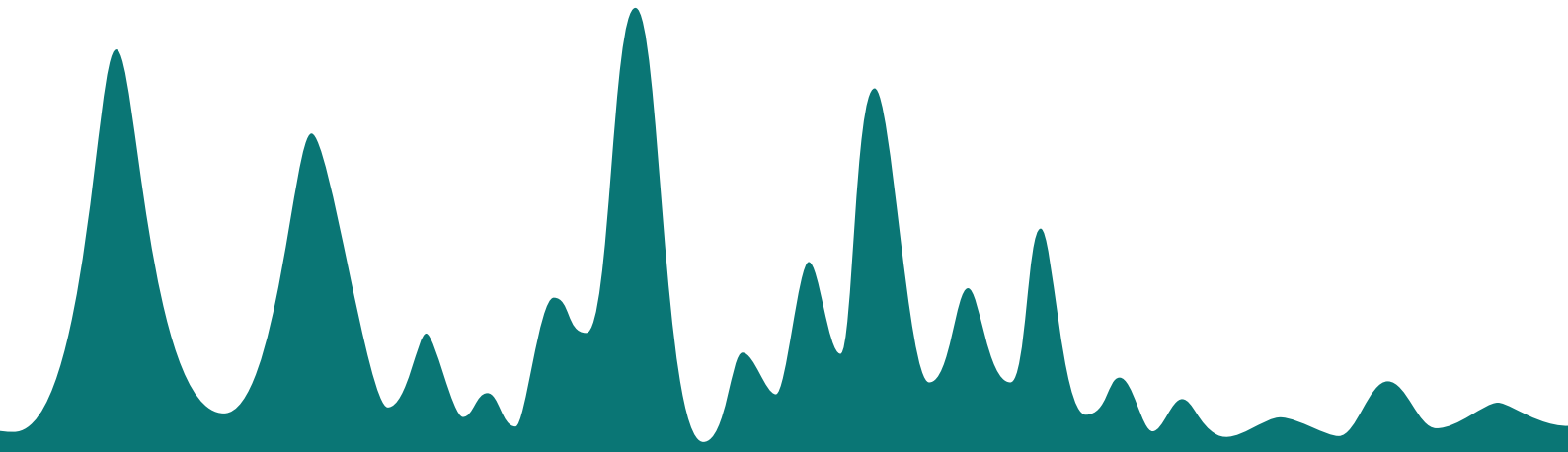
QPrEST

ELISA and LC-MS/MS supported by robust QPrEST standards are valuable cross-validation partners



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Introduction

Immunoassays have been the technology of choice for biomolecule analysis for many applications in research, diagnostics and epidemiology. ELISA, for example, was originally developed in 1971 and has evolved to a high sensitivity method that does not require complex and expensive equipment.

Sensitivity is being pushed to higher levels with detection by chemiluminescence and electrochemiluminescence. In addition, novel protein binders, miniaturization, and the development of multiplexing strategies, including multiple fluorochromes and other detection methods, ensure that ELISA will continue to play a major role in protein biomarker analysis.

There are, however, disadvantages in using immunoassays, including limitations in multiplexing and throughput. These disadvantages have driven the search for alternatives, such as mass spectrometry (MS), a gold standard method that has been used in research for decades.

The power of MS for the absolute quantification of proteins and peptides has meant that targeted MS is being applied in all phases of biomarker development, from discovery to validation.

LC-MS/MS is particularly attractive for high-throughput multiplex analysis needed to drive robust, time- and cost-effective data generation to support protein biomarker research and implementation in the clinic[1,2]. Obtaining quantitative data with high accuracy and precision can be a real challenge, however, and requires great care in method development, including the selection of internal standards and calibration methods.

The ability of ELISA and LC-MS/MS to deliver comparable data has led to their use in cross-validation studies. Here, we will look into the relative merits of ELISA and LC-MS/MS, including examples of head-to-head comparisons and the use of QPrEST standards to ensure the generation of accurate analyte concentrations using LC-MS/MS.

A comparison of ELISA and LC-MS

In a review of immunoassays and LC-MS technologies from 2016, Cross and Hornshaw looked into the advantages and disadvantages of these two analytical techniques for the quantification of biomolecules in biofluids[2]. **Table 1** shows a summary of their analysis.

