Performance qualification

ANAQUANT

SOLUTIONS FOR BIOANALYSIS

QP-M-001_V03

HCPprofiler standard qualification report

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HCPprofiler by ANAQUANT

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afaq ISO 9001 Qualité AFNOR CERTIFICATION

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1. Traceability

Version	Reason for modification	Release Date
001	Original version	29/03/2024
002	Data update	24/04/2024
003	Organization modification and adding part with HCPprofiler applications	11/07/2024

2. Scope

ANAQUANT has developed a HCPprofiler kit using quantitative LC-MS/MS analysis for accurate identification and quantification of HCPs in samples.

The developed HCPprofiler kit comprises an innovative standard and a unique data processing software. The standard consists of a water-soluble bead coated with peptides at different concentrations. The HCPprofiler standard (one bead) is added directly into the sample and provides an internal calibration curve. The present report assesses the standard's repeatability and stability over several months.

This report is a non-GMP qualification report assessing the HCPprofiler method for protein quantification, for example Host Cell Protein (HCP) quantification in the context of release and stability studies of drug substances and products. In addition, this method complies with the ICH Q2(R2) guideline on validation of analytical procedures, for intended use in quantitative impurity testing.

3. Summary of test parameters for non-GMP validation

This non-GMP method complies with the ICH Q2(R2) guideline on validation of analytical procedures for intended use in quantitative impurity testing, in the context of HCP clearance. In the present report, ANAQUANT validates accuracy, repeatability, intermediate precision, reproducibility, robustness, specificity, linearity, and working range. A method cross validation is also described.

Specificity of the HCPprofiler method is assessed by testing the extent to which other substances interfere with the quantification of a given analyte. Specificity is typically used to describe the ultimate state, measuring unequivocally a desired analyte.

Working range is defined as the concentration interval for which it has been demonstrated that the present analytical procedure has a suitable level of precision, accuracy, and linearity.

Linearity is assessed by plotting signals against analyte concentration and validated over a given working range provided that values obtained by the analytical are proportional to the theoretical sample values. The statistical method used here is a calculation of a regression line by the method of least squares. The coefficient of determination (R^2) should be ≥ 0.90. Spike-in levels that are outside of the lower and upper limits of quantification (LLOQ and ULOQ) are out of range.

Accuracy is how close a given set of measurements are to their true value (the value which is accepted either as a conventional true value or as an accepted reference value). HCPprofiler standard accuracy is evaluated using internal quality control protein spiked during sample preparation. Accuracy values should range between 0.8 to 1.2 for the quantitative analysis to be validated.

Precision is how close a set of measurements (from multiple samplings of the same sample) are to each other. Precision can be addressed at three levels as described below: repeatability, intermediate precision and reproducibility. In the present report, precision will be expressed as the coefficient of variation (CV) of a series of measurements (ICH Q2).

Repeatability refers to precision under the same operating conditions over a short time interval. In this report, yeast lysate protein quantification was performed in triplicate and resulted in average CVs ≤ 15%.

Intermediate precision corresponds to the evaluation of the impact of random events on the precision of the HCPprofiler standard, such as different HCPprofiler standard batches and different time points. The obtained average CVs were ≤ 15%.

Reproducibility was assessed in this report by using different instruments in different laboratories. The obtained average CVs were ≤ 25%.

Robustness refers to the ability of an analytical procedure to meet the expected performance requirements with deliberate variation of the analytical procedure parameters (Flow rate, injection volume, on-autosampler stability)

Method cross validation is a comparison of data from at least two different analytical methods (reference method and test method) or from the same method used by at least two different laboratories (reference site and test site) within the same study. The goal is to determine whether the obtained results are consistent.

4. Analytical procedure

4.1. Global HCPprofiler workflow

In order to detect HCPs, the sample undergoes a preparation process comprising denaturation, reduction, alkylation and enzymatic digestion (ANAQUANT operating procedure SV-O-016).

Protein separation is not performed as it may lead to an underestimation of HCPs. Indeed, some HCPs are bound to the drug product and would then not be identified if they are eliminated through a protein purification process.

The HCPprofiler standard is then added and samples were analysed in nanoLC-MS/MS (Figure 1).

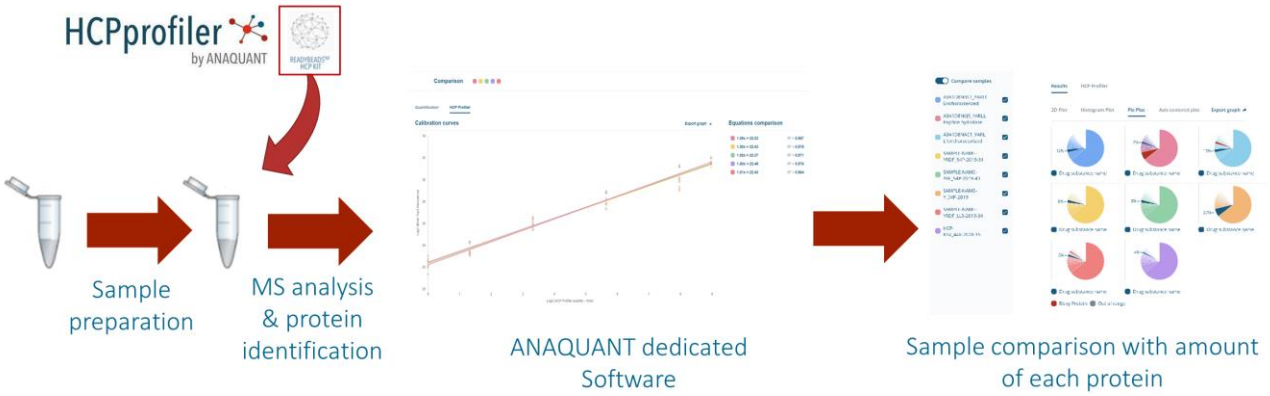

Figure 1: Schematic overview of the HCPprofiler analytical procedure

Calibration curves and protein quantification are obtained using ANAQUANT's dedicated HCPprofiler software.

4.2. Analytical procedure parameters

A chromatographic gradient is performed, using a PepMap™ RSLC C18 analytical column 2µm, 0.075mm ID X 500 mm column on a Vanquish NEO system liquid chromatography (Thermo Fisher Scientific, San Jose, CA)

Solvent A refers to a 0.1% formic acid solution in water, and solvent B refers to a 80% acetonitrile solution containing 0.1% formic acid; peptides are eluted with a 4%-50% acetonitrile gradient over 60 min followed by washing with solvent B at a flow rate of 300nL/min.

