Quantitative protein mass-spectrometry requires a standardized pre-analytical phase

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Abstract

Objectives: Quantitative protein mass-spectrometry (QPMS) in blood depends on tryptic digestion of proteins and subsequent measurement of representing peptides. Whether serum and plasma can be used interchangeably and whether *in-vitro* anticoagulants affect the recovery is unknown. In our laboratory serum samples are the preferred matrix for QPMS measurement of multiple apolipoproteins. In this study, we investigated the effect of different matrices on apolipoprotein quantification by mass spectrometry.

Methods: Blood samples were collected from 44 healthy donors in Beckton Dickinson blood tubes simultaneously for serum (with/without gel) and plasma (heparin, citrate or EDTA). Nine apolipoproteins were quantified according to standard operating procedure using value-assigned native serum calibrators for quantitation. Tryptic digestion kinetics were investigated in the different matrices by following formation of peptides for each apolipoprotein in time, up to 22 h.

Results: In citrate plasma recovery of apolipoproteins showed an overall reduction with a bias of −14.6%. For heparin plasma only −0.3% bias was found compared to serum, whereas for EDTA-plasma reduction was more pronounced (−5.3% bias) and variable with >14% reduction for peptides of apoA-I, A-II and C-III. Digestion kinetics revealed that especially slow forming peptides showed reduced formation in EDTA-plasma.

Conclusions: Plasma anticoagulants affect QPMS test results. Heparin plasma showed comparable results to serum. Reduced concentrations in citrate plasma can be explained by dilution, whereas reduced recovery in EDTA-plasma is dependent on altered proteolytic digestion efficiency. The results highlight the importance of a standardized pre-analytical phase for accurate QPMS applications in clinical chemistry.

Keywords: anticoagulants; matrix comparisons; pre-analysis; quantitative proteomics.

Introduction

Providing patients with accurate test results is of highest importance in clinical chemistry practice. Therefore, high-quality tests should be developed that are fit-for-clinical purpose, including a robust pre-analytical phase. This is certainly the case for the multiplex measurement of proteins as achieved with quantitative protein mass-spectrometry (QPMS) tests [1]. In QPMS absolute quantitation of proteins is dependent on the so-called bottom-up proteomics approach in which proteolytic degradation of the proteins generates protein specific peptides that can be quantified. The digestion of serum or plasma proteins by trypsin should result in the stable formation of quantifying and confirmation peptides to achieve accurate quantitative results. In serum and plasma samples the *in-vitro* coagulation process may influence protein digestion and ultimately the quantitation of proteins.

QPMS is anticipated to be increasingly used for multiplex protein quantitation and personalized medicine in clinical chemistry routine practice [1, 2]. In our laboratory we developed in-house tests for the detection of protein biomarkers in serum and plasma (apolipoproteins and antithrombin [3, 4]) and urine [5]. In medical laboratories a large variety of blood collection tubes is available for all diverging parameters used for patient diagnostics. This is also the case for biobanking and clinical research [6]. Thus far, serum has been used as the reference matrix for the quantitation of apolipoproteins or their proteoforms in

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blood in our laboratory [3, 4]. Plasma matrices are an important source of diagnostic study materials but it is currently unsure whether and to what extent results in the different (blood) matrices are interchangeable in the context of QPMS.

Digestion kinetics of blood-based biomarkers may be affected by variation in sample preparation conditions [7, 8]. Similarly, differences between the serum and plasma matrices have been reported from (discovery and qualitative) proteomics studies and initiatives for standardized blood collection procedures have already been proposed in the human proteome project (HUPO) [9]. Effects of the clotting process in serum or the complexing agents such as EDTA and citrate in the plasma matrix are known to cause changes in the blood composition [10]. These matrix differences may affect the proteolytic digestion in QPMS and cause inaccurate test results [9–12], but thus far, this has not yet been investigated.

Protein biomarkers can be measured in the different matrices from serum or plasma blood samples. In case of plasma samples, sodium citrate, lithium heparin or K_2EDTA are used as anticoagulants. Differences in the serum and plasma matrices exist, because of the clotting process taking place in the serum samples (due to clot activation by silica particles on the serum tube walls) whereas the coagulation is prevented in plasma. In the case of lithium heparin plasma, activation of antithrombin through the interaction with heparin, leads to inhibition of the coagulation cascade and prevention of fibrin formation. In K_2EDTA plasma coagulation is prevented differently through chelation of metal ions such as calcium, required for coagulation. Sodium citrate also forms complexes with metal ions and calcium, but in contrast to K_2EDTA its anticoagulation is reversible. Due to their specific properties the three plasma types are used for different purposes (clinical chemistry, hematology and coagulation tests in heparin-, EDTA- and citrated plasma, respectively).

Here, we evaluate the effects of sample matrix and pre-analysis on the quantitation of nine apolipoproteins in a structured manner [4]. Blood samples from 44 healthy donors were collected and the proteins were quantified in serum (with and without gel) and four different plasma matrices (lithium heparin, K_2EDTA without and with protease inhibitors (PIC) and sodium citrate). Our standard procedure for calibration using selected native serum calibrators [4] is applied for all matrices, and differences in results between pre-analytical conditions are assessed. Furthermore, effects of the plasma anticoagulants on apolipoprotein digestion were investigated as a potential cause for the observed matrix variations.

Materials and methods

Materials

Serum and plasma samples of 44 pseudo anonymized healthy donors were obtained through the Leiden University Voluntary Donor Service (LuVDS) which is coordinated by the biobank facility of the Leiden University Medical Center. Volunteers (14 male, 30 female, ages varying from 25 to 68 years) were phlebotomized at the clinical chemistry blood collection facilities. Blood was drawn in 6 different Beckton Dickinson (BD) blood tubes (Figure 1 and Supplementary Table ST1).