	Advantages	**Disadvantages**
ELISA	Ease of use Acceptance Equipment costs Throughput Sensitivity	Selectivity * Analyte detection * Sample volumes Cost per sample Reproducibility Multiplexing Assay time
LC-MS	Selectivity Sensitivity Sample throughput ** Sample volumes Cost per sample Reproducibility Multiplexing Extended compound range	Equipment costs Complexity Sample complexity

Table 1. Summary of advantages and disadvantages of ELISA and LC-MS based on review by Cross and Hornshaw, 2016 (Ref. 2).

*But note possibility of alternative protein binders.
**But sequential.

Highly sensitive immunoassays will remain the method of choice for simple and routine assays, and developments within the immunoassay field, such as new protein binders and detection methods, will continue to extend the power of this approach. Immunoassays are usually more sensitive than LC-MS/MS.

Although LC-MS/MS is a sensitive method, there are still limitations in the quantification of very low amount of proteins. It is also important to note that, despite the rapid development of methods based on MS, antibodies in themselves will also continue to be vital tools.

One example is stable isotope standards and capture by anti-peptide antibodies (SISCAPA), an MS-based method for protein quantification. Others include immunoaffinity mass spectrometry (IAMS) and protein immunoprecipitation combined with multiple reaction monitoring mass spectrometry (IP-MRM).

The complexity and cost of LC-MS continue to be major barriers to acquisition and Cross & Hornshaw believe that for this technique to make an impact, the benefits need to be communicated and methods must be developed to provide fast, easy and cost-effective sample preparation.

Approaches to targeted LC-MS/MS

Within MS-based proteomics, targeted MS/MS techniques such as selected reaction monitoring (SRM) and parallel reaction monitoring (PRM) are most equivalent to ELISA in that they are used for the detection and quantification of known analytes in biological samples with high sensitivity and specificity. Of these approaches, SRM performed on a triple quadrupole instrument generally provides higher sensitivity while PRM and other targeted high-resolution MS methods can provide good specificity without extensive validation of ideal pairs of precursor and fragment ions to avoid interference from peptide derived from other proteins in the sample.

Accurate absolute quantitation using targeted MS/MS involves compensating for variations in a rather complex workflow that includes:

1. Sample preparation including protein digestion using a suitable protease
2. Separation of peptides by liquid chromatography
3. Identification and quantification of peptides using SRM/PRM

Compensating for variations that can arise from sample preparation, matrix effects, and instrument variation generally involves adding an internal standard at some point in the workflow.

The most advanced and reliable internal standard is based on a stable-isotope-labeled (SIL) version of the full-length target protein (PSAQ). Shorter SIL peptides, which can include post-translational modifications (PTMs) and winged SIL (WiSIL) peptides extended on both ends with endogenous sequence to provide cleavage sites for the protease, are more readily available but may not reflect method variation. This has led to the development of QPrEST standards that come closer to the PSAQ gold standard by comprising long sections of the endogenous sequence that help to mimic the cleavage of the endogenous protein around the cleavage peptides.

A recent evaluation of different internal standards for SRM analysis of an analyte in cerebrospinal fluid indicated that QPrEST showed better accuracy than SIL and WiSIL peptides, indicating that this standard better reflects digestion conditions[3].

Direct comparison of ELISA and LC-MS/MS

A number of evaluations comparing ELISA and LC-MS/MS to determine biomolecule concentrations in complex biological fluids are shown in **Table 2**.

These examples involve cross-validation of the two methods using a cohort of patient samples and a range of MS approaches.

The two methods are generally in agreement based on the data analysis method used, indicating that they are very valuable as cross-validation partners.