MS analysis was performed on an Exploris 240 instrument (Thermo Fisher Scientific, San Jose, CA) in Data-Dependent Acquisition (DDA) mode. In this untargeted analysis process, MS and MS/MS spectra of thousands of peptides are acquired through the whole chromatographic separation range

4.3. Database search strategy

Once the experimental MS and MS/MS spectra were acquired, data process was performed using a software search engine that uses mass spectrometry data to identify proteins from protein databases. The different steps of the shotgun analysis process using a bottom-up strategy is represented in Figure 2.

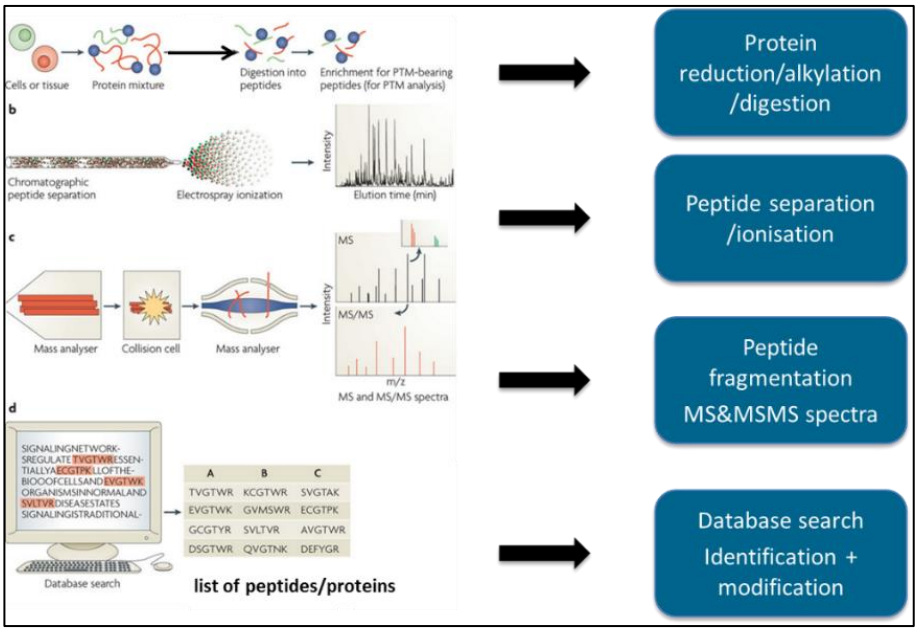

Figure 2: Schematic overview of the shotgun analysis with a bottom-up strategy.

Data processing was performed with Proteome Discoverer (from Thermo Fisher Scientific, San Jose, CA). ANAQUANT implemented a sequence database search strategy to identify peptides based on MS/MS spectra. Protein sequence data bank was derived from UniprotKB (http://www.uniprot.org), using the following features: trypsin cleavage, 2 missed cleavages allowed. Cysteine carbamidomethyl was set as a fixed modification whereas mono oxidation of methionine and pyroglutamic acid from N-ter glutamine were set as variable modifications. A decoy strategy was used and peptide validation was <1% FDR based on PSM score. In order to filter at protein level, only the proteins with at least two identified peptides comprising at least one specific peptide were validated.

4.4. Evaluation of individual protein quantification using HCPprofiler solution

In-house HCPprofiler solution was used to quantify each of the identified protein in the samples (Figure 3) Protein quantification was based on the Top3 strategy. This strategy previously demonstrated in the literature, that for 2 given proteins present in the sample in equal quantities, the respective means of the 3 best signals (3 best peptide responses) were equal and that HCPprofiler solution provides accurate quantitative results (Proteomics, Trauchessec and al., 2021).

HCPprofiler offers a robust and precise technique for analytical testing by combining two innovations:

- ANAQUANTS's READYBEADS technology, in which 54 peptides representing 18 proteins are coated on beads and provide a robust internal calibration curve ranging from 1 to 500 fmol injected (Figure 3). These 54 peptides were selected for their specificity. Their sequences do not correspond to mouse, CHO, human or any of the proteomes existing in public databases.
- ANAQUANT's in-house analysis software tool integrates the calibration curves to provide standardized quantification results.

Protein name	Peptide	Protein injected quantity (fmol) (to correlate to mean top 3 abundance)
ANAQUANT-1	Peptide 1 Peptide 2 Peptide 3	1.0
ANAQUANT-2	Peptide 1 Peptide 2 Peptide 3	1.0
ANAQUANT-3	Peptide 1 Peptide 2 Peptide 3	1.0
ANAQUANT-4	Peptide 1 Peptide 2 Peptide 3	2.5
ANAQUANT-5	Peptide 1 Peptide 2 Peptide 3	2.5
ANAQUANT-6	Peptide 1 Peptide 2 Peptide 3	2.5
ANAQUANT-7	Peptide 1 Peptide 2 Peptide 3	10
ANAQUANT-8	Peptide 1 Peptide 2 Peptide 3	10
ANAQUANT-9	Peptide 1 Peptide 2 Peptide 3	10

Protein name	Peptide	Protein injected quantity (fmol) (to correlate to mean top 3 abundance)
ANAQUANT-10	Peptide 1 Peptide 2 Peptide 3	50
ANAQUANT-11	Peptide 1 Peptide 2 Peptide 3	50
ANAQUANT-12	Peptide 1 Peptide 2 Peptide 3	50
ANAQUANT-13	Peptide 1 Peptide 2 Peptide 3	250
ANAQUANT-14	Peptide 1 Peptide 2 Peptide 3	250
ANAQUANT-15	Peptide 1 Peptide 2 Peptide 3	250
ANAQUANT-16	Peptide 1 Peptide 2 Peptide 3	500
ANAQUANT-17	Peptide 1 Peptide 2 Peptide 3	500
ANAQUANT-18	Peptide 1 Peptide 2 Peptide 3	500

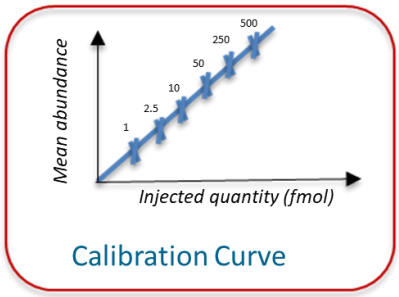

Figure 3: Description of the 54 peptides representative of 18 proteins coated on the READYBEADS technology to obtain a universal calibration curve

The added value of individual protein quantification lies in its ability to assess the quantity of the identified proteins, and therefore to process samples independently.

4.5. Qualification samples

The qualification of the HCPprofiler solution was assessed using BSA (Bovine Serum Albumin), an international standard, provided by the NIST (SRM 927e) as quality control spiked in yeast sample.

