Unraveling trypsin digestion, a continuing story: mitigating matrix effects for accurate MS-based quantification of serum apolipoproteins

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After unraveling the impact of trypsin digestion on *imprecision* of MS-based quantification of apolipoproteins (apo) A-I and B [1], understanding the impact of trypsin digestion on *bias* has been one of our major challenges. True quantification of endogenous proteins by MS-based targeted peptide measurements that agrees with the requirements for clinical use has, nonetheless, been stated as an elusive goal [2]. When quantification is based on peptide standards, the differences in peptide formation and decay rates during protein digestion are particular sources of bias [3]. The use of native, value-assigned, sera for external calibration, on the other hand, might provide the required trueness, as supported by the excellent agreement and correlation between LC-MS/MS and immunoturbidimetric analysis of apoA-I, apoB, apoC-II, apoC-III, and apoE, based on this, protein-centric, calibration approach [4].

However, several challenges remain before the method can be applied to clinical studies. First, the total error of 16.6% for quantification of apoA-I with the best-performing peptide was still above the minimal total allowable error based on biological variation (≤13.7%), with bias *and* calibration assigned as the major contributors (35 and 28%, respectively) [5]. Second, the use of a stable isotope-labelled intact protein (SIL-apoA-I) as internal standard revealed a non-systematic (random) bias among 100 patients samples (**Figure 1**), that was, moreover, highly consistent in the same sample measured over multiple days (**Figure 2**). These results particularly suggest matrix effects on the digestion completeness of both endogenous and SIL-apoA-I, despite the reached plateau and observed inter-specimen concordance in digestion time course experiments with various normo- and hypertriglyceridemic sera [6].

Third, the 1000-fold dilution during the digestion procedure makes the method less suitable for inclusion of lower abundance apolipoproteins or signature peptides. Finally, the current *manual* sample preparation is lengthy (including 20h overnight digestion) and laborious, and has furthermore been assigned as an important source of imprecision [5]; all issues that limit the application to large sample cohorts to validate the clinical performance of the apolipoprotein panel in cardiovascular disease risk assessment.

In this study, we describe a detailed optimization of digestion conditions by design of experiments. Besides emphasis on reducing matrix effects on the digestion completeness of apoA-I, the effect of various denaturation, reduction, alkylation, and digestion conditions on the peptide yield from multiple apolipoproteins (apoB-48, apoB-100, apoC-I, apoC-II, apoC-III, and apoE) were assessed. Lastly, the optimal digestion procedure was evaluated with respect to automation potential and the effect on assay simplicity, imprecision, and throughput.