All tubes were treated according to validated processing of the Clinical Chemistry Department. Serum tubes were kept at room

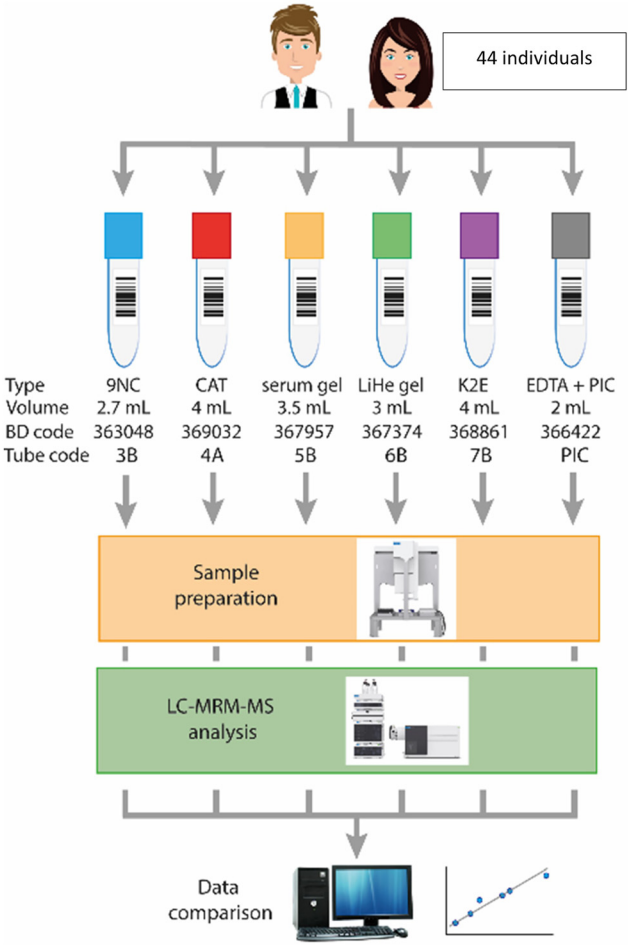

Figure 1: Total workflow for matrix comparison of serum and plasma samples from 44 healthy donors collected in Beckton Dickinson blood tubes for serum (4A and 5B, gel tube) and plasma (3B, sodium citrate; 6B, lithium heparin gel tube; 7B and PIC; K_2EDTA tubes without and with protease inhibitor cocktail). Samples were prepared by tryptic digestion of proteins on a Bravo automated liquid handling platform and proteotypic peptides were quantified by LC-MRM-MS/MS. Data of the different matrices were compared to the reference serum gel tubes 5B [4].

temperature for 60 min in order to allow clotting. Before centrifugation the plasma samples were inverted 6 times. Next, serum and plasma tubes were simultaneously centrifuged for 10 min at 2,350 *g* (Hettich Rotanta 460-RS). Multiple aliquots were prepared per matrix to prevent additional freeze/thaw cycles in future batch processing. All samples were subsequently stored at −80 °C until use (maximally 3 months for quantification of the 9 apolipoproteins by QPMS). All healthy donors gave broad consent. The biomaterial and associated clinical data of the 44 donors that have been collected via the LuVDS are released for research purposes only, after being approved by the IRB.

Apolipoprotein quantification (QPMS)

Apolipoprotein measurement was performed in duplicate according to the standard operating procedure for sample preparation and tryptic digestion of serum apolipoproteins, apoA-I, A-II, A-IV, (a), B, C-I, C-II, C-III and E as published earlier for 6 apolipoproteins [4, 13]. Specifications for LC-MS measurements of all apolipoproteins are summarized in Supplementary Table ST2. Samples were prepared on a 96-channel Agilent BRAVO automated liquid handling platform and measured with an Agilent 6495 triple quadrupole mass spectrometer. ApoE phenotyping was performed for all 44 healthy donors in the 6 matrices as originally described [4, 14].

Immunoturbidimetric assays (ITA) apoA-I and B

For comparison to the QPMS, apoA-I and B were also measured by single ITA measurements on a Cobas C502 system (Roche Diagnostics) in 5 matrices (serum 4A and 5B and plasma 6B, 7B and PIC).

Tryptic digestion kinetics

Kinetics for tryptic digestion of the 9 apolipoproteins and formation of all 22 peptides was investigated for 6 selected healthy donors (7, 12, 24, 33, 35 and 36). Digestion was stopped at 0, 0.5, 1, 2, 3, 6, and 22 h time points, all started at t0 by addition of trypsin and stopped by quenching with 0.6% (v/v) formic acid in 5% (v/v) methanol in MQ water [4]. Maximal storage time for digestion kinetics was 6 months.

Serum spiking with anticoagulants

In order to mimic the plasma situation, serum (from SST, 5B tubes) from the same 6 donors (above) was spiked with K_2EDTA, lithium heparin or sodium citrate. Lithium heparin and K_2EDTA were obtained from Sigma (product nr H0878 and 03660, respectively) and sodium citrate buffer was collected from BD 3B tubes. K_2EDTA concentration in the BD tubes 7B is present at 1.8 mg/mL whereas the BD 6B tubes contain 17 units lithium heparin per ml blood. K_2EDTA was prepared at 18 mg/L and 10 µL was evaporated to dryness under N_2 (g). By adding 100 µL serum 1 × K_2EDTA was obtained (1.8 mg/mL serum). Lithium heparin was prepared at 170 Units/ml MQ water and 10 µL (1.7 Units) was evaporated under N_2 (g). Addition of 100 µL serum resulted in 1 × lithium heparin. Also 10 × higher concentrations were tested for K_2EDTA and lithium heparin. Sodium citrate buffer (collected from the 3B tubes) was added to serum at the same standard ratio of 1:9 as applied in the 3B tubes preparing sodium citrate plasma. Maximal storage time for the spiking experiments was 12 months.

Data evaluation

Matrix comparison evaluation was performed based on 44 duplicate measurements in R (v 4.0.3) and data was plotted using ggplot2 package (v 3.3.3). Comparisons were made for the alternative matrices 4A, 6B, 7B, PIC and 3B to the gel serum (5B) matrix for the 9 apolipoproteins and 22 peptides using Deming regression to obtain slopes and intercepts of linear regression lines. Desirable TEa was set to the known values of 9.1, 11.6 and 24.1% for apoA-I, B and (a), respectively and to 20% for the other apolipoproteins. Bias and imprecision should not exceed TEa using the formula; Bias + 1.65 imprecision < TEa.

GraphPad Prism 9.0 was used for matrix comparisons and one-way ANOVA was applied with paired comparisons to obtain box and whisker plots with means and percentile distributions of the data for the 22 peptides of the 9 apolipoproteins (Supplementary Information).

Recalibration sodium citrate and K_2EDTA matrix results

Matrix specific recalibration was performed for the separate 7 batch files. One of the samples in each batch was used for recalibration of the other samples in the batch by assignment of the serum gel value (5B) to it and performing 1-point calibration. Thus, values were recalculated for apoA-I (for both peptides AKPAL and VQPYL) in all samples in the batch in the sodium citrate (3B) and K_2EDTA (7B and PIC) tubes.