The protein from the yeast samples (strain BY4741) were processed using HCPprofiler solution as described above (§4.1). Uniprot database corresponding to yeast proteome (Swiss-Prot reviewed "yeast" database containing 20,395 entries) was used for MS/MS spectra interrogation. All along the qualification report, data from five proteins (at different concentration contained in the low analytical working range) were specifically extract (Table 1).

Table 1: QC and Yeast Proteins from which the results were extracted from data to qualify the HCPprofiler solution

Uniprot accession	Description	MW (Da)	working range approx (fmol)
P02769	ALBU_BOVIN Serum albumin	69200	NA
P07246	ADH3_YEAST Alcohol dehydrogenase 3, mitochondrial	40300	1
P41940	MPG1_YEAST Mannose-1-phosphate guanyltransferase	39500	5
P0CX53	RL12A_YEAST 60S ribosomal protein L12-A	17800	10
P00560	PGK_YEAST Phosphoglycerate kinase	44700	35
P02994	EF1A_YEAST Elongation factor 1-alpha	50000	60

5. Validation Study

5.1. Specificity

Specificity is the ability to assess unequivocally the analyte in various matrices.

Each protein is identified on the basis of the detection of at least two peptides per protein, including at least one specific peptide signature per protein.

5.1.1. Technology specificity

ANAQUANT's READYBEADS technology, in which 54 peptides representing 18 proteins are coated on beads, provide a robust internal calibration curve. These 54 peptides were selected for their specificity. Their sequences do not correspond to mouse, CHO, human or any of the proteomes existing in public databases.

5.1.2. Absence of interference

Specificity of the HCPprofiler method is assessed by testing the extent to which various components of the sample interfere with the quantification process. Calibration curves were performed with the HCPprofiler standard in samples that present heterogenous matrices. Table 2 lists the 14 analyzed samples (yeast, vaccines, mRNA, cells culture samples…).

Table 2: List of samples matrices analysed with the HCPprofiler standard

Sample	Matrix complexity
MATRIX 1	Drug product: purified recombinant protein, simple matrix
MATRIX 2	Drug product: purified recombinant protein, simple matrix
MATRIX 3	Drug product: purified recombinant protein, simple matrix
MATRIX 4	Drug product: purified recombinant protein, simple matrix
MATRIX 5	Mix of several purified proteins + RNase, slightly complex matrix
MATRIX 6	Mix of several purified proteins + RNase, slightly complex matrix
MATRIX 7	Mix of several purified proteins + RNase, slightly complex matrix
MATRIX 8	Drug product: purified mAb, slightly complex matrix
MATRIX 9	Drug product: purified viral vector (AAV), slightly complex matrix
MATRIX 10	Partly purified viral vaccine, in Vero cell, moderately complex matrix
MATRIX 11	Human keratinocyte lysate, highly complex matrix
MATRIX 12	Human keratinocyte lysate, highly complex matrix
MATRIX 13	Human keratinocyte lysate, highly complex matrix
MATRIX 14	HELA cells lysate, highly complex matrix

Sample were processed according to the HCPprofiler standard protocol described in the Analytical procedure part.

To characterize samples' heterogeneity, total protein amount was estimated in each of the 14 matrix samples. Results indicate that the samples' total protein amount ranged from 8 ng to 42 µg (3 logs of magnitude, as shown in Figure 4), while the number of identified proteins ranged from a few dozens to several thousands (data not shown).

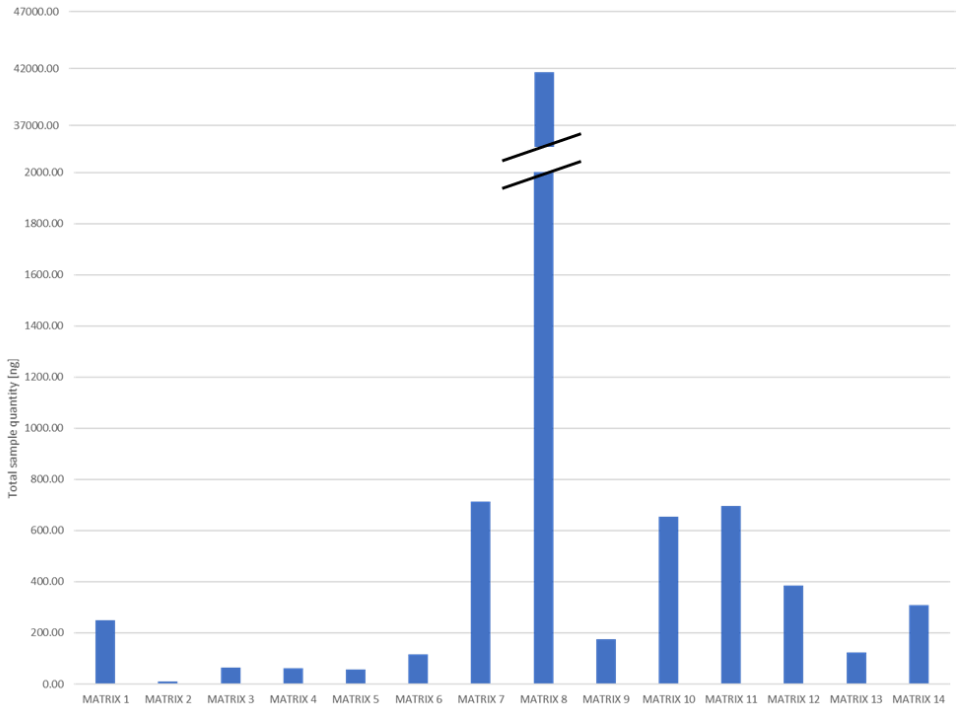

Figure 4: Characterization of matrices heterogeneity: respective total protein amount in each sample

HCPprofiler calibration curves were automatically obtained using the HCPprofiler software. The calibration curves were compared, based on several criteria: the slope, the y-intercept and the determination coefficient (Table 3).

Table 3 Assessment of HCPprofiler standard reproducibility in several matrices. Comparison of the calibration curves obtained in 14 different matrices

	Mean	Standard deviation	CV (%)	Lower limit	Upper limit
Slope	0.9	0.1	**6%**	0.9	1.0
Intercept	20.9	1.2	**6%**	19.7	22.1
R^2	1.0	0.0	**2%**	0.9	1.0

For each of these criteria, deviation between samples remained ≤ 6%, demonstrating that calibration curves are **repeatable in highly diverse and complex matrix samples. Hence, the presence of various components, in various amounts, are shown not to interfere with the HCPprofiler protein quantification method.**

In conclusion, HCPprofiler is a well-suited solution for quantification, ensuring reproducible calibration curves, whatever the matrix complexity. Furthermore, no component of the matrix interferes with the HCPprofiler standard, allowing accurate protein quantification regardless of the initial sample composition.

