined and indicated in bold.

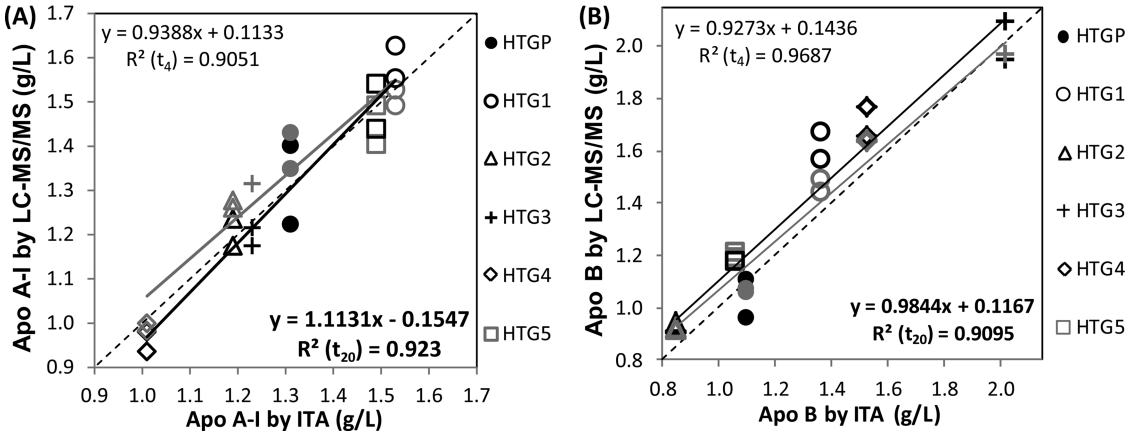

Figure 8. Comparison between Apo A-I (A) and Apo B (B) concentrations measured with the clinical immunoturbidimetric assay (ITA) and the LC−MS/MS method in six specimens with different triglyceride compositions. Calibration for the LC−MS/MS method was performed with three human serum calibrators at two concentration levels after 4 h (gray) and 20 h (black) of trypsin digestion. Concentrations are based on the average responses of four signature peptides.

native-protein calibrators, because acceptable recoveries are obtained for slowly as well as rapidly formed peptides, independent of the stage of digestion completeness. Moreover, recovery values for the slowly formed peptide FPEVDVLTK after 4 h trypsin digestion showed, despite the uncompleted digestion, less deviation from the expected values as well as less variation across the different specimens compared to 20 h trypsin digestion.

Method Comparison

The comparisons between absolute concentrations of Apo A-I and Apo B measured by LC−MS/MS and ITA in the six serum specimens are illustrated in Figure 8. Overall, absolute quantification in the different serum samples with the LC−MS/MS method after 4 h of trypsin digestion, using the average concentration of four signature peptides, correlated well with the ITA measurement for Apo B (R^2_{t4} = 0.9687) and somewhat less for Apo A-I (R^2_{t4} = 0.9051). Correlations (R^2) after 20 h of tryptic digestion were 0.9095 and 0.9230 for Apo B and Apo A-I, respectively. The best correlation for Apo A-I concentrations between LC−MS/MS and ITA was obtained when LC−MS/MS concentrations were based on measurement of the peptide AKPALEDLR after 4 h trypsin digestion (R^2_{t4} = 0.9709). The correlation of Apo B concentrations between LC−MS/MS and ITA, on the other hand, correlated very well for all four selected peptides, though better after 4 h than after 20 h trypsin digestion (R^2_{t4} = 0.96 ± 0.01 and R^2_{t20} = 0.89 ± 0.05).

Because the ratio of Apo B/Apo A-I might be used to improve cardiovascular risk prediction,[31,32] the Apo B/Apo A-I ratios obtained by LC−MS/MS and ITA were also compared and showed good correlation when based on the average concentration of four signature peptides ($([\text{Apo B}]/[\text{Apo A-I}])_{\text{LC-MS/MS}} = 0.94([\text{Apo B}]/[\text{Apo A-I}])_{\text{ITA}} + 0.07$; R^2 = 0.9327 after 4 h, and $([\text{Apo B}]/[\text{Apo A-I}])_{\text{LC-MS/MS}} = 1.10([\text{Apo B}]/[\text{Apo A-I}])_{\text{ITA}} - 0.008$; R^2 = 0.9534 after 20 h).

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Peptide Selection and Absolute Quantification

For the development of a clinically applicable method, bias and imprecision of Apo A-I and Apo B measurement by LC−MS/MS methods should meet the desirable analytical specifications. This requires extensive evaluation of the proteolytic digestion step to reduce all potential sources of variation. Suggestions for further reduction of uncertainties introduced by the enzymatic digestion step include the automation of the sample preparation procedure, the use of (internal) standards that correct for variations during digestion, or the selection of other, more robust, signature peptides and/or proteolytic enzymes.

For example, Hoofnagle et al. demonstrated a reduced digestion variability for different apolipoproteins in HDL when an intact labeled analog of Apo A-I was used instead of individual SIS peptides.[20] However, labeled analogs of intact proteins are not easily available and do not necessarily reflect an identical digestion behavior as native proteins.[28] In addition, the use of winged SIS peptides has not always shown to improve method performance compared to noncleavable SIS peptides.[51,52]

The results from this study, nonetheless, demonstrate the beneficial effects of the addition of SIS peptides prior to digestion. Because of the presence of the SIS peptide during the entire workflow, QGLLPVLESFK could be accurately quantified in all specimens despite the generally accepted rule for bottom-up quantitative proteomics studies to exclude peptides with N-terminal glutamine residues. Moreover, QGLLPVLESFK showed the highest absolute MS intensity after LC−MRM-MS analysis of all selected peptides from Apo A-I (Figure 1). Exclusion of peptides with N-terminal glutamine residues prior to method development could, therefore, be undesirable for quantification purposes, particularly if the peptide signal intensity is crucial for peptide selection (e.g., for the quantification of low-abundance proteins).

