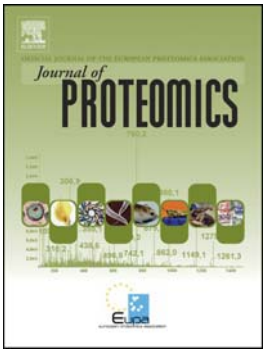

A multicentric study to evaluate the use of relative retention times in targeted proteomics

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A MULTICENTRIC STUDY TO EVALUATE THE USE OF RELATIVE RETENTION TIMES IN TARGETED PROTEOMICS

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[£], equal contribution; FC designed the experiment. FC, NCC and AP prepared the PME10 sample. VV, FC and AP analyzed the data and wrote the manuscript. The rest of the authors performed the proteomics experiments.

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Abstract

Despite the maturity reached by targeted proteomic strategies, reliable and standardized protocols are urgently needed to enhance reproducibility among different laboratories and analytical platforms, facilitating a more widespread use in biomedical research. To achieve this goal, the use of dimensionless relative retention times (iRT), defined on the basis of peptide standard retention times (RT), has lately emerged as a powerful tool. The robustness, reproducibility and utility of this strategy were examined for the first time in a multicentric setting, involving 28 laboratories that included 24 of the Spanish network of proteomics laboratories (ProteoRed-ISCIII). According to the results obtained in this study, dimensionless retention time values (iRTs) demonstrated to be a useful tool for transferring and sharing peptide retention times across different chromatographic set-ups both intra- and inter-laboratories. iRT values also showed very low variability over long time periods. Furthermore, parallel quantitative analyses showed a high reproducibility despite the variety of experimental strategies used, either MRM (multiple reaction monitoring) or pseudoMRM, and the diversity of analytical platforms employed.

KEYWORDS:

Proteomics; liquid chromatography; targeted proteomics; reproducibility; standardization; inter-laboratory validation; multiple reaction monitoring.

Introduction

Quantitative analysis of specific target proteins present in complex proteomes has turned out to be one of the most attractive tools available in modern proteomics[1-3]. Collectively known as targeted proteomics, these techniques track the presence/absence of protein-specific peptides (proteotypic peptides[4, 5]) in the samples studied and, under specific experimental conditions, are amenable to produce absolute quantification data[6-12].

Despite having low resolution and medium mass accuracy, triple quadrupole mass spectrometers are the instrument of choice to perform targeted analysis[13]. These instruments offer excellent sensitivity, high selectivity and dynamic range as well as high speed to monitor in a single liquid chromatography coupled to mass spectrometry (LC-MS) analysis tens or hundreds of proteotypic peptides corresponding to many different proteins. In this targeted approach (Multiple Reaction Monitoring, or MRM), often described as tandem mass spectrometry *in space*, peptides are selected in the first quadrupole or Q1 according to the m/z (mass to charge) ratio of the precursor ion,

fragmented by collision-induced dissociation (CID) in the collision cell, Q2 or second quadrupole and finally, predefined peptide-specific fragments are selected in the second mass filter (Q3 or third quadrupole), followed by the measurement of the intensity of the transmitted ions or transitions. Other mass spectrometers perform targeted experiments as well, but peptide selection and fragmentation is usually accomplished with ions trapped in the same place, with multiple separation steps taking place over time (tandem mass spectrometry *in time*)[14, 15]. Methods developed using the last type of mass spectrometers are often described as pseudoMRM methods.

However, monitoring too many transitions in a single LC-MS targeted experiment could increase the total cycle time up to a point where there are no sufficient data points to reconstruct with adequate resolution the chromatographic elution profiles of the targeted peptides, affecting to the global quality of the data. In these cases, targeted proteomics strategies may benefit from a great enhancement in efficiency if targeted peptides are monitored solely around the expected retention time (scheduled approaches)[16, 17]. Concatenating multiple retention time windows, each corresponding to different sets of target scheduled peptides, the otherwise limited number of peptides that can reasonably be measured in one LC-MS run can be greatly increased, without compromising the accuracy of quantitation or the sensitivity of the detection. Additionally, targeted signal acquisition in the expected time window provides additional experimental evidence supporting that the detected signal is actually generated by the selected peptide and fragments. Therefore, scheduled approaches favour a significant increase of the number of individual proteotypic peptides that can be reasonably analysed per LC-MS run, extending the number of transitions measured per proteins and/or the number of different proteins studied[18, 19].

Selection of proteotypic peptides is usually based on previous data either from repositories (PeptideAtlas, SRM Atlas)[20, 21] or obtained in a discovery, non-targeted analysis, typically using LC-MS *shotgun* experimental approaches that, in addition to peptide sequences, also provide information about the chromatographic retention times of the peptides of interest. At first glance, translation of peptide retention times from the discovery phase to a targeted proteomics protocol should be straightforward. In practice, empirically measured peptide retention times (RT) are only valid for each specific experimental set up resulting from the influence of many different parameters, such as solvents, gradient, column length, dead volumes in the LC system, type of stationary phase used, column aging and many others. To solve this issue, Escher and

collaborators proposed a normalized, dimensionless retention time value (named iRT score) for peptides as a useful tool for transferring and sharing peptide retention times across chromatographic set-ups both intra- and inter-laboratories[22]. The iRT score for a given peptide is calculated and normalized relative to a set of synthetic peptides and its value should remain stable across a wide range of LC configurations.

The use of retention time standards allows the normalization of peptide RT to a "universal" scale, that can be useful to facilitate sharing of targeted analysis methods across different experimental setups and laboratories, pinpoint wrong signal assignments to particular peptides, or design scheduled targeted methods[23]. We think that these are crucial issues in the context of projects that require the coordination of multiple participants, such as the Human Proteome Project[24, 25].

To assess the robustness and reproducibility of iRT values in the context of a multi-centric study, the Spanish network of proteomics laboratories ProteoRed-ISCIII [26](www.prb2.org/es/proteored) has coordinated a new multicentric study, PME10, in which a total number of 28 (n=24 from ProteoRed-ISCIII) different laboratories have taken part. The global objective of PME10 was to evaluate the suitability of retention time standards to improve targeted proteomic analyses. To achieve this goal, participants were requested to estimate relative retention times (iRT) from a provided set of retention time standards and their empirically measured retention times. Then an averaged set of relative RT values is obtained and used as a global iRT scale to assess its capability of predicting empirical RTs in hypothetical scheduled assays. For that purpose, a single centralized pre-digested sample was analysed under different chromatograpic conditions.

Finally, in the context of the Spanish Human Proteome Project[25] (spHPP-Chromosome 16) we evaluated the accuracy and reproducibility of a quantitative analysis of 16 peptides belonging to a set of chromosome 16 – encoded proteins previously detected in MCF7 cell line, and for which validated MRM methods had been already set up in the context of the HPP project.

PME10 adds up to previous multicentric experiments illustrating ProteoRed´s commitment with the improvement of the accuracy, reproducibility and robustness of different proteomic methodologies[27-29]. Previous multicentric experiments have evaluated the sensitivity, efficacy and robustness of targeted proteomic approaches [30-32] but this is the first inter-laboratory study, to our knowledge, that has combined this methodology and the evaluation of normalized chromatographic retention times.

MATERIALS AND METHODS

A more detailed description of the materials, methods and instrumentation used is provided as Supplementary information (supplementary Table 3).