5.2. Working range

HCPprofiler standards are intended to be used in a global proteomic analytical approach for the analysis of complex matrices with no prior knowledge of the proteins present in the samples. Therefore, evaluation of the individual protein response linearity is not relevant. The working range is defined as the concentration interval for which it has been demonstrated that the HCPprofiler analytical procedure has a suitable level of precision, accuracy and linearity for the quantification of HCPs. Sample preparation (*e.g.*, dilutions) should be taken into account to fall within the working range.

5.2.1. Linearity

The linear relationship between analyte concentration and response, and the corresponding definition of the working range are described below.

Linearity of the calibration curve is evaluated in each single sample injection with acceptance criteria on slope and R^2. The aim of the experiment is to evaluate the linearity of the response of one representative protein with an averaged molecular weight (69KDa) over the working range.

To perform the linearity test, known protein amounts, in this case bovin serum albumin (BSA), have been spiked-in at six different known quantities, covering the working range (Figure 5Figure 5), in a complex matrix (yeast lysate). HCPprofiler standard was added in each sample (n=6).

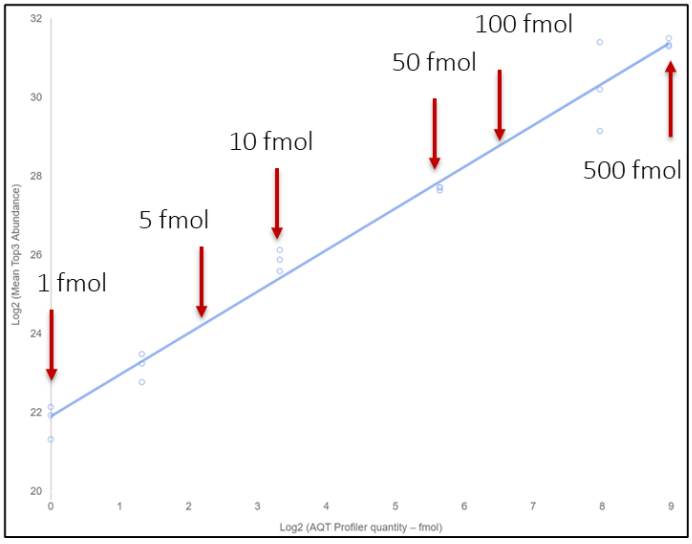

Figure 5: Spiking levels of BSA in the 6 yeast lysate sample preparations

The linearity has been evaluated with a plot of signals as a function of BSA injected quantities (fmol) by calculation of a simple linear regression (Figure 6). The calculated coefficients of determination provide mathematical estimates of the linearity.

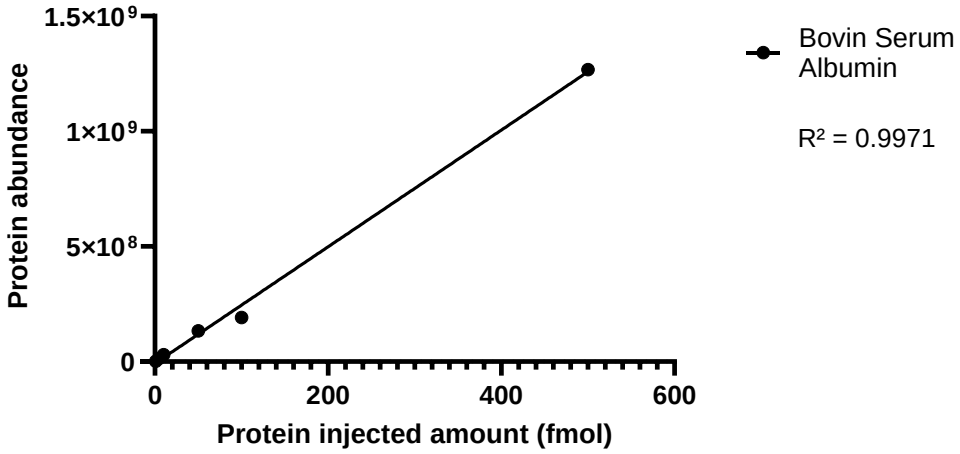

Figure 6: Linearity validation of the HCPprofiler quantification method, based on the quantification of BSA spiked in a complex matrice of yeast lysat

A linear regression of the measured BSA abundances was performed and the calculated coefficient of determination (R^2) was close to 1 (0.9971), thereby validating linearity.

5.2.1. Lower and upper limits of quantification (LLOQ and ULOQ)

LLOQ and ULOQ are defined in each individual test sample by the HCPprofiler internal standard calibration curve obtained with the READYBEADS technology. The coefficient of determination (R^2) should be ≥0.90. Proteins quantities that are below LLOQ and above ULOQ are not quantified as they are out of range. These quantification limits are not protein specific but are linked to the universal quantification method, based on the 54 peptides coated on each HCPprofiler standard and not present in any known sequenced proteome, as described before. ANAQUANT's HCPprofiler solution has been shown to provide a linear quantification range for injected protein quantities from 1 to 500 fmoles.

5.3. Accuracy

The accuracy of HCPprofiler solution was assessed using BSA (Bovine Serum Albumin), an international standard, provided by the NIST, added into yeast lysate to correspond to 50 fmol (R1) and 400 fmol (R2) of injected protein.

Results are presented in Table 4. Samples are yeast lysates supplemented with different production batches of HCPprofiler standard processed over a period from 2019 to 2024 with an experimental replicate number that correspond to a quality control value (R1 and R2). For each sample, the BSA concentration determined is compared to its theoretical concentration by calculating error and accuracy of the quantitative analysis.

Table 4: BSA quantification results with different batches of HCPprofiler standard. Comparison of calculated and theoretical BSA amounts by calculation of percent errors and accuracies

Sample	Calculated Value (fmol)	Theoretical value (fmol)	Percent error (%)	Accuracy (%)
B807-R1	42	50	16	84
B807-R2	329	400	18	82
B836-R1	59	50	-19	119
B836-R2	447	400	-12	112
B951-R1	44	50	11	89
B951-R2	405	400	-1	101
C845-R1	58	50	-16	116
C845-R2	352	400	12	88

Accuracies obtained with these 4 HCPprofiler standard batches ranged from 119% to 82%, which corresponds to a confidence interval of 20%. These results indicate a high level of quantification accuracy for the HCPprofiler standard, robust despite variations in standards' batch, time and BSA amounts.

5.4. Precision

The precision of HCPprofiler standard has been investigated using yeast lysate samples spiked with BSA and HCPprofiler standard before analysis and data treatment with the HCPprofiler software.