Ref.	Application	ELISA	LC-MS/MS	Standard	Comments
4	Biomarkers for chronic kidney disease progression in plasma.	Sandwich ELISA.	MRM	SIS added after digestion.	MRM significantly correlated (R = 0.5–0.9) with results from respective ELISA.
5	HSP90α	Sandwich ELISA.	MRM, PRM	SIL peptides added after digestion.	PRM data correlate better with ELISA measurements than SRM data and shows comparable sensitivity to immunoassay.
6	Predictive test for diabetic kidney disease.	ELISA using CaptSure technology.	IAMS/MRM	Labeled peptides added before digestion. Calibrator standard processed in parallel.	Includes upstream immunocapture step. Rigorous comparison of data for 100 samples a high correlation (R = 0.97). Analysis includes Bland-Altman plot.
7	Cerebrospinal fluid amyloid species.	Sandwich ELISA.	MRM	Labeled amyloid peptide.	No digestion needed. R2 values for Abeta 1-40 (0.8) and Abeta 1-42 (0.6)
8	Biomarker candidates in plasma.	Sandwich ELISA. Capture antibody is also used in the IP-MRM assay.	IP-MRM/ SISCAPA	SISCAPA – proteotypic tryptic peptides and corresponding spiked SIL internal standards captured by antibodies.	IP-MRM and ELISA measurements for all target proteins, except one, were highly correlated (r = 0.67−0.97).
9	Circulating ATPase inhibitory factor 1 (IF1).	Competitive ELISA with polyclonal antibody.	RefMRM	SIL peptides added after digestion.	IF1 plasma levels obtained using both techniques were strongly correlated (r = 0.89, $p < 0.0001$), while the Bland-Altman plot did not indicate any major statistically significant differences.

Table 2. Examples of comparisons between ELISA and LC-MS/MS in the literature.

Case Study:
Measurement of apolipoproteins in plasma samples using ELISA and LC-MS/MS + QPrEST standards

To compare the performance of ELISA and LC-MS/MS using QPrEST standards and their suitability for cross-validation, the two techniques were used to measure a panel of four apolipoproteins (A1, D, H, M) in plasma samples. Schematic workflows for the two methods are shown in **Figure 1**.

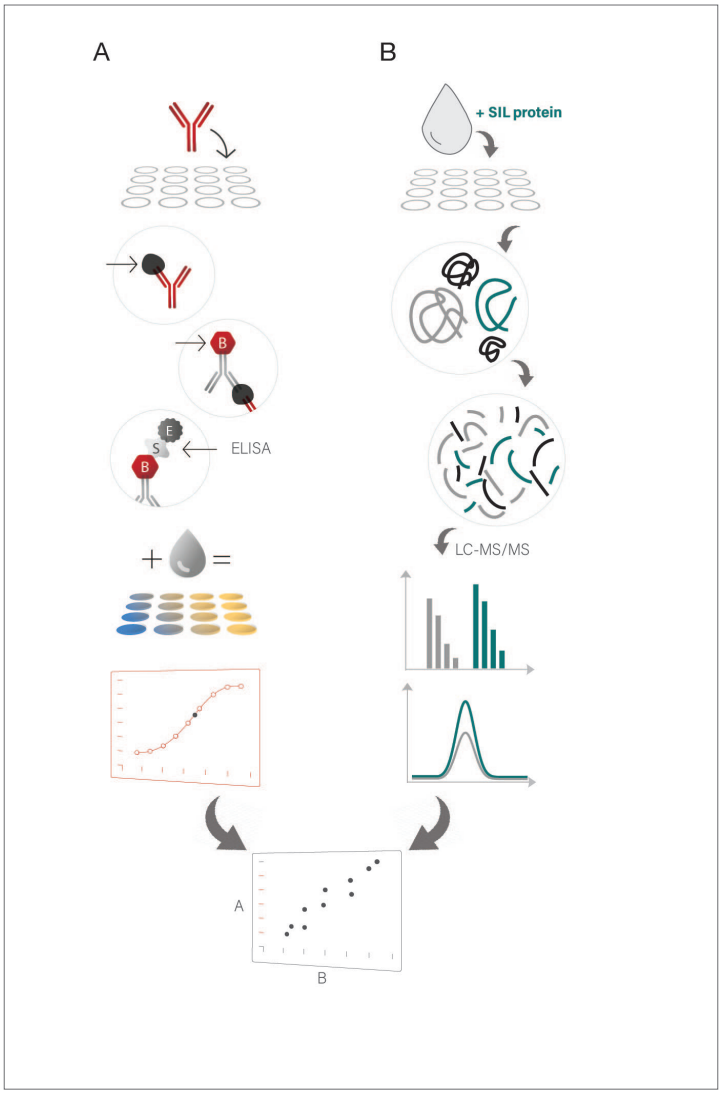

Figure 1. Schematic workflows for (A) ELISA, and (B) LC-MS/MS using SIL protein standards.