1) Materials

Each participant received a dried study sample containing a mixture composed of: a) 20 micrograms of a tryptic digest of MCF7 human breast cancer cell line total proteome; b) the MSRT1 calibration mix peptide standard mixture, kindly supplied by SIGMA-ALDRICH (St. Louis, MO), composed by 14 isotopically labeled synthetic peptides (average of 4000 fmol/peptide, Table 1) and c) 16 isotopically labeled heavy peptide standards (400 fmol/peptide, Table 1) corresponding to 16 human proteins for which SRM methods had been set up and validated by the Spanish chr16 spHPP consortium (data not shown). Peptides were labeled either with $^{13}C_6$ $^{15}N_1$ Leu (+7Da), $^{13}C_6$ $^{15}N_2$ Lys (+8Da) or $^{13}C_6$ $^{15}N_4$ Arg (+10Da). MSRT1 calibration peptide mixture is a LC-MS platform standard intended to test chromatographic parameters such as LC resolution, peptide elution profiles, and retention time prediction. It has been designed to span a wide range of chromatographic elution times. The amounts of individual peptides vary to display relatively similar electrospray responses. Normalized retention time (iRTs) values relative to the previously described Biognosys standards[22] were also provided (Supplementary Table 1).

In addition, every participant also received a second dried aliquot of pure MSRT1 peptide standards (average 3000 fmol/peptide), and of the labeled peptide standards mixture (800 fmol/peptide, Supplementary table 2). Samples were sent ready for LC-MS analysis.

2) Sample Preparation Protocol:

Participants were suggested to dissolve the dried peptide mixtures in a small volume of 20-30% Acetonitrile, 0.1% formic acid in water, and to dilute this stock solution with 0.1% formic acid in water to reduce the acetonitrile concentration to 2-5% at the moment of the analysis. Participants were suggested to inject 0.2-1 μg of MCF7 digest, 40-200 fmol MSRT1 peptides and 4-20 fmol of heavy peptide standards per run.

3) Analysis conditions:

a. Chromatography:

PME10 participants were recommended to analyse the sample using three different liquid chromatography (LC) gradients of increasing length. More precisely, a linear gradient of 0-40% acetonitrile in water in 60, 90 and 120 minutes was recommended, while relatively permissive conditions were allowed in other LC parameters. Specific analysis conditions are detailed in Supplementary table 3, but a brief outline of the conditions is as follows: C18-based reversed phase chromatographic columns internal diameters ranged from 50 μm to 2.1 mm; flow rates were 220 nL/min to 300 μL/min; sample load: 0.4-2 μg. In general, most of the participants employed nanoLC conditions (flow rates 220-500 nL/min) while only two laboratories used microLC conditions (flow rates 700-1000 nL/min). Finally, a unique participant employed standard analytical LC conditions (300 μL/min)

b. Mass spectrometry:

According to the type of instrumentation (Supplementary table 3), 13 triple quadrupole mass spectrometers were used, including 9 ABSciex 5500 Qtrap instruments. The rest of instruments used included 10 different versions of the Thermo Orbitrap family, 2 ABSciex 5600 TripleTOF QTOF mass spectrometers, two three dimensional ion-traps (Bruker AmaZon Speed) and finally, one Thermo QExactive instrument. The list of precursors and fragment ion m/z values to be monitored as well as the recommended dwell times, declustering potentials and collision energies were made available to the participants either as tables (Supplementary tables 4a and 4b) or skyline files (not shown). For MSRT1 peptides the transitions listed are the ones suggested in the Sigma-Aldrich webpage. The consensus SRM parameters for 16 heavy (standard) - light (endogenous) peptide pairs corresponding to 16 proteins encoded in the chromosome 16, plus nine additional non isotopically labeled peptides, was obtained from the Spanish spHPP-Chromosome project, totalling 164 transitions. Therefore, the list includes the transitions corresponding to at least one labeled standard peptide for each of the 16 Chromosome 16-encoded proteins.

Some participants adapted the acquisition parameters to the specific instrument and experimental setups, either for SRM or other targeted approaches (pseudoSRM). Participants using Thermo Orbitrap (OT) instruments were suggested to apply parallel reaction monitoring (PRM) acquisition methods similar to those described in PME8 study[29]. In some cases, for analysis in OT or other instruments, it was necessary to shorten the list of targeted precursor ions. In such cases, the analysis was limited to the 14 MSRT1 standard peptides, plus the 16 chromosome 16-encoded protein peptide

heavy-light pairs. Due to the limited amount of sample available, optimization of acquisition parameters was beyond the aim of the study.

4) Study design:

Samples were prepared and distributed to the 28 participants (24 from Spain, and one from Russia, Sweden, Switzerland and United States, respectively). The participants were invited to analyse at least one run of the sample containing the pure MSRT1 standard and three independent runs of the PME10 sample for each of the three suggested gradient lengths, summing a total of 12 LC-MS runs. Extracted ion chromatograms (XIC) corresponding to target peptides were first used to obtain their specific retention times and next to calculate the corresponding iRT values. Furthermore, for quantification purposes, we asked the participants to calculate the light/heavy (L/H) ratios for each of the signals detected for the 16 peptides included in the labeled standard mixture.

5) iRT Calculations:

Participants were suggested to submit the data through a standardized spreadsheet file for a centralized analysis. In order to normalize all observed retention times to a common iRT scale, participants were requested to plot the reference MSRT1 iRT values (Supplementary table 1), against their observed experimental retention times for each of the recommended LC-MS runs (60, 90 and 120 min). Reference MSRT1 iRT values were calculated according to the iRT scale defined by Escher et al[22]. Calculation of a linear regression fit in each plot (figure 1) results in a linear equation for each run, in the form:

$$iRT = m \cdot RT_{(obs)} + b$$

The equations were used, together with the experimental retention times, to calculate the iRTs of all the observed peptides in two variants: an *external* estimation, where the conversion equation was obtained from plotting the MSRT1 iRT values versus the measured RTs of the pure MSRT1 peptides; and an *internal* estimation, where the equation was obtained from plotting the MSRT1 iRT values versus the RTs of the MSRT1 peptides spiked in the cell extract (see an example in fig.1).

The iRT values from all participants and gradients were collected, filtered for outliers (values outside the range defined as 1.5 times the inter-quartile range extending from quartiles one and three respectively), averaged for every peptide and used to

reconstruct a global (in the scope of the multi-centric experiment) iRT scale on which the predictive capability of measured RTs can be tested.

4- Quantification of MCF7 proteins

For each isotopically labelled heavy peptide corresponding to the Chromosome 16 – encoded proteins, participants were requested to calculate the light to heavy ratio (L/H), for each of the three technical replicas of each LC gradient. Averaged L/H ratios were calculated considering all values, as well as standard deviations. Finally, absolute amounts, expressed as fmol of protein per μg of MCF7 total proteome, were calculated. As in previous ProteoRed Multicentric experiments (PME), we suggested the participants to submit their results for a centralized analysis. Templates as well as specific details were made available through the ProteoRed webpage (http://www.legacy.proteored.org/).