5.4.1. Repeatability of HCPprofiler standard

Triplicates were performed, using 3 different HCPprofiler standard's batches (9 samples). After MS acquisition and data processing, calibration curves were automatically obtained with the HCPprofiler software for each sample. Results are presented on Figure 7. A comparison of the equations of the calibration curves allows to assess repeatability of the HCPprofiler standard.

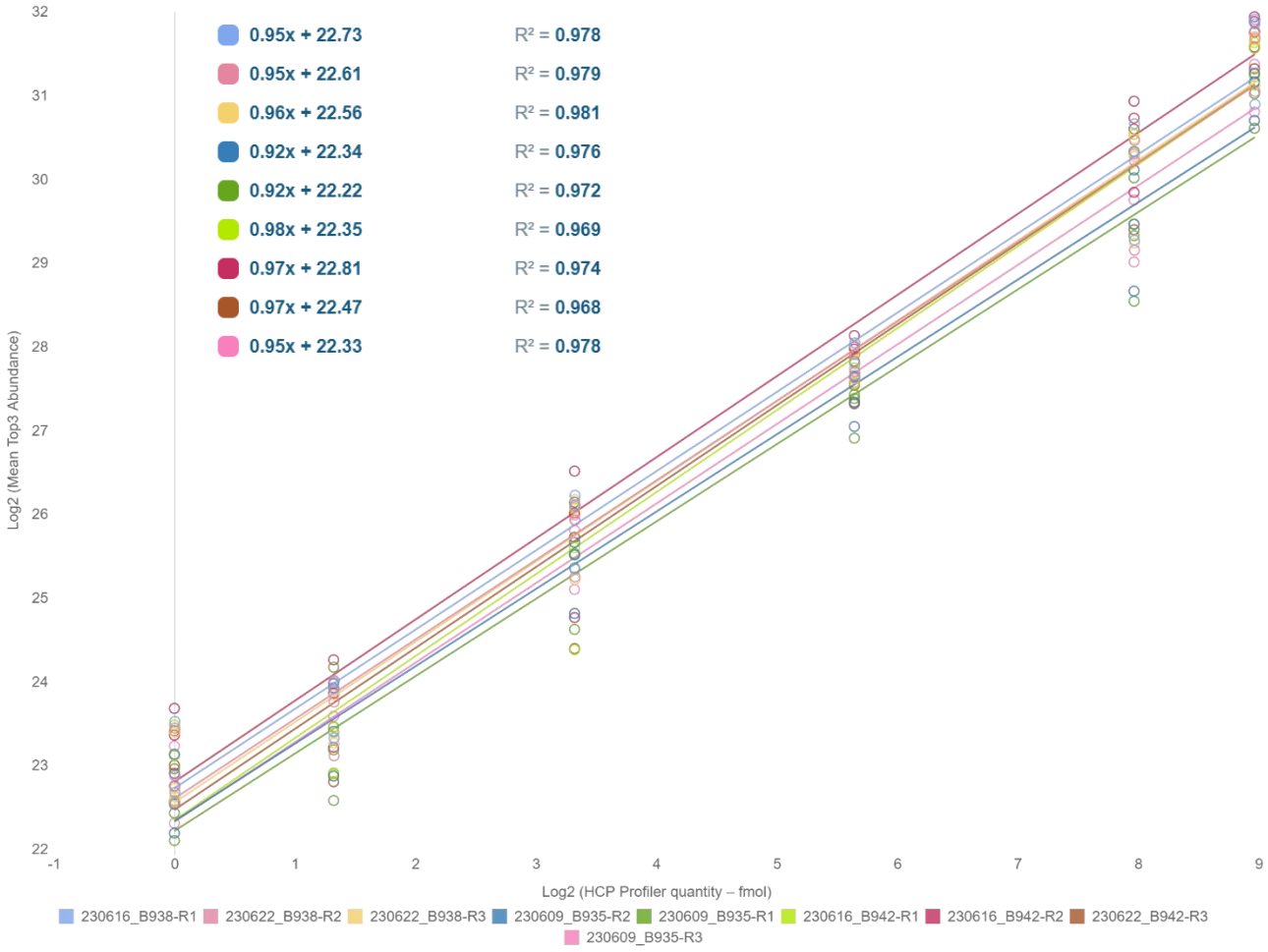

Figure 7: Calibration range obtained for all 9 replicates (3 replicates of 3 batches: B935, B938, B942) and associated linear range equations

The coefficients of variation (CV) were 2% for the slope and 1% for the intercept, while the CV for R^2 was 0.5%, which meets repeatability requirements.

5.4.2. Repeatability of quantification by HCPprofiler standard

Intra-batch repeatability of the HCPprofiler standard was assessed by yeast lysate proteins quantification in triplicates (n=3). Presented results are focus on five yeast proteins between 1 to 100 fmoles injected. Data were processed as previously described and proteins were quantified using the HCPprofiler software.

Table 5: Intra-batch comparison of yeast protein quantification performed in triplicate

Sample	Yeast proteins injected quantity [fmol]					Number of quantified proteins	Total quantity [ng]
	ADH3	MPG1	RL12A	PGK	EF1A		
B942-R1	1.3	7.1	20.6	62.3	92.1	985	95.6
B942-R2	1.0	5.3	16.3	48.2	72.1	931	70.6
B942-R3	1.1	6.3	18.8	56.0	80.1	984	82.2
Mean	1.1	6.2	18.6	55.5	81.4	967	92.8
CV	13%	14%	12%	13%	12%	3%	15%

These results demonstrate the high repeatability of intra-batch HCPprofiler standard proteins quantification. The calculated variation coefficients range from 12 to 14%, notably low around the LLOQ, 1 fmol injected. Total proteins amounts were estimated with a variation of 15% for an average of 967 quantified proteins.

5.4.3. Intermediate precision

The intermediate precision was assessed by evaluating the repeatability of quantification using three HCPprofiler standard batches (B935, B938, and B942), each **manufactured on different dates**. The **analyses of the different batches were performed on separate dates**. Inter-batch evaluation was carried out by quantifying yeast lysate proteins in three replicates (R1, R2, and R3) for each batch (n=9), **with each replicate from the same batch being run on the same day**. Data were processed as previously described, and proteins were quantified using the HCPprofiler software.