Results

Variation in concentration obtained by QPMS in serum and plasma matrices

Concentrations obtained through QPMS by LC-MRM-MS/MS for the serum and plasma samples were compared to assess the effects of the pre-analysis on the quantitative results. Comparisons were made between serum gel tubes (5B) and serum (4A), lithium heparin plasma (6B) K_2EDTA plasma (7B and PIC) and sodium citrate plasma (3B) using linear regression analysis and the obtained slopes and biases are shown in Table 1 (with highlighted slopes that significantly deviate from 1; the line of identity). Total errors (TE) for the apolipoproteins (and peptides) should not exceed the TEa, which is defined as 24.1, 9.8 and 11.6% for apos(a), A-I and B, respectively, based on the desirable analytical quality specifications in the Westgard database [15], accessed 15-02-2022. TEa for apos A-II, A-IV, Cs and E was set to 20% based on state-of-the-art technology.

The two serum matrices as well as the lithium heparin plasma matrix (5B, 4A and 6B) are interchangeable for

Table 1: Matrix comparisons for apolipoproteins in QPMS.

Protein	Peptide	4A			6B			7B			PIC			3B		
		r	Slope	Bias	r	Slope	Bias	r	Slope	Bias	r	Slope	Bias	r	Slope	Bias
Apo(a)[b]	**LFLEP**	0.999	0.987	1.4	0.999	0.978	−1.3	0.998	0.920	−3.0	0.998	0.922	−4.1	0.999	0.822	−14.0
	GISST	0.998	0.982	2.2	0.999	0.988	4.1	0.999	0.957	−0.2	0.999	0.976	−0.2	0.998	0.846	−9.3
	GTYST	0.998	0.999	1.9	0.998	0.979	−2.2	0.999	0.957	−2.2	0.999	0.955	−3.4	0.998	0.852	−15.1
ApoA-I	**AKPAL**	0.951	0.945	0.4	0.972	0.888	−2.1	0.954	0.858	−1.6	0.954	0.870	−3.7	0.955	0.786	−14.9
	VQPYL	0.969	0.935	0.8	0.970	0.910	−2.5	0.929	0.788	−13.7	0.936	0.785	−15.1	0.970	0.781	−14.7
ApoA-II	**SPELQ**	0.960	0.975	0.7	0.964	0.954	−2.7	0.876	0.784	−18.8	0.825	0.616	−22.3	0.965	0.824	−14.6
	EQLTP	0.958	1.003	1.7	0.975	0.901	−1.9	0.892	0.690	−19.6	0.843	0.711	−21.0	0.957	0.861	−13.6
ApoA-IV	**LEPYA**	0.969	0.981	0.8	0.977	0.995	0.2	0.965	0.966	1.1	0.968	0.965	−2.6	0.974	0.805	−14.9
	LTPYA	0.960	0.983	1.9	0.960	0.900	0.6	0.971	0.946	−0.2	0.958	0.931	−1.5	0.949	0.833	−14.1
	SLAPY	0.950	0.991	1.8	0.968	0.940	0.1	0.960	0.943	−1.5	0.951	0.914	−0.9	0.955	0.853	−14.6
ApoB	**TGISP**	0.988	0.983	0.5	0.977	0.948	−2.4	0.973	0.954	−2.6	0.969	0.909	−4.0	0.977	0.792	−16.0
	TEVIP	0.982	1.033	−0.3	0.977	0.974	−2.4	0.971	0.942	−3.2	0.967	0.918	−4.7	0.978	0.810	−15.3
	FPEVD	0.981	1.019	−0.3	0.961	0.940	−3.3	0.959	0.964	−5.3	0.964	0.911	−8.4	0.949	0.768	−15.2
ApoC-I	**EFGNT**	0.964	0.930	2.0	0.961	0.962	3.1	0.953	0.862	−5.0	0.951	0.855	−6.0	0.951	0.819	−12.6
	TPDVS	0.968	1.045	1.0	0.972	1.000	1.9	0.952	0.902	−4.7	0.958	0.921	−6.9	0.968	0.878	−12.5
ApoC-II[c]	**ESLSS**	0.990	1.014	0.8	0.988	0.994	−0.1	0.990	1.012	−2.5	0.981	1.011	−3.2	0.988	0.783	−26.8
	TYLPA	0.984	1.026	3.1	0.983	1.027	3.5	0.979	1.029	2.0	0.981	0.983	−2.6	0.983	0.828	−23.8
ApoC-III	**GWVTD**	0.984	0.988	1.5	0.983	1.012	1.7	0.956	0.797	−14.6	0.946	0.772	−17.7	0.982	0.866	−11.3
	DALSS	0.979	0.955	2.7	0.975	0.974	2.3	0.956	0.891	1.5	0.960	0.880	0.5	0.973	0.793	−12.1
ApoE	**SELEE**	0.989	1.045	2.1	0.981	1.042	3.6	0.975	0.919	−5.3	0.970	0.898	−6.8	0.989	0.849	−13.5
	LGPLV	0.979	0.973	1.7	0.982	0.963	3.3	0.967	0.947	−0.4	0.948	0.920	−0.9	0.952	0.800	−15.4
Avge (All)		4A % Bias (±SD)			6B % Bias (±SD)			7B % Bias (±SD)			PIC % Bias (±SD)			3B [a] % Bias (±SD)		
apos/peptides		1.4	0.9		0.2	2.5		−4.8	6.4		−6.4	6.8		−13.9	3.8	

Correlations (r), slopes and biases of Deming regression are given for comparisons between serum gel (5B) and serum (4A), lithium heparin (6B), K_2EDTA (7B), K_2EDTA with PIC (PIC) and sodium citrate (3B) for 44 samples (in duplicate). Results are shown for all 9 apolipoproteins for the quantifying peptides (in bold) and confirmation or additional peptides. ApoE phenotyping peptides were not included. Biases are highlighted between 5 and 10% ( ) and >10% ( ). Average % bias for all 9 apolipoproteins (21 peptides) or for [a]8 apolipoproteins and 20 peptides (with apoC-II excluded). For calculation in R, values for Apo(a) < 5 nmol/L and for Apo C-II < 5 mg/L were excluded because bias for apo(a)[b] was strongly affected near the detection limit, whereas in the case of apoC-II[c] 0- values were obtained by calibration artefact (due to high intercept). Quantification peptides for the nine apolipoproteins are written in bold.

apolipoprotein quantitation, with marginally, but not significantly larger variation in slope and bias observed for the lithium heparin plasma matrix 6B (slopes 0.930–1.045 for 5B vs. 4A compared to 0.888–1.042 for 5B vs. 6B, respectively and biases −0.3 to 3.1% for 5B vs. 4A compared to −3.3 to 3.6% for 5A vs. 6B, respectively (Table 1)). Contrarily, for the two K_2EDTA matrices (7B and PIC) significantly lower slopes (≤0.797) and more negative biases were found for peptides from apoA-I, apoA-II and apoC-III (VQPYL – ApoA-I ≤ −13.7% bias, EQLTP and SPELQ – ApoA-II both peptides ≤ −18.8% bias and GWVTD – ApoC-III ≤ −14.6% bias). Moreover, for apoB FPEVD, apoC-I EFGNT and TPDVS, apoE SELEE, biases between 5.0 and 8.4% and less profound deviations in slope (0.855–0.964) were found for these two K_2EDTA matrices, indicating that these matrices are not interchangeable with the native serum matrix. Similarly, consistently lower slopes (average 0.82) and negative biases (average −13.9%) were found for the citrate plasma matrix (3B). Importantly, slopes and biases were even lower for the two apoC-II peptides in the citrate plasma matrix; however, this may be attributed to calibration artefacts due to high intercepts.