RESULTS AND DISCUSSION

Sharing chromatographic methods for proteomic analyses among laboratories is not straightforward, given the widely diverse array of instruments and experimental setups (e.g., from shotgun to targeted approaches), as many variables must be precisely tuned and integrated. In this regard, methods allowing a reliable prediction of peptide RT might be of great benefit. The use of peptide RT as the reference parameter for method sharing has demonstrated relevant restrictions, even when variables, such as gradient, column dimensions and type of stationary phase, or LC-system dead volumes were relatively controlled, as was also observed in this study..Figure 2 shows the RT values for two different sets of synthetic peptides (fig. 2a, 2b) obtained in different laboratories with a 90 min gradient and a relatively limited range of instrumental setups. Similar, results were obtained upon use of 60 and 120 minutes gradients (Supplementary figures 2 a-d). Data variability or distribution is illustrated by the standard deviation (SD) and the interquartile range (IQR) that in 90 min gradients were in the range of 8.5 and 10.5, respectively, in most cases. With respect to IQR, which depicts the range required to cluster the central 50% of the RT values, most of the values grouped around 10 minutes. Analysis of the results from 60 and 120 min gradients, demonstrated small, albeit consistent changes in the dispersion values (supplementary figures 2a-d). Thus, SD and IQR values were in the range of 7 and 7.5 for 60 min gradients and, 10 and 14 for 120 min gradients, respectively. A different landscape results upon RT normalization (iRTs). Each participant laboratory reported the estimated sets of iRT peptide values in each of the LC-MS runs in both, external and internal settings (see Materials and methods). Figures 3 a-d summarize the results

obtained, which in this case have been plotted considering all the three different gradients from each laboratory, either using external or internal conversion equations. Similar observations were made when gradient-dependent results were represented (data not shown). Overall, both SD and IQR values were in general smaller than those from RT and enhanced the reproducibility of the measured iRTs across different gradients and laboratories. On the other hand, no particular instrument dependence in the calculation of iRTs was observed. This statement was particularly evident when considering the MSRT1 peptide set, perhaps reflecting that these peptides have been selected due to their suitability as standards for LC-MS based experimental approaches. Moreover some MCF7 peptides were poorly or incorrectly detected, compromising the overall quality of the study (e.g., peptide GHYTEGAELVDSVLDVVR).

iRT values remain stable over time

Once established the stability of IRTs among laboratories and experimental conditions (figures 3a-d), we wondered about the potential deviations that may result when the analyses are repeated over time. To specifically address this question, a small representative subset of six laboratories repeated the analysis after one year, following essentially the same working scheme as initially designed. Our results clearly demonstrated that data dispersion of the new set of normalized iRTs (figures 4 a-d), is significantly lower than that corresponding to the non-normalized RTs (supplementary figures 4 a-f), as suggested by both, SD and IQR. We also found a significant reduction in data dispersion, probably reflecting the restricted number of participant laboratories (n=6 in this 2nd phase *versus* n=28 in the 1st phase). Nonetheless, median values calculated from iRTs are very similar to those obtained in the first inter-laboratory experimental phase, demonstrating that iRT values remain stable over time.

Relative retention time scales and iRT score for predicting and transferring RT

The two compiled sets, external and internal iRTs for each MCF7 peptide across gradients, replicates and laboratories were first filtered for outliers, as defined in the materials and method section. Next, the outlier-filtered sets were averaged to obtain a final, global iRT score for each of the MCF7 peptides. This scale was then used to illustrate the usefulness and robustness of these relative retention times for empirical RT prediction and transfer across LC set ups both spatially, in different laboratories, and temporally, over a long periods of time.

For each of the three gradients, the iRT sets (internal and external) were used to predict RTs, by interpolating (in the plot that correlates MSRT1 iRT values with MSRT1

empirical RTs) in the opposite direction to obtain a predicted RT from a fixed iRT scale for all the monitored MCF7 peptides. Then the predictive capability of this approach was accepted when the absolute value of the difference between the predicted and the empirical RTs that had been initially measured for every peptide lies within discrete time windows of up to 1 to 5 minutes. This simulation of a scheduled assay scenario, where a certain peptide signal could be monitored at a predicted RT value with a restricted time window, can be represented quantitatively (in terms of the percentage of laboratories that would successfully detect the peptide signal for all LC-MS runs of a certain gradient) using a heatmap format as shown for 90 min gradients in Figure 5 a-b. Similar predictive results were obtained for 60- and 120 gradients (Supplementary figures 5 a-d). According to our results, the majority of the participant laboratories could successfully detect the monitored peptides signals within an acceptable time window range for scheduled approaches. Similar results were obtained during the second set of analyses performed one year later, and using the same original iRT scale (data not shown).

Overall, these results provide a solid foundation in support of the use of relative retention time scales as a valuable tool for sharing and transferring RTs, not only across different laboratories but also over long time periods, addressing thus a key issue that otherwise hinders the spread and reuse of implemented scheduled targeted methods.

Targeted Quantitative analysis of MCF7 peptides.

Finally, in addition to the main interest of PME10 multicentric study, that was focused on the utility of iRTs to standardize and universalize targeted measurements, 16 peptides from chr16 proteins were simultaneously quantified in a MCF7 cell extract spiked with known quantities of the corresponding heavy versions as internal references. The results obtained are shown as fmol/μg in figure 6, including (figure 6a) or excluding (figure 6b) the quantitative results corresponding to the peptide GVVDSEDIPLNLSR. The results show remarkably low inter-laboratory %CV for most of the peptides, with the reasonably expected exception of the peptides at the lowest and highest concentration. Since all the isotopically labeled standard peptides were spiked in at similar concentrations, the larger variability observed at the extreme concentrations may be partly explained by the larger error expected in the determination of the ratios at values more divergent from 1:1. Due to the high quality of these quantitative results, this multicentric study did not evaluate the expected improving effect that the use of scheduled MRM methods, developed taking into

account the iRT values, would have on the quantitative data. In fact, we estimated that the eventual improvement would be minimal and poorly indicative of the puissance of the scheduled approaches. A possible explanation for these highly reproducible results is that the centralized preparation of the samples used in this study could have had a positive impact on the quality of the quantitative results. A more usual situation, in which every participant laboratory had prepared independently the sample, would unquestionably increase the dispersion of the quantitative results. An interesting example can be observed when quantitative results obtained in the first set of data (27 participant laboratories, fig. 6a/b) is compared with the second set of quantitative results obtained one year later (supplementary figures 6 a/b). In both cases, the samples were prepared in a centralized way but in different periods. According to this situation, quantitative results were highly similar but significant differences are evident, both in terms of absolute quantitative values as well as in terms of data dispersion. On the other hand, no significant differences, neither in accuracy nor in variability, were observed when comparing the MRM measurements on triple quadrupole instruments or other pseudo-MRM targeted approaches. In summary, the high reproducibility of the quantitative data demonstrated the reliability of the MRM approaches for the quantitative analysis of proteotypic peptides in complex samples.

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ABBREVIATIONS: iRT, dimensionless relative retention time; RT, retention time; LC-MS, liquid chromatography coupled to mass spectrometry; MRM, multiple reaction monitoring; CID, collision-induced dissociation; SRM, single reaction monitoring; HPP, Human Proteome Project.

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PME10 STUDY SAMPLE COMPOSITION		
MCF7 tryptic digest	**MSRT1 calibration mix (average 4000 fmol/peptide)[a]**	**MCF7 standard peptides (K8,R10) (average 400 fmol/peptide)[b]**
1mg/mL (V=20 mL)	RGDSPASSP[K] GLV[K] LGGNETQV[R] AEFAEVS[K] SGFSSVSVS[R] ADEGISF[R] DISLSDY[K] LVNEVTEFA[K] DQGGELLSL[R] GLFIIDD[K] LGEYGFQNA[L] YWGVASFLQ[K] TDELFQIEGLKEELAYL[R] AVQQPDGLAVLGIFL[K]	VSLEDLYNG[K] TAGTIC[CAM]LETF[K] EGHLSPDIVAEQ[K] SLEEIYLFSLPI[K] GEATVSFDDPPSA[K] IQDYDVSLD[K] FEELTNLI[R] NALANPLYC[CAM]PDY[R] AAPEASGTPSSDAVS[R] AEAGDNLGALV[R] GVVDSEDIPLNLS[R] YLTVATVF[R] TVLDPVTGDLSDT[R] SPEVLSGGEDGAV[R] SNLVDNTNQVEVLQ[R] TAAALAPASLTSA[R]

Table 1. PME10 Study sample description. [a] Each MSRT1 calibration mix synthetic peptide (n=14) has been isotopically labeled either with ($^{13}C_6$, ^{15}N) leucine (+7 Da), ($^{13}C_6$, $^{15}N_2$) lysine (+8 Da) or ($^{13}C_6$, $^{15}N_4$) arginine (+ 10 Da). Amino acid in brackets denotes site of label incorporation. [b] Stable isotope labeled proteotypic peptides corresponding to 16 chromosome 16 encoded proteins. Peptides are labeled with ($^{13}C_6$, $^{15}N_2$) lysine (+8 Da) or ($^{13}C_6$, $^{15}N_4$) arginine (+ 10 Da). Amino acid in brackets denotes site of label incorporation; [CAM] corresponds to carbamidomethylated cysteine.