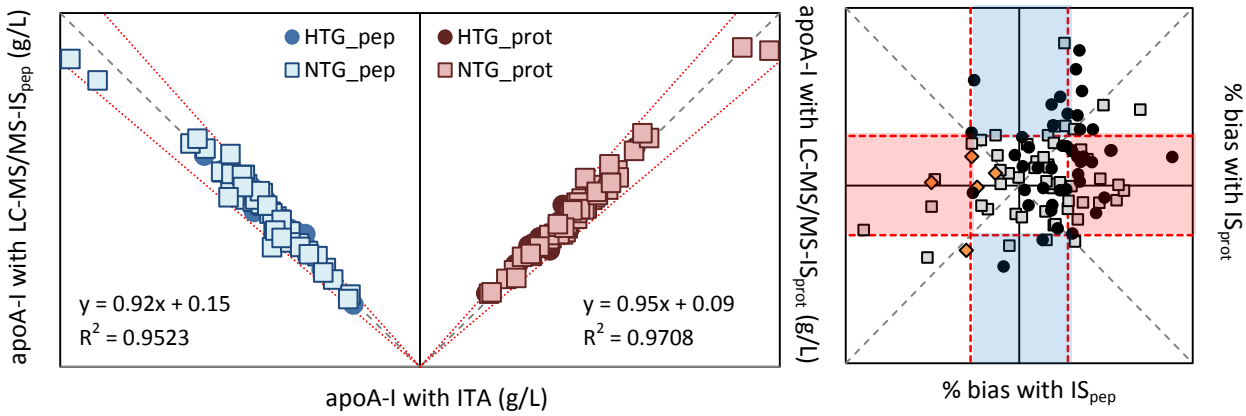

Figure 1: Comparison between quantification of apoA-I in 100 normotriglyceridemic (NTG) and hypertriglyceridemic (HTG) sera with immunoturbidimetric analysis (ITA, x-axis) and LC-MS/MS based on the best-performing peptide with either SIL-peptide internal (IS_{pep}, left y-axis, in blue) or SIL-apoA-I as internal standard (IS_{prot}, right y-axis, in red). The red lines indicate the total allowable error boundaries (13.7%).
The panel at the right compares the % bias of LC-MS/MS analysis with IS_{pep} (x-axis) and IS_{prot} (y-axis) relative to ITA for 54 NTG (□) and 46 HTG (●) sera (average of n=2) or relative to value-assigned target values (orange diamonds, average of n=30 for IS_{pep} and n=22 for IS_{prot}). The red lines indicate ±5.6% bias boundaries; data points in the blue regions have a bias outside ±5.6% with IS_{prot} and inside ±5.6% with IS_{pep}; data points in the red regions have a bias outside ±5.6% with IS_{pep}, and inside ±5.6% with IS_{prot}.

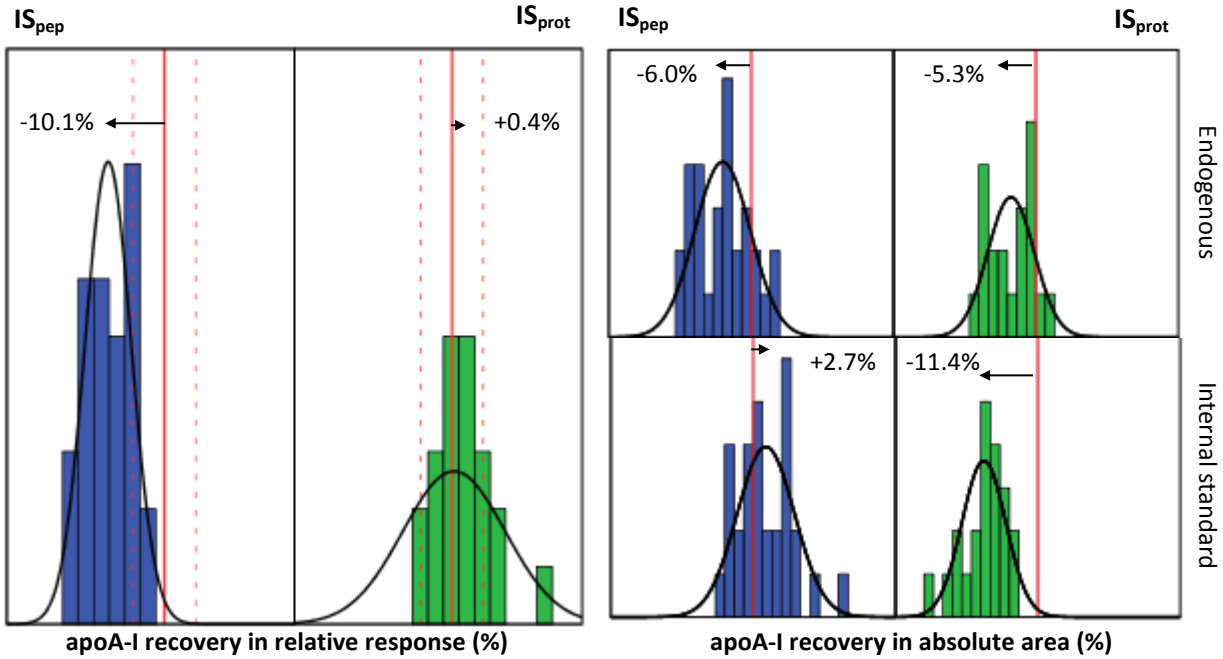

Figure 2: The effect of internal standard type on the recovery of apoA-I, based on repeated measurement of one specific sample with either IS_{pep} (n=30 over 10 days, in blue) or IS_{prot} (n=22 over 8 days, in green) using the best-performing peptide. The left panel illustrates the bias distribution of the calculated concentration compared to the target value (indicated by the red line ±5.6%). The right panel illustrates the bias distribution of the absolute MS area for the endogenous peptide (upper panel) and SIL-peptide (lower panel) compared to the expected MS area based on daily normalization with three serum-based calibrators.

References

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