In Supplementary Table ST3 the TE calculations confirm that the sodium citrate results exceed TEa for almost all peptides. For K_2EDTA and PIC matrix this is also the case for the deviating peptides of apoA-I (VQPYL), A-II (SPELQ and EQLTP) and C-III (GWVTD). Also for the quantifying peptide of apoA-I, AKPAL the (low) TEa of 9.1% is exceeded for the matrices 7B and PIC but also for lithium heparin (6B). The results of the matrix comparison were further confirmed through one-way ANOVA (Supplementary Table ST4A and B). Examples of the observed variation in slopes and the % of bias are shown for apo(a), A-I and B in Figure 2A and B, indicating the deviations for apo(a) and B occur only for the citrate plasma. In case of the apoA-I peptide VQPYL such deviations in slope and biases are

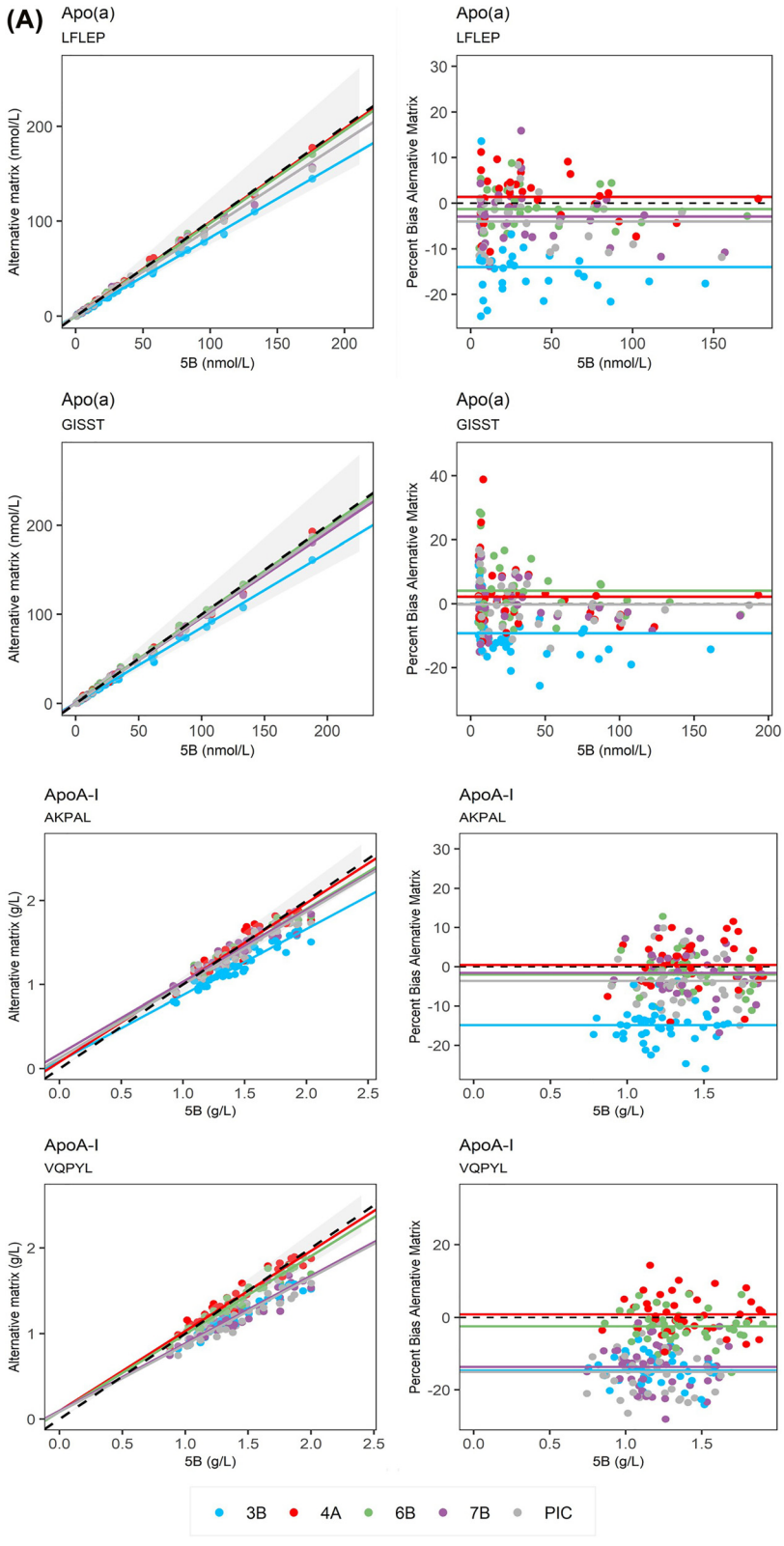

Figure 2: Matrix and peptide comparisons. (A) Examples of matrix comparisons for quantifying and confirmation peptides, respectively of apo(a) (LFLEP and GISST) and apoA-I (AKPAL and VQPYL) and (B) apoB (total) (TGISP and TEVIP) for the serum gel tubes (5B) as compared to the 5 alternative matrices (4A, 6B, 7B, PIC and 3B). Dotted lines; line of equality. Gray area indicates the total allowable errors (TEa) of 9.1% for apoA-I, 24.1% for apo(a) and 11.6% for apoB. Accompanying Bland Altman bias plots show % difference from the average as compared to the serum gel (5B) for apo(a), A-I and B. (C) Peptide comparisons for apo(a), apoA-I and apoC-III and accompanying bias plots. Apo(a), A-I and C-III concentrations for the quantifying peptides (LFLEP, AKPAL and GWVTD, x-axis) and the confirmation peptides (GISST, VQPYL and DALSS, y-axis).