Figure 1. Representative example obtained after plotting MSRT1 Peptide Standard experimental retention time values (y-axis, using a 90 min gradient) against MSRT1 reference iRT values (x-axis).

Figure 2. Measured retention times (RT) for MSRT1 (a) and MCF7 (b) peptide sets employing a consensus 90-min gradient. For each peptide the following information is included: Standard deviation (SD); Interquartile range (IQR), defined as the range necessary to cluster the central 50% of RT values; the number of different laboratories detecting each specific peptide (#Labs); values within the Q1-1.5IQR, Q3+1.5IQR range are represented as whiskers; boxes represent IQR; numbers inside boxes indicate the median of the RT values.

Figure 3. Normalized retention times (iRT) for MSRT1 (a and b) and MCF7 (c and d) peptides. Data from the three different gradients (60,90 and 120 min) were considered, either using an external (a and c) or an internal estimation (b and d). For each peptide the following information is included: Standard deviation (SD); Interquartile range (IQR), defined as the range necessary to cluster the central 50% of iRT values; values within the Q1-1.5IQR, Q3+1.5IQR range are represented as whiskers; boxes represent IQR; numbers inside boxes indicate the median of the iRT values. Dashed vertical lines (fig. 3a, 3b) indicate iRT values provided by SIGMA.

Figure 4. iRT values remain stable over time. Normalized retention times (iRT) from a second phase of the multicentric analysis (participant laboratories n=6) are shown for MSRT1 (a and b) and MCF7 (c and d) peptide sets. Data from all different gradients (60, 90 and 120 min) were considered, either using an external (a and c) or an internal estimation (b and d). Statistical analysis parameters are depicted as in figure 3 (Standard deviation (SD), Interquartile range (IQR), whiskers, boxes and numbers inside boxes). Dashed vertical lines (fig. 4a, 4b) indicate iRT values provided by SIGMA.

Figure 5. Evaluation of the capacity of iRT scores to predict and to facilitate the transference of peptide RT. The efficacy of RT prediction for a hypothetical scheduled approach is shown as heat maps. This simulated scenario is recreated by subtracting from the predicted RT the experimental RT originally measured for each peptide and gradient. The intensity of gray indicates the percentage of laboratories that would individually successfully detect the peptides in time windows from 1 to 5 minutes. Results are shown for 90 min gradients in Figure 5 either using external (fig. 5a) or

internal (Fig 5b) calibration. Similar results obtained for 60- (supplementary Fig. 5a, 5b) and 120-gradients (supplementary Fig. 5c, 5d) are also shown.

Figure 6. Targeted Quantitative analysis of MCF7 peptides. MCF7 peptides (n=16) quantification was estimated using either a MRM or a pseudo-MRM experimental approach (number of participant laboratories n=28). Data are represented with (figure 6a) or without (figure 6b) data from GVVDSEDIPLNLSR peptide. The following information is shown: standard deviation (SD); boxes represent Interquartile range (IQR), defined as the range necessary to cluster the central 50% of iRT values; values within the Q1-1.5IQR, Q3+1.5IQR range are represented as whiskers; numbers inside boxes indicate the median of the iRT values.