Peptide selection should furthermore preferably avoid peptides for which poor cleavage efficiency is expected.[29] In our study, peptide selection was mainly based on the PABST ranking in the Peptide Atlas Database. This ranking favors frequently observed peptides gathered from the literature or MS/MS databases, whereas prediction from empirical rules plays only a minor role.[53] Moreover, consideration of the peptide cleavage efficiency based on common rules for missed cleavages[46,47] is not incorporated in the PABST ranking. Other approaches for the selection of signature peptides during the development of bottom-up MS-based methods for absolute protein quantification should, therefore, be considered.

In addition to the use of alternative internal standards, or signature peptides, the use of other proteolytic enzymes can be considered. Krastins et al., for example, reported the use of the enzyme Glu-C for the digestion of Apo A-I to generate peptide sequences with more desirable assay performance.[54] Although the use of other enzymes could result in peptides with more favorable digestion kinetics or LC−MS/MS properties, these enzymes still require thorough evaluation of the digestion process.

The main requirement for peptide selection for absolute protein quantification based on native protein calibrators is the assurance of consistent interspecimen digestion efficiency. Consistent interspecimen digestion is most likely for rapidly formed peptides. We, therefore, consider the use of (1) serum-based protein calibrators, (2) rapidly formed signature peptides, and (3) short digestion times essential to reduce the uncertainty introduced by the digestion step and make the development of a quantitative proteomics workflow amenable to implementation in routine clinical chemistry laboratories.

4. CONCLUSION

The results presented in this study did not reveal a direct effect of serum triglyceride concentration on (1) tryptic digestion efficiency and (2) absolute quantification of Apo A-I or B. However, the results clearly point out that the enzymatic digestion step required for bottom-up protein quantification introduces a source of uncertainty that is not yet fully understood. Monitoring of the digestion kinetics for multiple peptides from one protein, as performed in this study, has proven useful for thorough understanding of peptide cleavage and stability, apparently the most important parameters for the selection of quantotypic peptides.

The differences in formation and stability of various peptides from the same protein, as shown in this study, indicate the relevance to monitor multiple signature peptides during method development and validation. A thorough evaluation of the performance of multiple peptides throughout the entire workflow will likely support the selection of the most robust and reliable peptides for final quantification. Similar to the recommendation to monitor multiple ions in MRM-based assays to detect potential interferences in the MRM measurement,[55,56] monitoring of correlations between different peptides from the same protein seems inevitable as a quality control of sample-to-sample digestion consistency and the occurrence of polymorphisms.[9] Hence, any relative deviation in response ratio between different peptides can be observed and subjected to further evaluation.

For absolute quantification of Apo B, the four initially selected signature peptides appear suitable for absolute quantification, especially if trypsin incubation time is reduced to 4 h. Prolongation of trypsin incubation does not increase the peptide recovery of the three rapidly formed signature peptides TEVIPPLIENR, TGISPLALIK, and SVSLPSLDPASAK. Moreover, for all peptides, including the slowly formed FPEVDVLTK, variations in peptide correlation and protein recoveries in the various serum samples increase after incubations longer than 4 h.

The results of the absolute quantification of Apo A-I are more difficult to interpret. From the five initially selected peptides, one peptide (DYVSQFEGSALGK) appeared unsuitable for absolute quantification, despite its use in other quantitative MS methods.[9,20,39] For the remaining four peptides, variations increased with prolonged incubation times, similar as for Apo B. However, the Apo A-I-derived peptides showed poorer correlations among the different serum samples, although average results correlated well with an immunoassay routinely used in the clinical chemistry laboratory. Except for AKPALEDLR, peptides with missed cleavages were identified for all Apo A-I-derived peptides and were still detectable after 28 h of trypsin digestion, indicating that complete proteolysis was not achieved even after prolonged digestion.

Hence, MS-based absolute quantification of Apo A-I requires further investigation to reduce sample-to-sample variation and ensure accurate quantification. Alternatives that will likely reduce the introduced variations include the automation of the sample preparation, the use of intact protein isotope-labeled internal standards and/or the selection of other signature peptides. Herein, assessment of absolute peptide recovery, necessitating SIS peptides with an exactly determined content,

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will deserve particular attention to further assess peptide cleavage and trypsin digestion efficiency in various specimens. For future studies, the consideration of peptide cleavage before (final) selection of signature peptides appears crucial to allow short trypsin digestion times and improve the robustness and accuracy of the test results.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Table 1. Sequences and MRM settings of the selected signature peptides from Apo A-I and Apo B. Supplemental Table 2. Final MRM list for screening of proteolytic background peptides of Apo A-I. Supplemental Figure 1: The absolute MS-responses of the Apo A-I derived peptides during trypsin digestion in nine different specimens. Supplemental Figure 2: The absolute MS-responses of the Apo B derived peptides during trypsin digestion in nine different specimens. Supplemental Figure 3: The % MS response peptides with modifications or missed cleavages relative to the target peptide response among the nine different specimens during trypsin digestion. Supplemental Figure 4: The MS-response ratios of the Apo A-I derived peptides during trypsin digestion in nine different specimens with SIS peptides added prior to digestion. Supplemental Figure 5: The MS-response ratios of the Apo B derived peptides during trypsin digestion in nine different specimens with SIS peptides added prior to digestion. Supplemental Figure 6: Recovery of Apo A-I (A) and Apo B (B) in six different specimens using the LC−MS/MS procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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