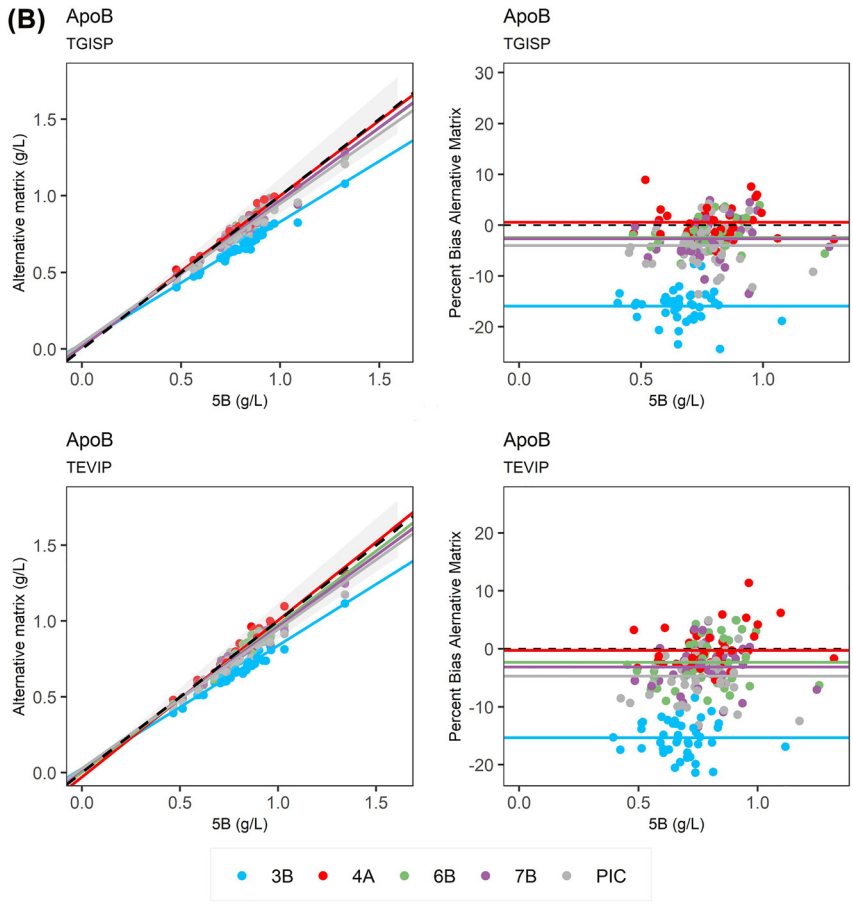

Figure 2: Continued.

observed for both the K_2EDTA (7B and PIC) and citrate plasma matrices. All matrix comparisons (to 5B) and the peptide comparisons can be seen in the supplementary material (Supplementary Figures S1A and B). Furthermore, box and whisker plots showing distribution of all 44 samples in the 6 matrices can also be seen in the supplementary material (Supplementary Figures S2A and B). ApoE phenotyping was identical in the 44 samples in all six matrices whereby 29 showed the E3/E3 phenotype, twelve E3/E4, two E2/E3 and one E4/E4 (data not shown).

One of the strengths of QPMS is the possibility for intrinsic quality control. Our methods are designed to rely on the quantitation of at least two peptides per protein, thus allowing the use of a quantifying and qualifying peptide. The latter serves as agreement of quantitation. Interestingly, the first indication for discordant results in plasma matrices came from interpeptide comparisons. Table 2 shows the correlations (r), slopes and biases for the 11 interpeptide agreements of the 9 apolipoproteins. In most cases correlations with $R > 0.95$, slopes close to 1 and limited biases <6% are observed for 10/11 peptide comparisons for both serum (5B, 4A) and the citrate and heparin plasma matrices (6B, 3B). Only for apoC-I, the peptides EFGNT and TPDVS showed poorer peptide agreement (with reduced correlations and slopes and higher %bias) for all 6 matrices. This lower correlation of apoC-I peptides was also observed in our work describing the original multiplex apolipoprotein assay [4]. Figure 2C shows the peptide agreements for the apo(a), A-I and C-III peptides. In the case of apo(a) a maximal bias of 3.7% is found for the two peptides LFLEP and GISST for the heparin plasma matrix (6B) (Table 2). However, for the two K_2EDTA plasma matrices the peptides of apoA-I (AKPAL and VQPYL) and apoC-III (GWVTD and DALSS) show a prominent disagreement with a negative bias ≤ −14% and a positive bias ≥20%, respectively (Figure 2C, Table 2). Despite the lower concentrations in the sodium citrate samples (3B), interpeptide agreement in this matrix is comparable to that in 5B, 4A and 6B with an overall average bias of −0.4% ± 3.7% SD (Table 2). Since the apoA-II peptides, SPELQ and EQLTP both showed similar deviations in the