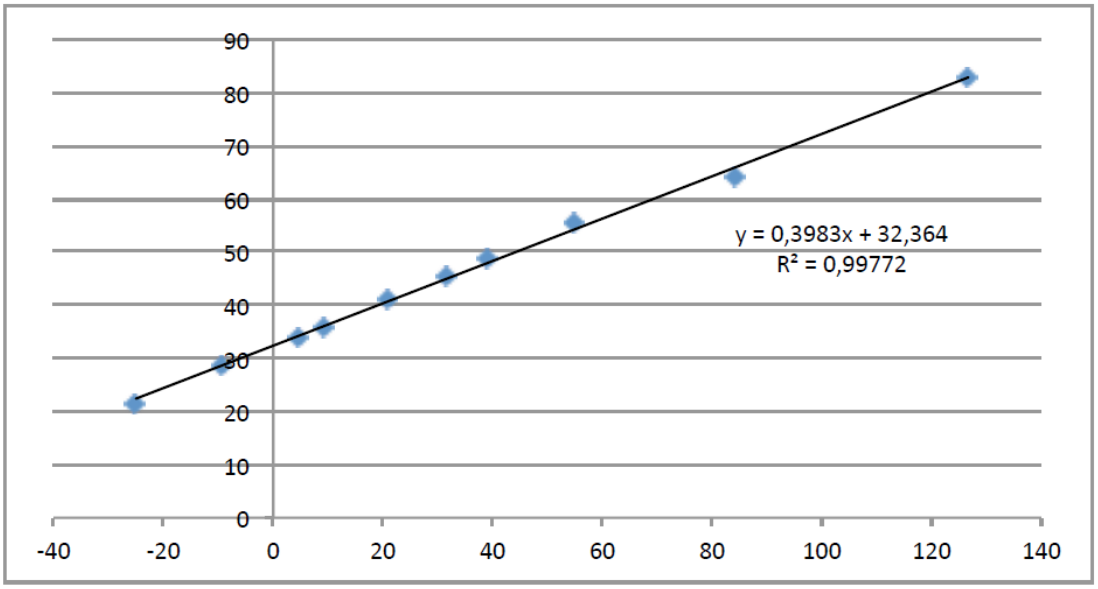


Figure 1

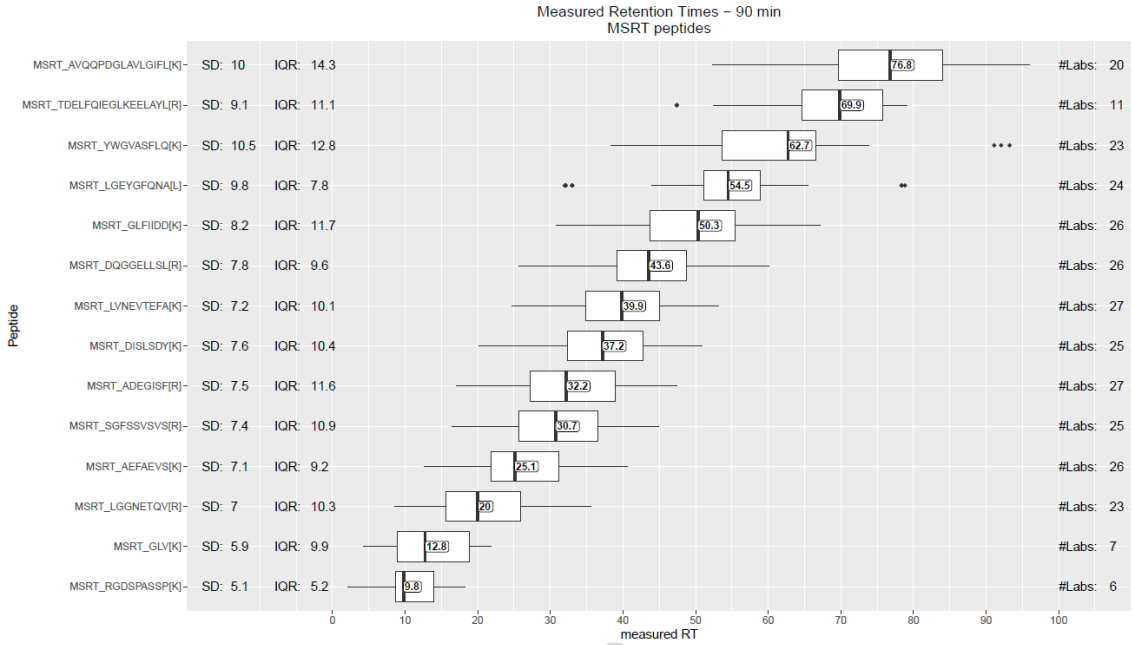


Figure 2a

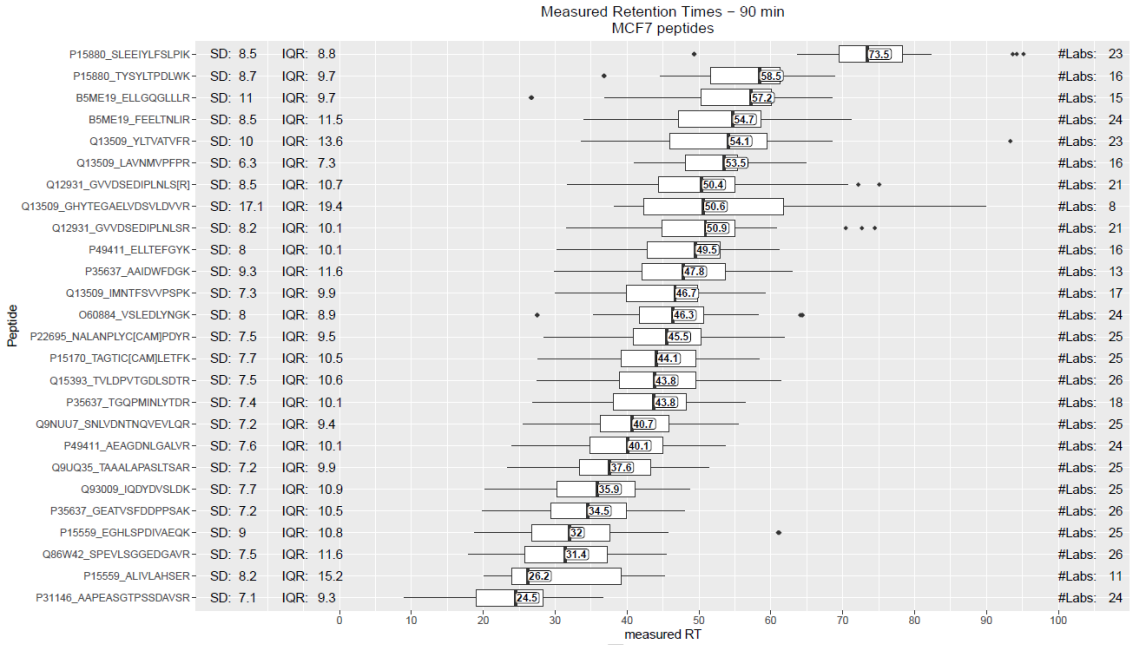


Figure 2b