Table 6: Inter-batch comparison of yeast protein quantification performed in triplicate for each of the three batches

Sample	Yeast proteins injected quantity [fmol]					Number of quantified proteins	Total quantity [ng]
	ADH3	MPG1	RL12A	PGK	EF1A		
B935-R1	1.0	6.7	18.1	67.1	101.4	749	101.4
B935-R2	1.1	6.0	16.7	58.6	85.0	743	92.3
B935-R3	0.9	6.0	17.1	50.7	82.1	689	78.5
B938-R1	1.1	5.8	17.6	53.8	79.0	965	84.2
B938-R2	1.1	6.1	19.3	57.8	87.2	927	83.4
B938-R3	1.1	6.1	18.2	58.5	78.9	916	90.0
B942-R1	1.3	7.1	20.6	62.3	92.1	985	95.6
B942-R2	1.0	5.3	16.3	48.2	72.1	931	70.6
B942-R3	1.1	6.3	18.8	56.0	80.1	984	82.2
Global Mean	**1.1**	**6.2**	**18.1**	**57.0**	**84.2**	**877**	**86.5**
Global CV (%)	**10%**	**8%**	**7%**	**10%**	**10%**	**13%**	**11%**

The calculated variation coefficients range from 7 to 10%. Total proteins amounts were estimated with a variation of 11% for an average of 877 quantified proteins.

Figure 8 shows the distribution of the coefficients of variation obtained for yeast proteins quantified in these 9 samples using density curves. The width of each curve reflects the approximate frequency of data points in each region (violin plot).

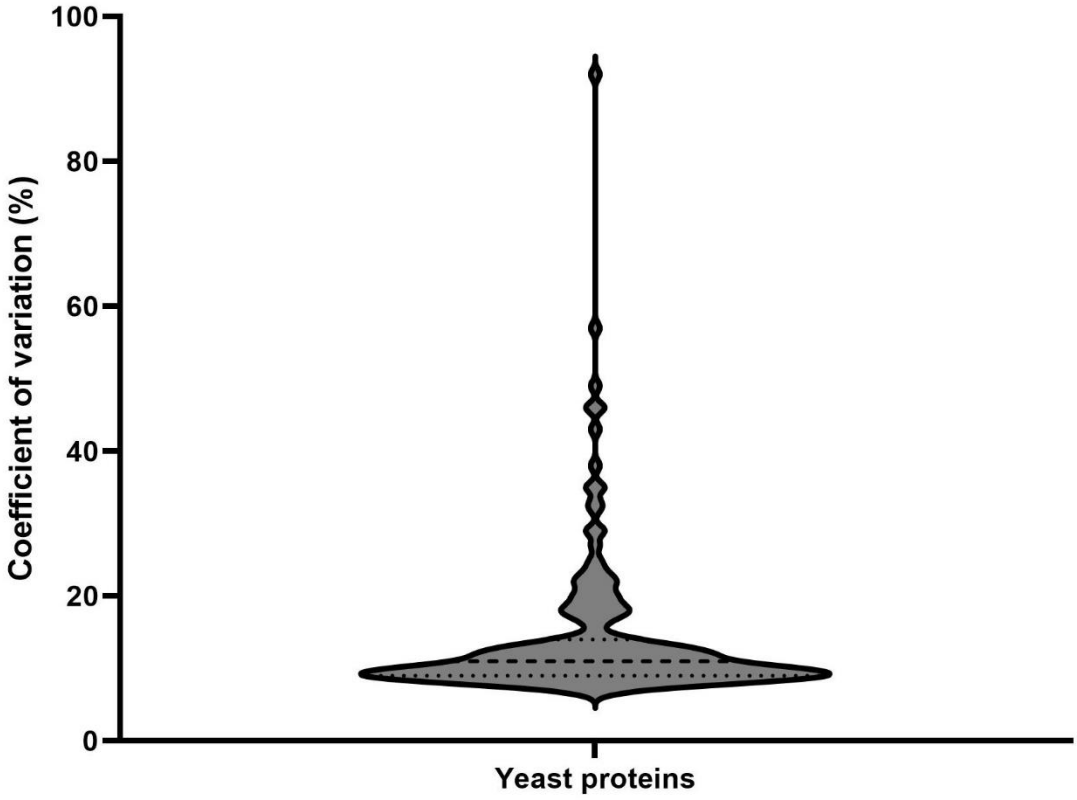

Figure 8: Violin plot of coefficient of variation for yeast proteins quantification in 9 samples.

The results presented above validate the intermediate precision of protein quantification across 3 batches of HCPprofiler standard, with a mean of 14%, a median of 11% with quartiles of 9 and 14%.

5.5. Reproducibility

Reproducibility was assessed through the quantification of yeast lysate proteins in two technical replicates using four HCPprofiler standard batches (n=8) analyzed on 2 instruments (Exploris 240 (Thermo) and Qexactive HF (Thermo)) in two different laboratories (user-induced bias is thus also accounted for).

Data were processed as previously described and proteins were quantified using the HCPprofiler software.

5.5.1. Reproducibility of HCPprofiler standard

Figure 9 shows the comparison of the calibration curves obtained on two different analysis instruments.