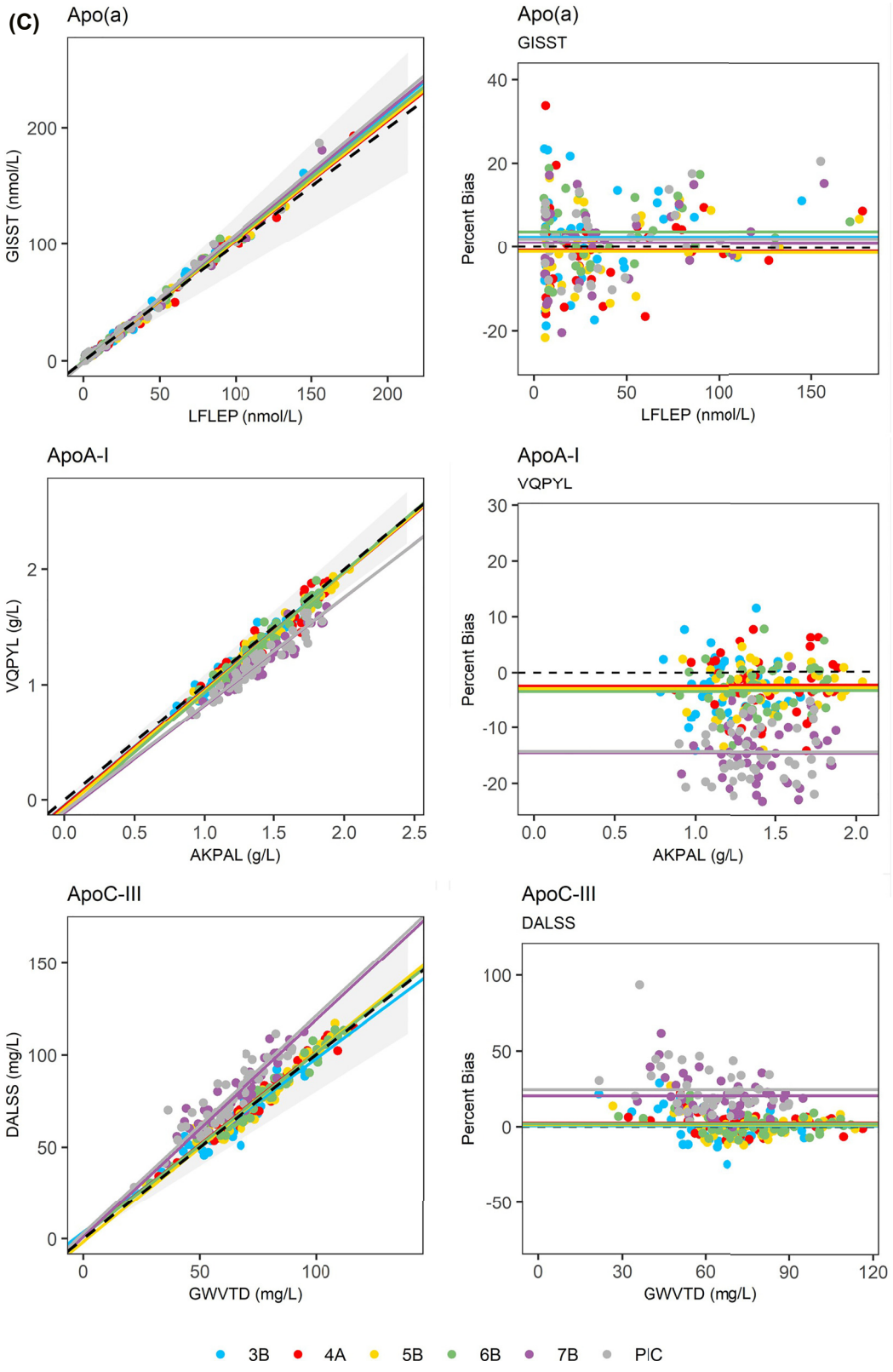

Figure 2: Continued.

Table 2: Deming regression comparison for interpeptide agreement between confirmation and quantifying peptides, for all nine apolipoproteins showing correlation (r), slopes and % bias in all six matrices.

Protein	Quantifying Peptide	Qualifying Peptide	5B			4A			6B			7B			PIC			3B		
			r	Slope	%Bias	r	Slope	%Bias	r	Slope	%Bias	r	Slope	%Bias	r	Slope	%Bias	r	Slope	%Bias
Apo(a)[a]	LFLEP	GISST	0.997	1.040	−1.1	0.996	1.035	−0.8	0.997	1.051	3.7	0.996	1.080	1.1	0.993	1.099	2.0	0.996	1.070	2.4
ApoA-I	AKPAL	VQPYL	0.982	1.023	−2.9	0.959	1.012	−2.4	0.975	1.047	−3.4	0.945	0.934	−14.6	0.954	0.927	−14.4	0.964	1.013	−2.6
ApoA-II	SPELQ	EQLTP	0.976	0.971	−2.1	0.980	0.998	−1.2	0.970	0.913	−1.4	0.933	0.859	−3.0	0.937	1.099	−0.4	0.967	1.015	−1.0
ApoA-IV	LEPYA	LTPYA	0.968	0.971	−0.3	0.974	0.973	0.8	0.976	0.881	0.1	0.968	0.947	−1.7	0.984	0.937	0.7	0.972	1.009	0.5
ApoA-IV	LEPYA	SLAPY	0.968	0.971	−0.3	0.974	0.973	0.8	0.976	0.881	0.1	0.968	0.947	−1.7	0.984	0.937	0.7	0.972	1.009	0.5
ApoB	TGISP	TEVIP	0.989	0.987	1.3	0.990	1.037	0.4	0.983	1.013	1.3	0.987	0.966	0.8	0.984	0.997	0.5	0.984	1.008	2.0
ApoB	TGISP	FPEVD	0.989	0.987	1.3	0.990	1.037	0.4	0.983	1.013	1.3	0.987	0.966	0.8	0.984	0.997	0.5	0.984	1.008	2.0
ApoC-I	EFGNT	TPDVS	0.947	0.771	−9.7	0.936	0.867	−10.5	0.929	0.799	−10.6	0.934	0.806	−9.6	0.903	0.824	−10.2	0.919	0.821	−9.5
ApoC-II[b]	ESLSS	TYLPA	0.975	0.973	0.7	0.975	0.984	2.6	0.979	1.006	5.8	0.976	0.987	6.5	0.973	0.946	3.5	0.972	1.031	3.8
ApoC-III	GWVTD	DALSS	0.972	1.031	1.3	0.980	0.997	2.5	0.973	0.994	1.9	0.917	1.179	20.4	0.909	1.182	24.4	0.942	0.943	0.6
ApoE	SELEE	LGPLV	0.979	1.026	−1.1	0.978	0.956	−1.6	0.978	0.949	−1.5	0.970	1.060	3.9	0.945	1.056	5.0	0.970	0.974	−3.6
Avge (All)			5B % Bias (± SD)			4A % Bias (± SD)			6B % Bias (± SD)			7B % Bias (± SD)			PIC % Bias (± SD)			3B % Bias (± SD)	
Apos/Peptides			−1.2	3.2		−0.8	3.6		−0.3	4.3		0.3	8.9		1.1	9.7		−0.4	3.7	

Biases > 14% (for apoA-I and GWVTD) are indicated for 7B and PIC (K_2EDTA matrices). For apoC-I a negative bias (≈10%) is seen for the peptide TPDVS in all six matrices. For calculation in R, values for Apo(a) < 5 nmol/L and for Apo C-II < 5 mg/L were excluded because bias for apo(a)[a] was strongly affected near detection limit, whereas in the case of apoC-II[b] 0- values were obtained by calibration artefact (due to high intercept).

matrix comparisons of the K_2EDTA plasma (7B and PIC) to serum gel (5B) these apoA-II peptides show a relatively good peptide agreement (bias ≥ −3.0%) (Tables 1 and 2).

Assessment of tryptic digestion kinetics in serum and plasma matrices

As stated, the technique of QPMS through bottom-up proteomics relies on the transition of the measurand from intact protein to peptide. This enzymatic process is catalyzed by the protease trypsin. However, it is widely known that trypsin does not cleave at all sites with equal efficiency [16]. The peptides that showed most profound deviation in results between K_2EDTA and serum matrices are known by experience to be slower forming peptides [4, 17], suggesting that the digestion efficiency in the K_2EDTA matrices is impaired. This is further confirmed by the generally higher bias observed for the PIC (protease inhibitor cocktail) matrix compared to the regular K_2EDTA matrix without PIC (7B) (Table 1).