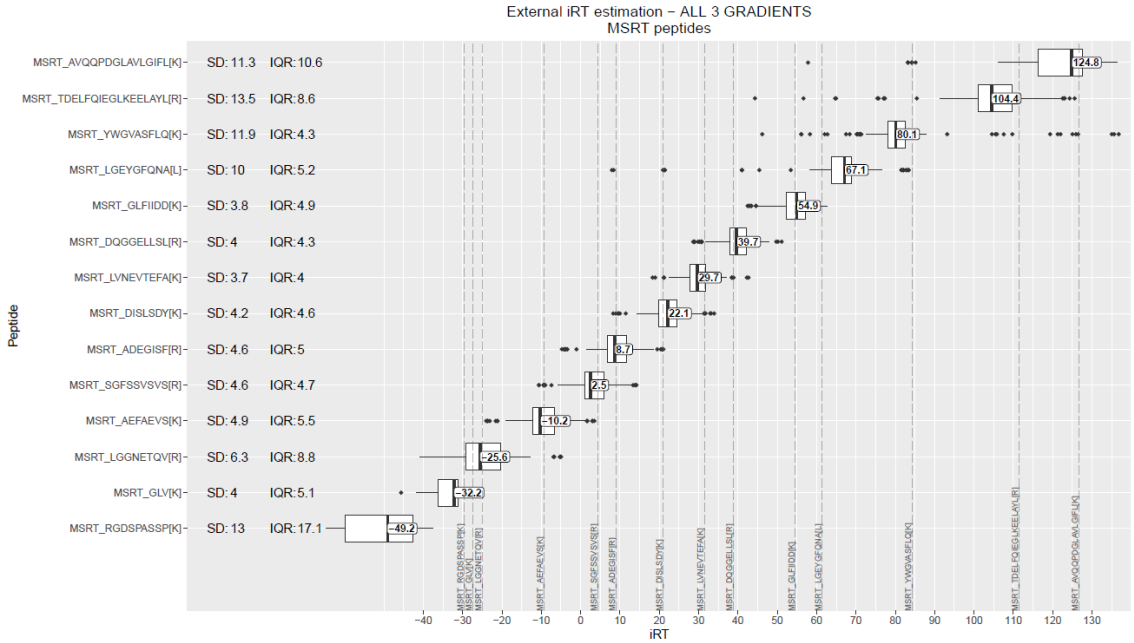


Figure 3a

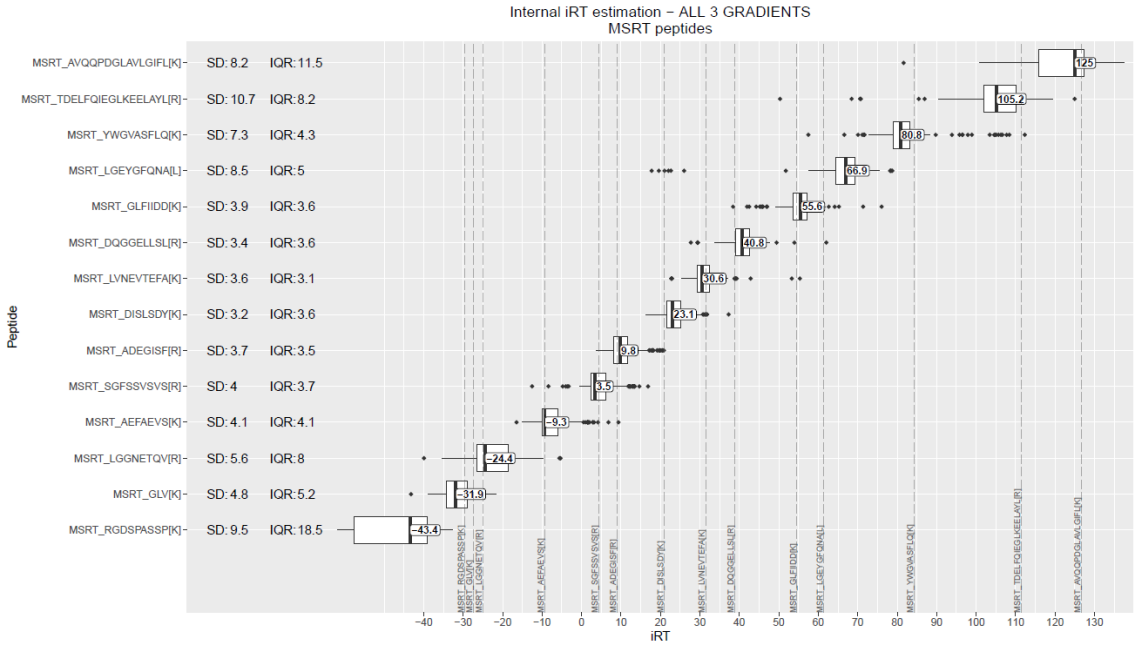


Figure 3b

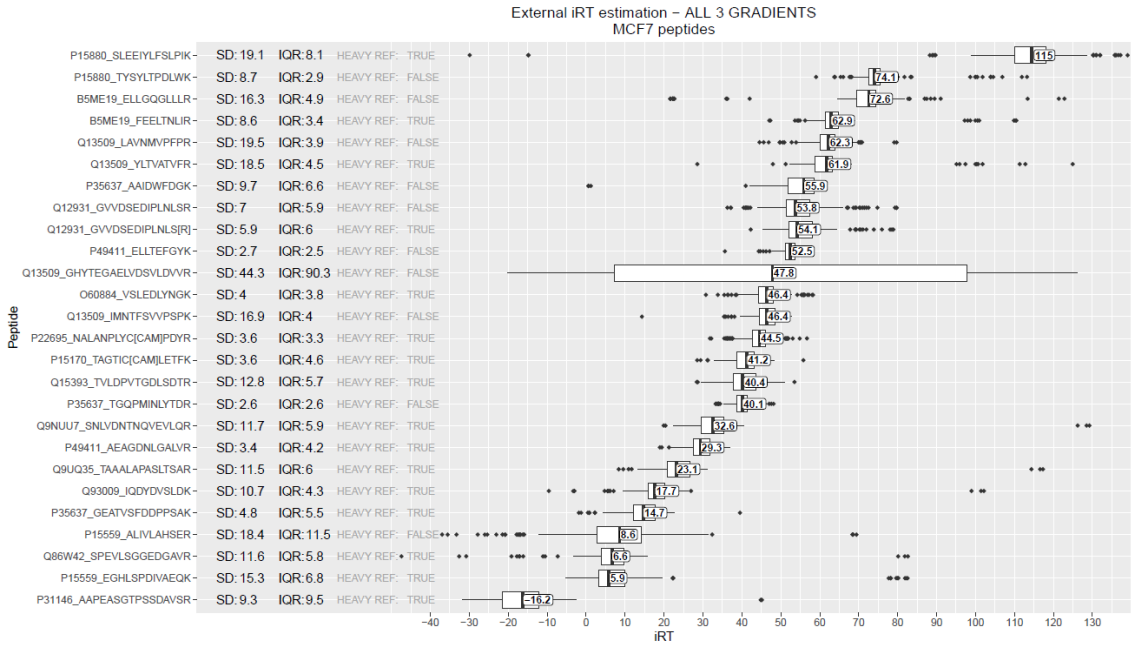


Figure 3c

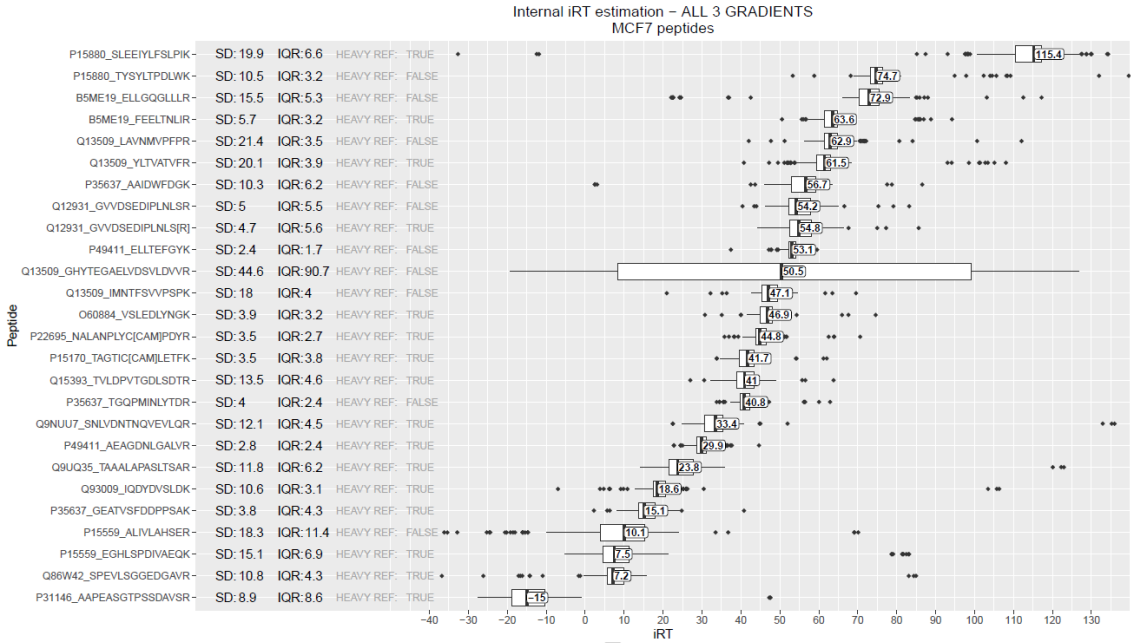