A) **ELISA:** Plasma sample and serially diluted standard proteins are added to individual wells of a plate pre-coated with monoclonal antibodies (mAb) specific for the protein of interest. The protein is detected by adding a biotinylated detection mAb followed by streptavidin-conjugated enzyme and a colorimetric substrate. The colorimetric change, which is directly proportional to the amount of protein, is measured using a plate reader. Measured absorbance of the protein standard samples is plotted against known concentrations and the calibration curve is used to calculate the analyte concentration in the sample.

B) **LC-MS/MS:** A known amount of SIL protein standard is mixed with the plasma sample. The sample containing plasma proteins and the SIL standard is digested with trypsin and the mix of peptides is analyzed by PRM LC-MS/MS. Generated heavy to light ratio is used to calculate the absolute concentration.

The two methods can be used for cross-validation (A vs B) at the bottom of the figure.

Materials and methods

Protein quantification by ELISA

Four apolipoproteins (A1, D, H, M) were quantified in 30 human plasma samples from healthy individuals using ELISAPRO kits (Mabtech AB, Sweden). The analysis was performed according to the manufacturer's protocol. Two technical replicates were prepared for each plasma sample. The readings were performed on a Versa Max (Molecular Devices, CA, USA) reader and data was analyzed using the Soft Max analysis program (Molecular Devices, CA, USA).

Protein quantification by LC-MS/MS

Heavy isotope-labeled QPrEST standards (Atlas Antibodies AB, Sweden) were designed to target apolipoproteins APOA1, APOD, APOH and APOM. The standards were pooled and spiked into the same 30 human plasma samples. Spiked plasma samples were digested with ProteinWorks eXpress Direct Digest Kit (Waters Corporation) using a 5-step protocol including reduction and alkylation steps, and post-digestion cleanup was performed with ProteinWorks µElution SPE Clean-up Kit (Waters Corporation). Three technical replicates were prepared for each plasma sample.

Peptide samples were analyzed by LC-MS/MS in parallel reaction monitoring (PRM) mode on a Q Exactive Plus MS instrument (Thermo Fisher Scientific). The peptides monitored by the PRM method are shown in **Table 3**. Data processing was performed using Skyline and the average heavy-to-light peptide ratio for each protein in each sample was used to calculate the plasma concentrations.

QPrEST standard	QPrEST sequences with tryptic peptides
QPrEST APOA1	SKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQEEMELYRQ KVEPLRAELQEGARQKLHELQEKL
QPrEST APOD	GKCPNPPVQENFDVNKYLGRWYEIEKIPTTFENGRCIQANYSLMENGKIK
QPrEST APOH	EPGEEITYSCKPGYVSRGGMRKFICPLTGLWPINTLKCTPRVCPFAGILENGAVRYTTFEYPN TISFSCNTGFYLNGADSAKCTEEGKWSPELPVCAPIICPPPSIPTFATLRVYKPSAGNN SLYRDTAVFECLPQHAMFGNDTITC
QPrEST APOM	KDGLCVPRKWIYHLTEGSTDLRTEGRPDMKTELFSSSCPGGIMLNETGQGYQRFLLYNRSPHPPEK CVEEFKSLTSCLDSKAFLLTPRNQ

Table 3. Sequences of the QPrEST standards. The peptides used for quantification of APOA1, APOD, APOH and APOM are marked in blue and orange.

Results

The data from ELISA and LC-MS/QPrEST for the four apolipoproteins were highly correlated as shown by regression analysis (**Figure 2**) and these results were comparable with examples in the literature – see **Table 1**.

However, the regression line gradients for ApoD, ApoH and ApoM were not close to 1, indicating that one or other of the methods was inaccurate, for example due to incorrect calibration. This discrepancy, together with limitations in the use of regression analysis, was addressed using ApoH as an example.