The digestion kinetics for the peptides from the 9 apolipoproteins was investigated for 6 donors in four different matrices (serum gel (5B), lithium heparin (6B), sodium citrate (3B) and K_2EDTA (7B) plasma). For peptides VQPYL – apoA-I, GWVTD – apoC-III, and SPELQ – apoA-II, clearly impaired formation is observed, while the formation of EQLTP – apoA-II is generally slow, but does not seem to deviate between the matrices (Figure 3). Digestion time courses for all peptides are shown in Supplementary Figure S4. Ten of the 21 peptides shown form rapidly within the first 2 h of digestion (ApoA-I, AKPAL; A-IV, LEPYA, LTPYA; (a) LFLEP, B, TGISP, TEVIP; C-II, ESLSS, TYLPA; C-III, DALSS and E, LGPLV). Because the incubation time for the multiplex assay is 3 h the % of peptide signal formed at 3 h relative to the 22 h time point (which is regarded complete) is presented in Supplementary Table ST6, further confirming the delayed formation of peptides.

Spiking of serum samples with anticoagulants

To assess whether the deviating results for the various matrices are solely caused by the addition of the anticoagulants, (gel) serum samples (5B) were spiked with lithium heparin, K_2EDTA and sodium citrate at the same concentration (or dilution) as in the BD tubes 6B, 7B and 3B. Only the (dilution by) sodium citrate caused a similar average of 11.9 ± 4.0% reduction compared to the sodium citrate (3B) matrix itself, whereas (no significant) reduction was seen for lithium heparin or K_2EDTA (1.7 ± 2.6% and 2.2 ± 2.2% for 6B and 7B, respectively). However, addition of a 10-fold increased K_2EDTA concentration reduced

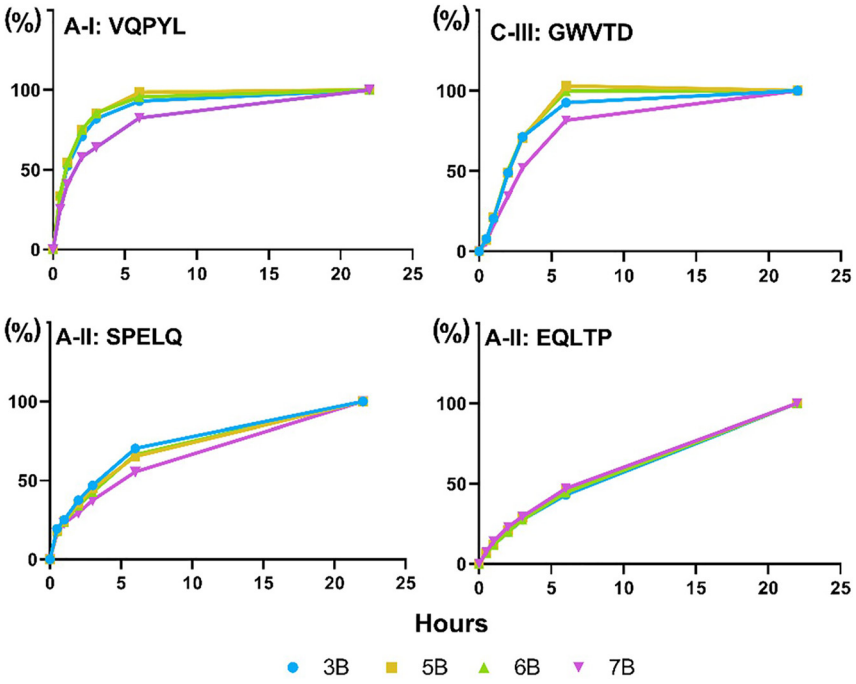

Figure 3: Trypsin digestion kinetics for selected peptides of apoA-I (VQPYL), C-III (GWVTD) and A-II (SPELQ and EQLTP) in serum (5B) and plasma (3B, 6B and 7B) of six healthy donor samples. All data are normalized to the 100% relative responses (RR) for each matrix at the 22 h time point.

apoA-I peptide VQPYL (17.6 ± 4.8%) compared to AKPAL and apoC-III peptide GWVTD (22.6 ± 4.3%) compared to DALSS. Both apoA-II peptides, SPELQ and EQLTP were decreased 19.9 ± 5.7% and 21.9 ± 4.7% in comparison to the control 5B serum samples, respectively.

Immunoturbidimetric (ITA) measurements of ApoA-I and B

Immunoturbidimetric measurement are independent of proteolytic digestion and matrices can – to some extent – be exchanged. Roche ITA measurements for both apolipoproteins A-I and B are CE-marked for serum, lithium heparin plasma and K_2EDTA plasma. The exchangeability of these matrices was confirmed through the measurements of the 44 samples from tubes 5B, 4A, 6B, 7B and PIC (Supplementary Figure S3). The K_2EDTA (PIC) samples show the largest difference from the mean (0.044 and 0.029 g/L) for apoA-I and B, respectively and slopes of 0.97 for both (Supplementary Table ST5).

The need for matrix-specific calibrators in QPMS

Native, matrix-based calibrators are the ideal choice during QPMS test development and application, as their digestion behavior mimics that of the clinical samples. The calibrators used for our QPMS test for apolipoprotein quantitation are serum-based, and thus the serum samples show the least bias, while larger bias is observed for the plasma samples. To assess whether matrix-based calibration indeed diminishes the observed variation in quantitation, the apoA-I concentrations of the QPMS measurements were recalculated using one of the samples as calibrator, with its value assigned by the result of the serum gel matrix (5B). A major correction was observed for the sodium citrate plasma (Figure 4, Table 3). Moreover, equivalent results were obtained for peptides AKPAL and VQPYL in the K_2EDTA matrices both with and without PIC, indeed indicating the need for matrix-based calibrators.

Discussion

The pre-analytical phase is important for accurate test results, and pre-analytical mistakes have been described as potentially the most important source of erroneous patient results [1, 18]. Specifically, the selection of the correct blood collection tube is of utmost importance to achieve accurate clinical chemistry results. Variation in results between matrices (both with and without anticoagulants) has been reported for numerous general clinical chemistry analytes [19–24], cytokines [25], and various metabolites [26, 27].