Figure 3d

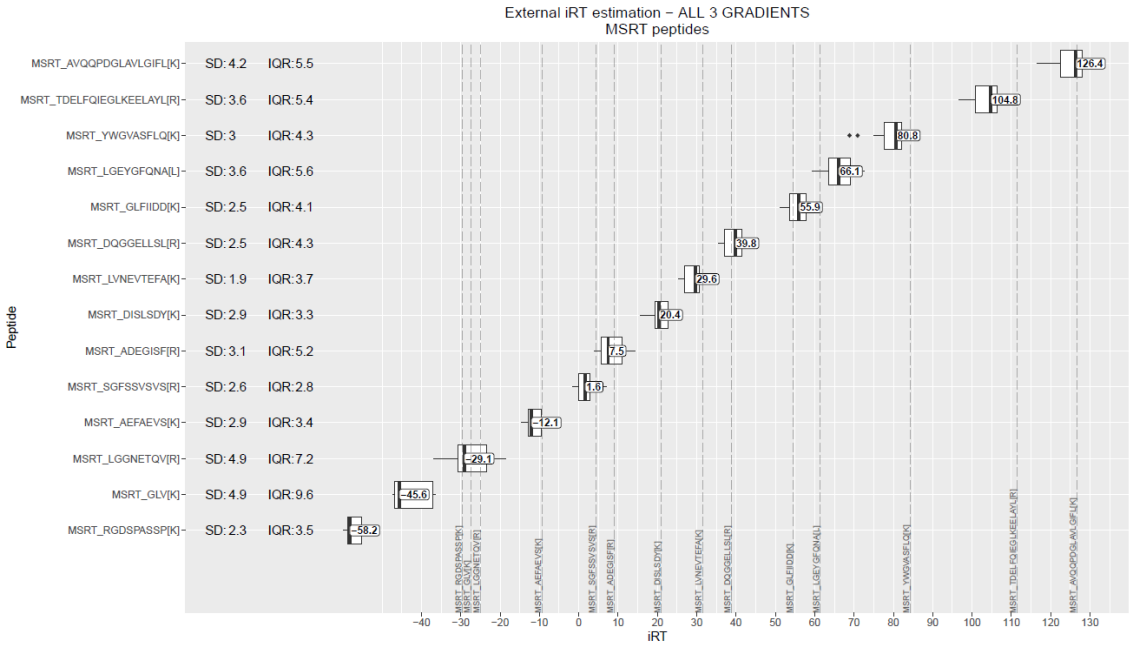


Figure 4a

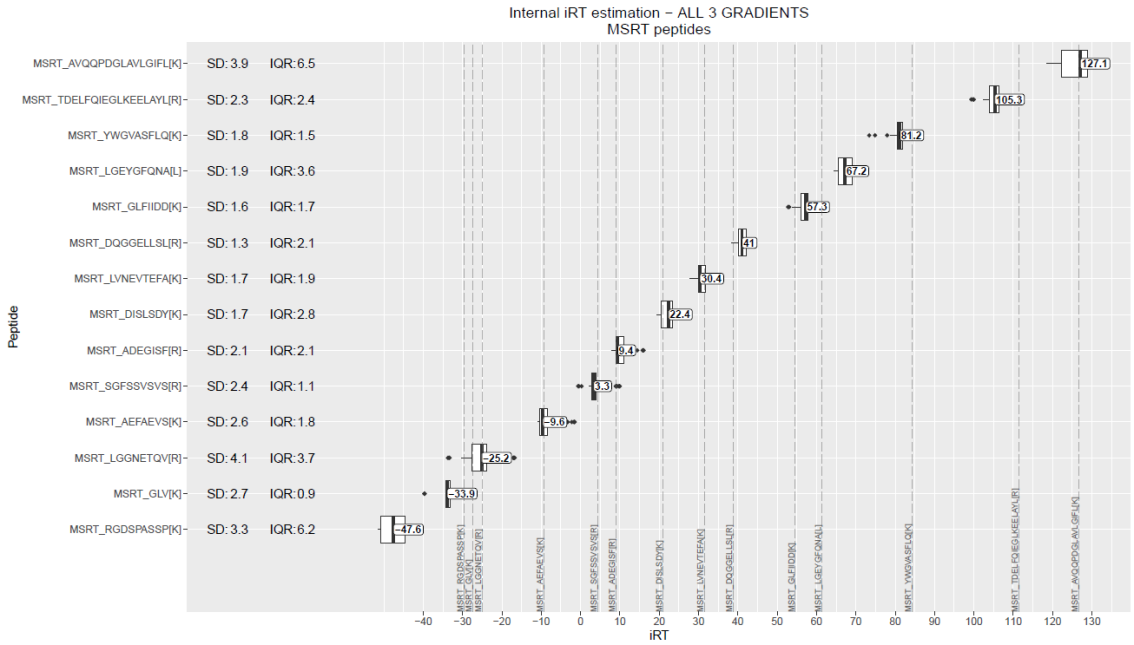


Figure 4b

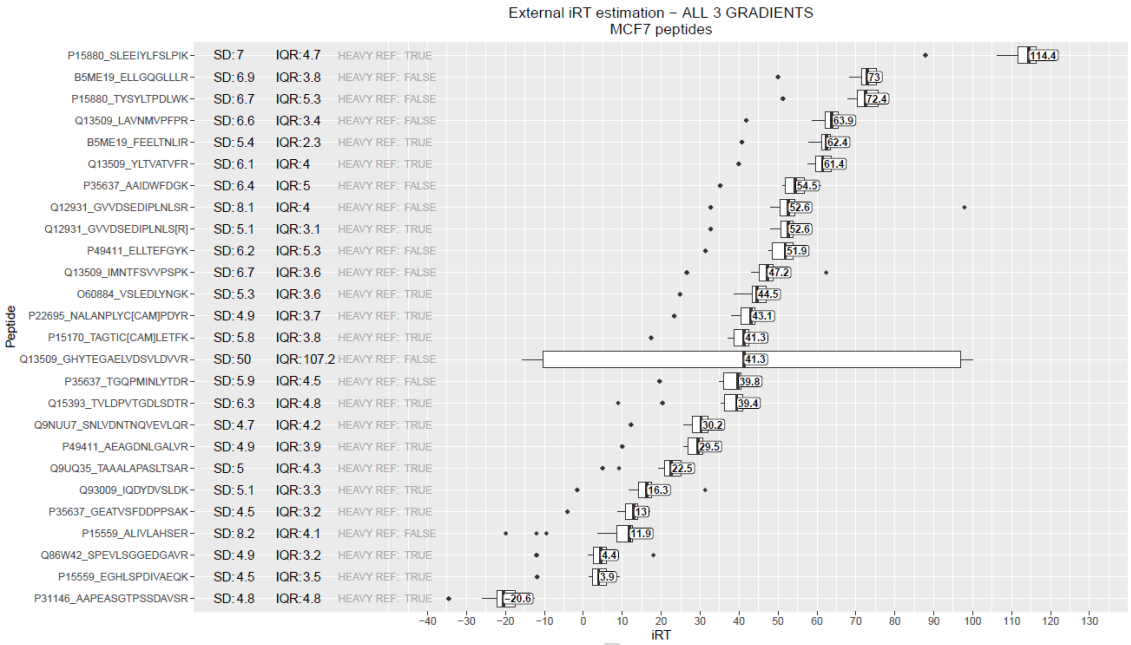