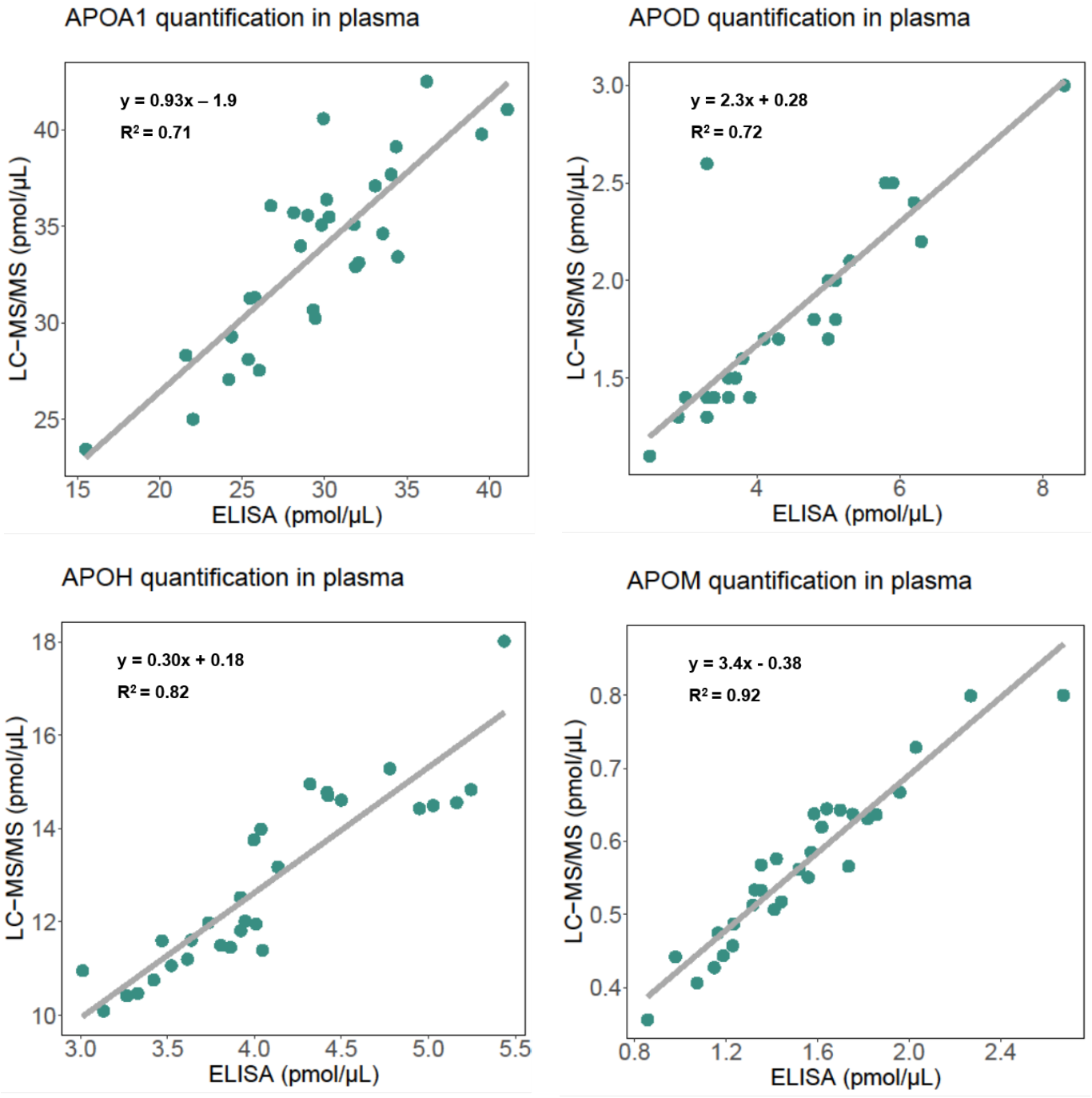

Figure 2. Correlation of plasma protein concentrations determined by the QPrEST LC-MS/MS method and ELISA for APOA1, APOD, APOH and APOM. LC-MS/MS data is shown on the x-axis and ELISA data is shown on the y-axis. The regression equation and correlation coefficient (R2) are presented for each protein.

A more stringent approach to method comparison

The discrepancy between the data from ELISA and LC-MS/QPrEST for ApoH was investigated by re-quantifying the ELISA standard by LC-MS/MS and a PRM approach with corresponding QPrEST as internal standard.

Re-quantification of the ELISA standard resulted in a regression line with a gradient close to 1, indicating that the two methods give similar absolute values overall (**Figure 3**).