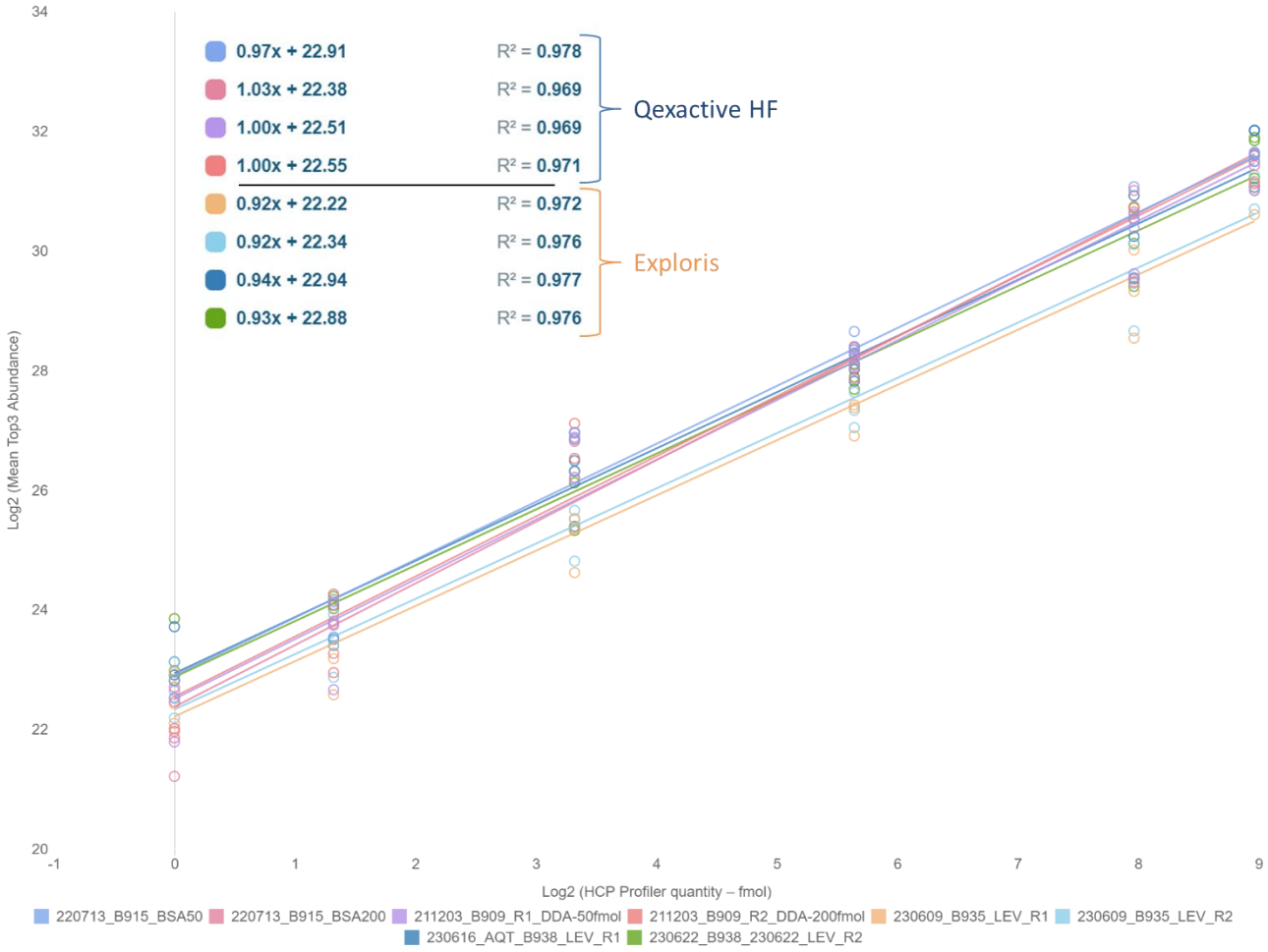

Figure 9: Calibration curves from Exploris 240 and Qexactive HF instruments comparison

Variations between the equations are 4.4% for slope, 1.3% for y-intercept and 0.4% for determination coefficient. Calibration curves are therefore highly reproducible between the 2 instruments.

5.5.2. Reproducibility of protein quantification

Yeast lysate proteins were also quantified during the previous reproducibility test. The quantification results are presented in Table 7 with a focus on five yeast proteins (1 to 100 fmoles injected). The four replicates on each instrument come from at least two independent runs and two independent HCPprofiler beads batches.

Table 7: Yeast lysate protein quantification with HCPprofiler solution using 2 different MS instruments

Instrument analysis	Yeast proteins injected quantity [fmol]					Number of quantified proteins	Total quantity [ng]
	ADH3	MPG1	RL12A	PGK	EF1A		
Qexactive	0.9	7.0	14.2	46.2	64.7	752	92.5
	1.1	8.5	19.4	54.1	66.5	726	113.3
	1.0	5.8	13.8	47.3	61.4	698	79.7
	0.9	6.4	13.8	48.0	63.7	674	83.1
Exploris	1.0	6.7	18.1	66.9	101.1	749	110.8
	1.1	6.0	16.7	58.5	84.8	743	101.4
	0.9	6.2	17.2	54.9	81.8	801	91.1
	0.9	6.2	17.8	60.6	93.9	753	92.5
Mean	**1.0**	**6.6**	**16.4**	**54.6**	**77.2**	**737**	**95.6**
CV	**9%**	**13%**	**13%**	**13%**	**20%**	**5%**	**13%**

The variation coefficient is calculated for each protein (n=8) and ranges from 9 to 20%. Total proteins amounts were estimated with a variation of 13% for an average of 737 quantified proteins.

Figure 10 shows the distribution of the coefficients of variation for yeast proteins quantified in these 8 samples using density curves. The width of each curve reflects the approximate frequency of data points in each region (violin plot).

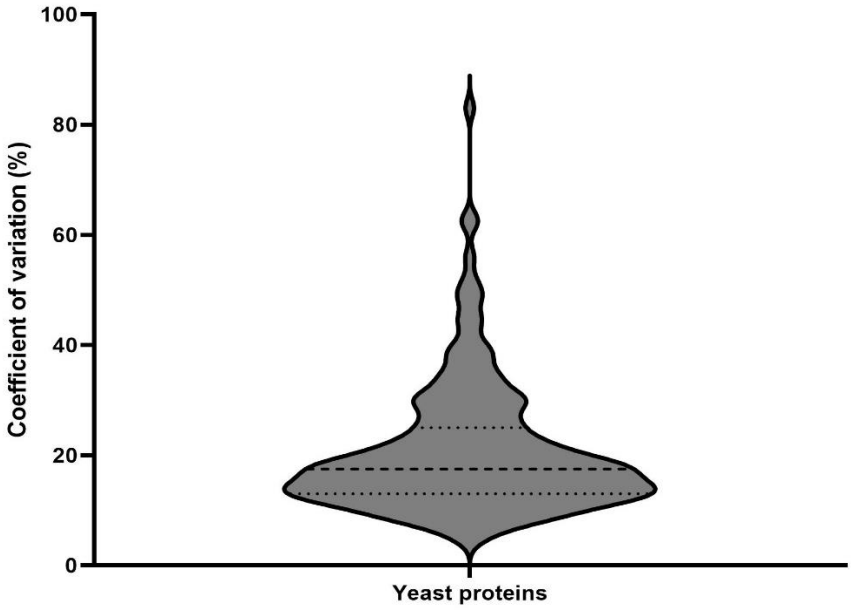

Figure 10: Violin plot of coefficient of variation for yeast proteins quantification in 8 samples.

The results presented above validate the reproducibility of protein quantification using 2 different instruments in 2 different laboratories, with a mean of 21%, a median of 17% with quartiles of 13 and 25%.

5.6. Robustness

Overall, the previous results validate the robustness of HCPprofiler analytical procedure. Indeed, the method proved reliable despite different HCPprofiler standard batches used at different times (§5.3, 5.4), in different matrices (§5.1), on different instruments in different laboratories, by different operators (§5.5) and over a wide working range.

The robustness of the HCPprofiler standard is validated as the method meets the expected performance requirements during normal use.