Quantitative protein mass spectrometry (QPMS) has several advantages over conventional immunoassays. It allows for the definition of protein measurands at the

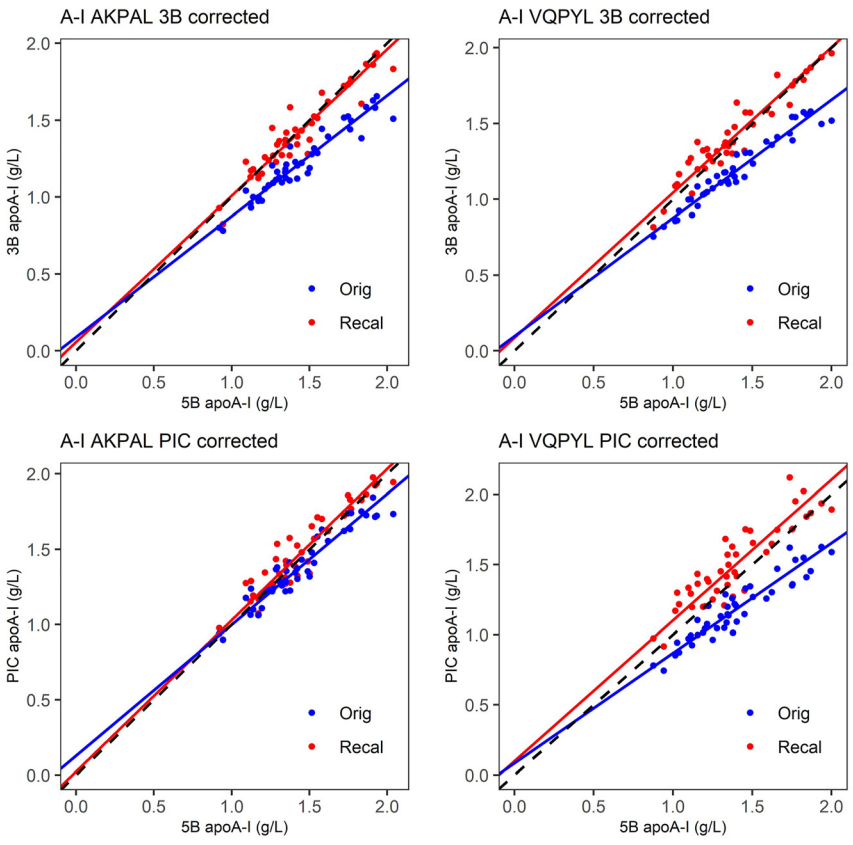

Figure 4: Matrix specific recalibration. Top, recalculation of the sodium citrate results (blue) with one point calibration in the 7 batches for 44 samples (red) for apoA-I peptide AKPAL (left) and VQPYL (right). Bottom, recalibration for the K_2EDTA (PIC) results for apoA-I with blue and red (as above) showing adjustment for AKPAL (left) and VQPYL (right). Dotted lines, line of equality (to serum gel (5B).

Table 3: Matrix comparisons for apoA-I in sodium citrate (3B) and K_2EDTA (PIC) plasma matrix as compared to serum gel (5B) for both peptides (AKPAL and VQPYL).

ApoA-I	Matrix comparison to serum gel (5B)				Peptide comparison	
Correction	**3B Sodium Citrate**		**PIC K_2EDTA**		**AKPAL vs VQPYL**	
	AKPAL slope	VQPYL slope	AKPAL slope	VQPYL slope		slope
					5B original	1.023
original	0.786	0.781	0.870	0.785	PIC original	0.927
one point	0.952	0.962	1.012	1.003	PIC one point	1.021

Original slopes of the matrix comparisons are given for 3B vs. 5B and PIC vs. 5B with strongest deviations (<0.80) highlighted. Recalculation of 3B and PIC results were performed using one point correction in each batch (7 batches/44 samples). On the right side, correction for peptide comparison between AKPAL and VQPYL (apoA-I).

molecular level, combined with multiplexed and antibody-independent quantitation, and facilitates in-house method development. However, to ensure accurate test results, the total analytical process should be evaluated, including matrix-dependent variation. In previous profiling and proteomics studies such variation has been recognized [11, 12, 28], and it may be caused by serum or plasma contamination by platelets, erythrocytes or coagulation factors [10], but also altered proteolytic activity, impacting the peptide production, has been reported [29].

In the present study we show for the first time the influences of the varying matrices in blood collection tubes on the targeted quantification of nine apolipoproteins (A-I, A-II, A-IV, (a), B, C-I, C-II, C-III and E) by QPMS using a

native serum-based calibration strategy. Through the comparison of QPMS measurements of samples collected from 44 healthy donors, it could be revealed that apolipoprotein concentrations were interchangeable between serum tubes with and without gel (5B and 4A). Moreover, they were highly similar to the lithium heparin plasma (6B) matrix. Contrarily, measured apo concentrations were overall lower in sodium citrate plasma matrix (3B, average –13.9%, Table 1) attributable to the dilution of the blood samples by the sodium citrate buffer. The most variable results were obtained from the K_2EDTA plasma matrix (both 7B and PIC). Four peptides (apoA-I VQPYL, apoA-II SPELQ, apoA-II EQLTP and apoC-III GWVTD) showed highly variable results (bias ≤ –13.7%), while other peptides were more moderately reduced (between –5.0 and –8.4%) in the 7B and PIC tubes (apoB FPEVD, apoC-I EFGNT and TPDVS and apoE SELEE). The proteinase inhibitors cocktail (PIC) caused a reduction of measured concentrations for (almost) all peptides as compared to the K_2EDTA matrix without the PIC (7B). These results are in line with an earlier comparison in which no additional protection from degradation was observed for apolipoproteins, even after longer sample processing duration (2 and 6 h) [11].

Because QPMS relies on the proteolytic digestion of proteins into their representing proteotypic peptides, we hypothesized that the variation observed for the K_2EDTA matrices was caused by altered protein digestion kinetics. Digestion time courses indicate that the slower forming peptides, particularly apoA-I VQPYL, apoC-III GWVTD and apoA-II SPELQ form even slower in the K_2EDTA matrix 7B compared to serum (5B), see Figure 3, while apoA-II peptide EQLTP showed very slow digestion kinetics in all matrices. To assess whether the effects are solely due to the anticoagulant, K_2EDTA was spiked into the serum matrix 5B. A 10 × addition indeed reduced concentrations of the four slow-forming peptides, but this was not confirmed when a 1 × addition was used. We therefore speculate that the K_2EDTA does not have a direct effect on proteolytic digestion by trypsin, but does so indirectly.

Matrix-specific calibration is a widely accepted strategy to overcome matrix-specific variation [30]. Also for QPMS matrix-specific calibration is required for accurate quantitation, as was shown through the use of a provisional single-point recalibration for the citrate and K_2EDTA matrices.

Importantly, in the same samples in which QPMS showed matrix-specific variation, we could confirm that no such differences were observed for the ITA methods for apoA-I and B (Supplementary Figure S3). Therefore, IA may in certain conditions be favored over QPMS by LC-MRM-MS, for instance in studies where samples have been collected with inconsistent blood collection conditions.

Overall, we here demonstrate that quantification of proteins from variable matrices by targeted LC-MRM-MS/MS approach using bottom-up proteomics is not necessarily interchangeable and depends on (tryptic) digestion kinetics in the serum and plasma matrices. Matrix-specific calibration and reference ranges will be required for adequate and reliable patient classification.

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