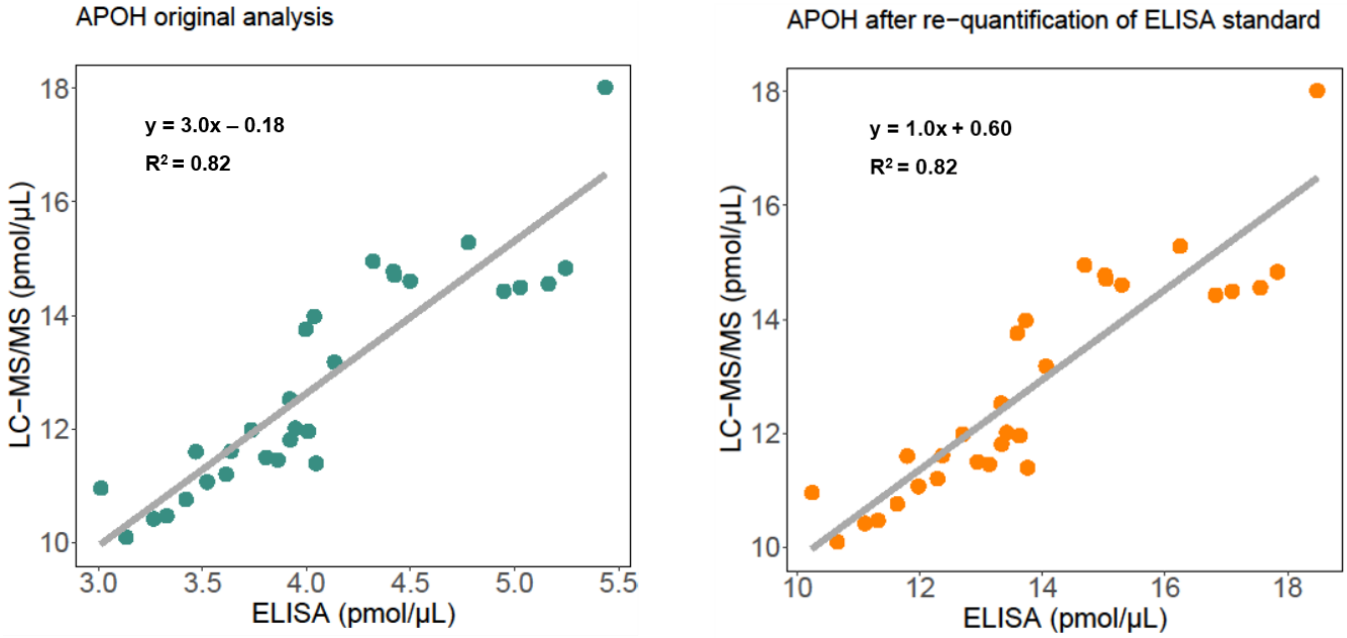

Figure 3. Effect of re-quantifying ELISA standard on regression analysis.

On the limitations of regression analysis

Limitations in the use of linear regression and correlation to compare analytical methods were also addressed. Correlation describes the linear relationship between two sets of data but not their agreement and since correlation investigates the relationship between one variable and another rather than the difference, this approach is not recommended for cross-validation of analytical methods[10].

Applying the least-squares approach commonly used is problematic in that the reference and the test methods both contain error, which violates one of the statistical assumptions in regression analysis that the independent variable (the reference method) should be measured without error and this limitation has led to the use of alternative regression methods[11].

The correlation coefficient and regression technique can also be misleading when assessing agreement, because they evaluate only the linear association of two sets of data. In addition, R2, the coefficient of determination only indicates the proportion of variance that the two variables have in common.

Bland and Altman plot

To overcome limitations in regression analysis, Bland and Altman devised a plot to describe the agreement between two quantitative measurements[12]. This approach is widely used as a supplement to regression analysis and the scatter plot[13], a practice that is also recommended by the Clinical and Laboratory Standards Institute (CLSI; 14).

The Bland and Altman plot displays every difference between two paired methods against the average of the two methods (**Figure 4**). In the case of ApoH analysis, the differences between the ELISA data and LC-MS/MS data were plotted against the mean from the two methods. This approach highlights any relationships between measurement error and the "true value", which in the absence of a reference method is assumed to be the mean from the two methods.

For example, before standard re-calibration, the difference between the two methods increased with the true value and the highest concentration fell outside the general recommendation of Bland and Altman that 95% of the data points should lie within ± 1.96 standard deviations (SD) of the mean difference[12].This in itself could indicate an upper limit for the cross-validation of the two methods that is not obvious from the regression (**Figure 3**).

Re-quantification of the standard reduced the overall bias (limit of agreement, ± 1.96 SD) and all points essentially fell within the limits. In addition, there was no discernible pattern in the Bland and Altman plot, indicating that the proportional error seen before re-calibration had been reduced by re-calibrating the standard. This proportional error could also be seen in the regression (**Figure 3**, left; gradient << 1), but this is not always the case[10].