5.7. Method cross validation

A method cross validation was performed by comparing the protein quantification obtained using our HCPprofiler based calibration curve to those obtained using a reference method. Here, the reference method was a SRM (Selected Reaction Monitoring) targeted LC-MS/MS method using labeled peptides as proteins specific standards for absolute proteins quantification. This reference method is to date the gold standard for protein quantification by mass spectrometry. Here, the targeted method used is focused on 82 peptides representative of 74 critical HCPs that were extracted from an in-house HCP database and other proteins (including the immunogenic PLBL2 lipase, the HTRA1 protease and the BIP proteins) based on a review of the literature.

Two types of CHO samples were used: a purification intermediate sample of a therapeutic protein, and a drug product sample. That way, different datasets sizes, in terms of HCPs abundance, were analyzed.

In the purification intermediate sample, more than 200 CHO host cell proteins were simultaneously identified and quantified using the HCPprofiler solution. Among these proteins, 59 correspond to HCPs that were also screened by the gold standard SRM method (Figure 11).

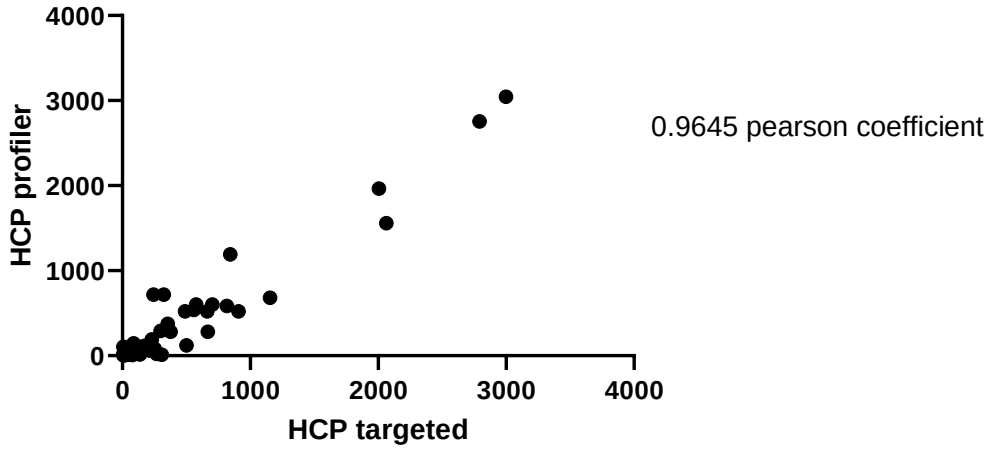

Figure 11 : Correlation between HCPs ppm quantity measured using HCPprofiler method (y-axis) and SRM method (x-axis) in a purification intermediate sample

Furthermore, 6 HCPs identified and quantified by the HCPprofiler method in a purified protein sample from CHO cells were also screened using the gold standard targeted SRM method (Figure 12).

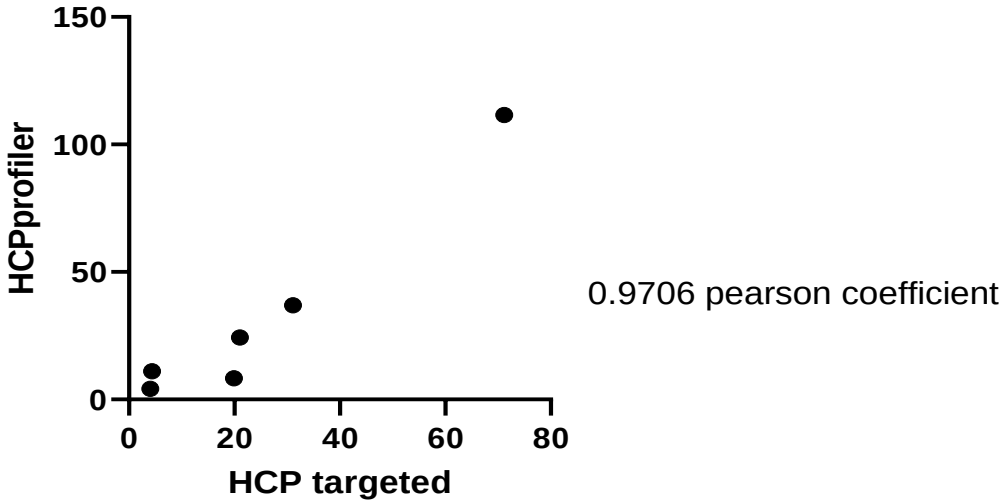

Figure 12 : Correlation between HCPs ppm quantity measured using HCPprofiler method (y-axis) or SRM method (x-axis) in a drug product sample

Figure 11 and 12 show how measured HCP quantities correlate in both methods. Pearson coefficients show a strong positive correlation:

- Figure 11: r = 0.941–0.979; p <0.05
- Figure 12: r =0.749 – 0.997; *p* <0.05

Thus, validating that HCPprofiler quantification compares to the gold standard method for protein quantification in LC-MS/MS, in both types of samples.

Altogether, the results show that the HCPprofiler solution is validated to quantify HCPs all along the DSP to the highest standards.

6. HCPprofiler application

6.1. Quantification of HCPs in a therapeutic peptide DS

An approximately 15 kDa therapeutic peptide drug substance (DS) was processed and analyzed in triplicate using the HCPprofiler quantification method, as detailed in section 4.1.

Depending on the technical triplicate, between 17 and 21 host cell proteins (HCPs) were identified. Fifteen proteins were above the method's quantification limit (1 fmol injected), two were at the quantification limit, and four were below it. The following table presents the overall HCP quantification results in ppm, focusing on five different HCPs, each quantified at different levels to demonstrate method repeatability.

Table 8: HCPprofiler quantification of HCPs in a DS triplicate

Accession	Description	Protein quantity in DS – R1 (ppm)	Protein quantity in DS – R2 (ppm)	Protein quantity in DS – R3 (ppm)	corresponding fmol injected amount range	CV %
Protein 1	ECOLI Large ribosomal …	948	1109	1009	220	8
Protein 2	ECOLI small ribosomal …	199	187	208	40	5
Protein 3	ECOLI small ribosomal …	124	143	116	25	11
Protein 4	ECOLI Ribosome …	32	32	33	10	2
Protein 5	Nitrate/nitrite ….	9	10	10	1	4
	Global HCPs quantity (ppm)	**2741**	**3125**	**2738**		**8**

Global HCP quantification with a variation coefficient below 10%, along with individual HCP quantification results showing variation coefficients between 2% and 21% for proteins quantified above the detection limit, demonstrates the repeatability of the HCPprofiler method. This method is well-suited for analyzing HCPs in therapeutic peptide drug substances.

7. Conclusion

HCPprofiler standard solves the reproducibility problems and user-induced biases while it ensures stability over several months. It appears a valuable tool to ensure accuracy and reproducibility in quantitative studies despite matrices complexity.