These results highlight the importance of using the right approach to data analysis and also correct calibration of methods to ensure that method comparison is reliable. This can involve re-quantifying method standards, for example by using QPrEST standards, which have been used to validate antibodies for a range of applications[15].

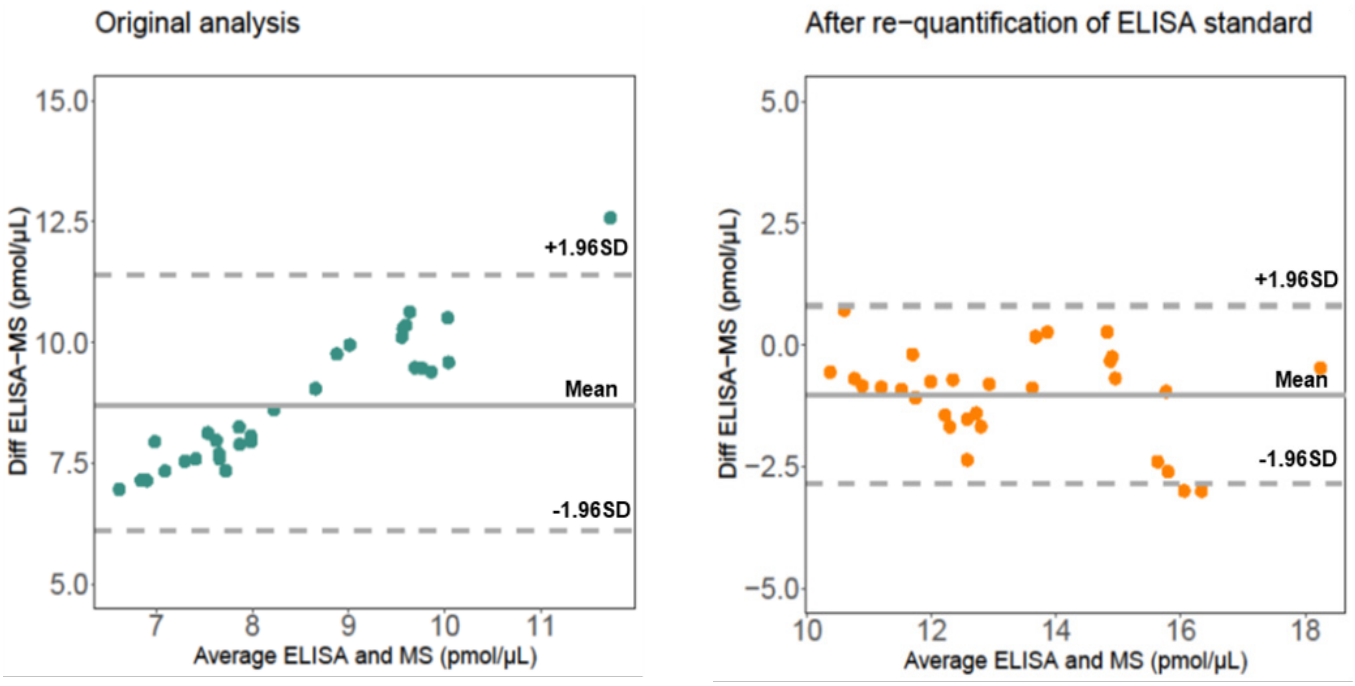

Figure 4. Bland-Altman plots showing comparison of data from ELISA and LC-MS/MS before and after re-quantification of ELISA standard.

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Conclusions

- High sensitivity ELISA remains the gold standard for determining levels of many analytes, especially, in simple and routine assays.
- LC-MS/MS has a number of advantages over ELISA that can make it a more suitable method for complex analysis such as targeted proteomics, including selectivity, sample throughput, sample volumes, cost per sample, reproducibility and multiplexing.
- A number of studies show that ELISA and LC-MS/MS in the form of MRM or PRM generate comparable data, making these very valuable methods for cross-validation.
- The choice and quantification of standards is critical to ensuring accurate data.
- Method comparison should include the use of Bland-Altman plots to ensure that the level of agreement between methods is acceptable.
- SIL proteins, such as QPrEST, support a quantitative method orthogonal to ELISA for platform cross-validation in translational and clinical research.

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