Figure 4c

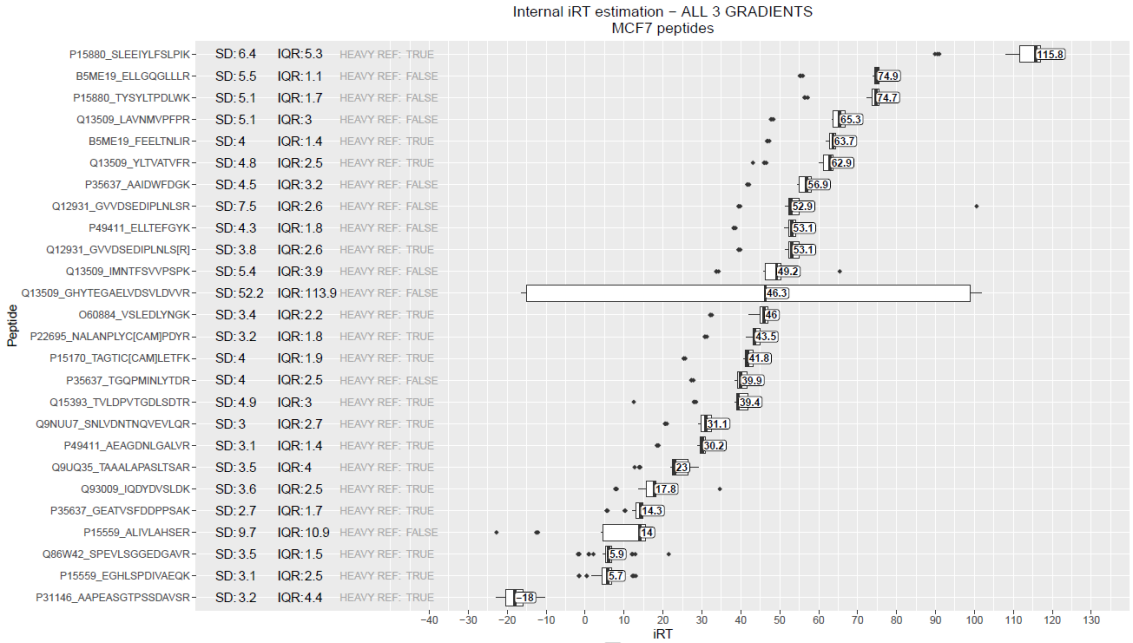


Figure 4d

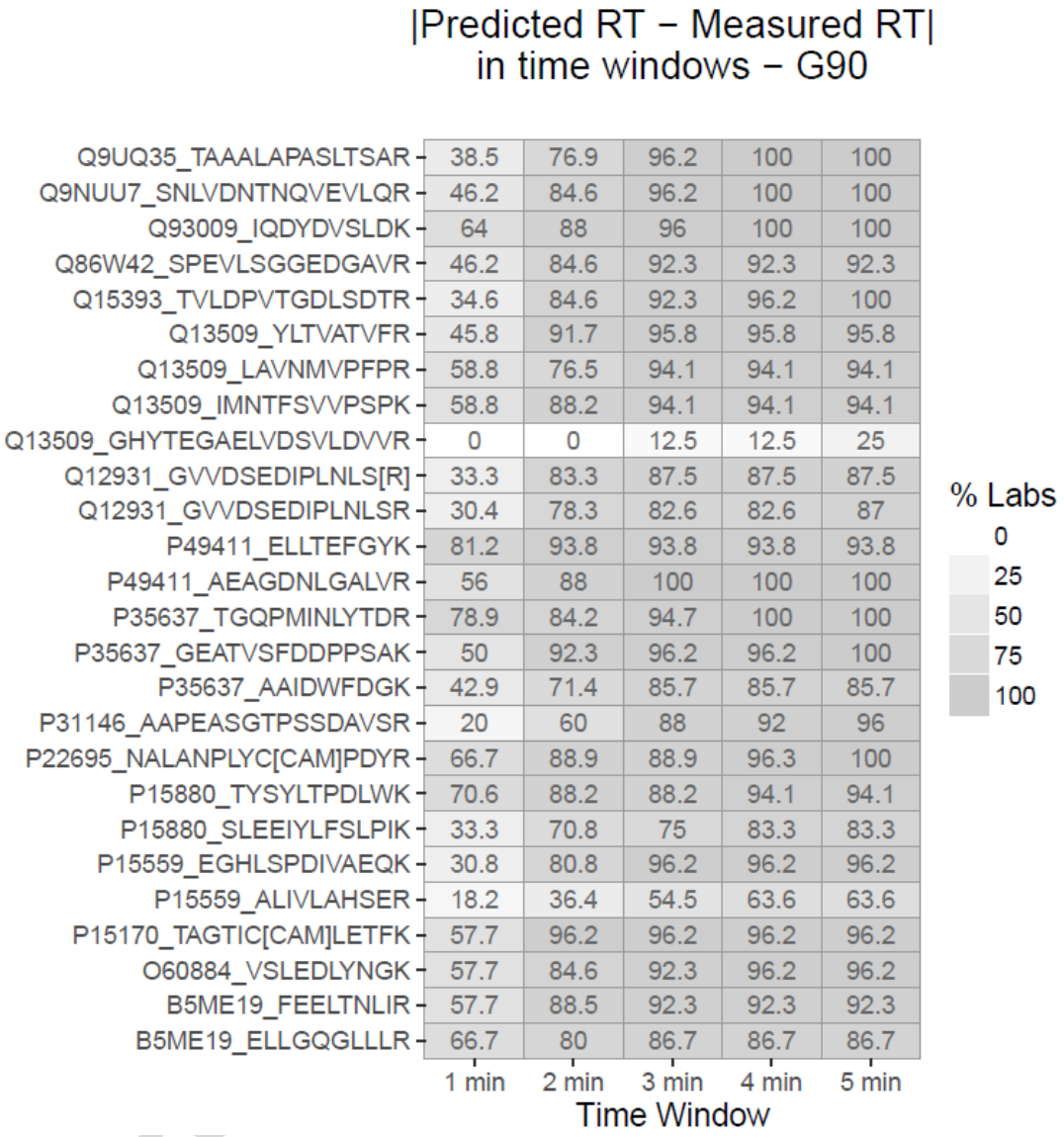


Figure 5a

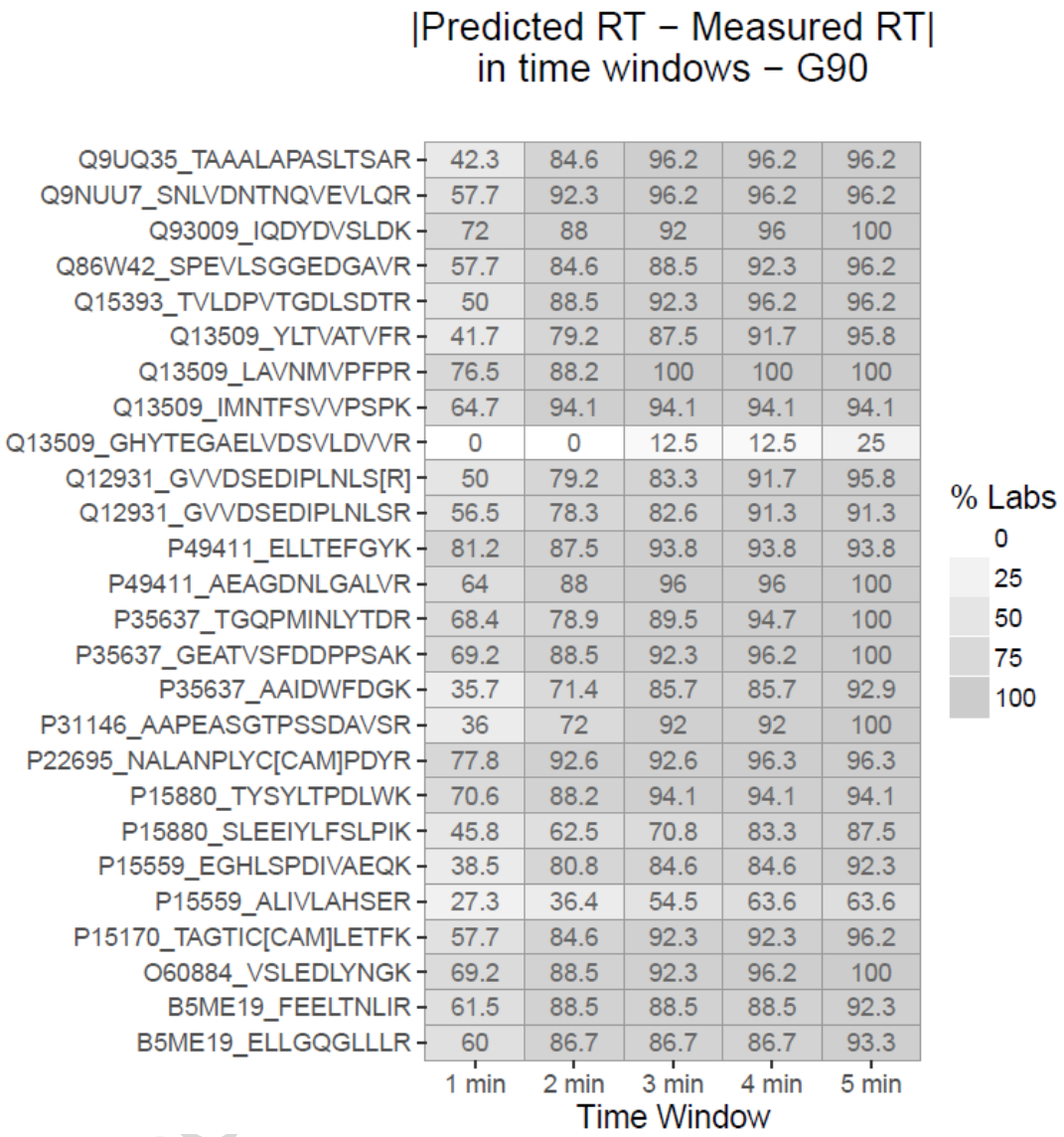


Figure 5b

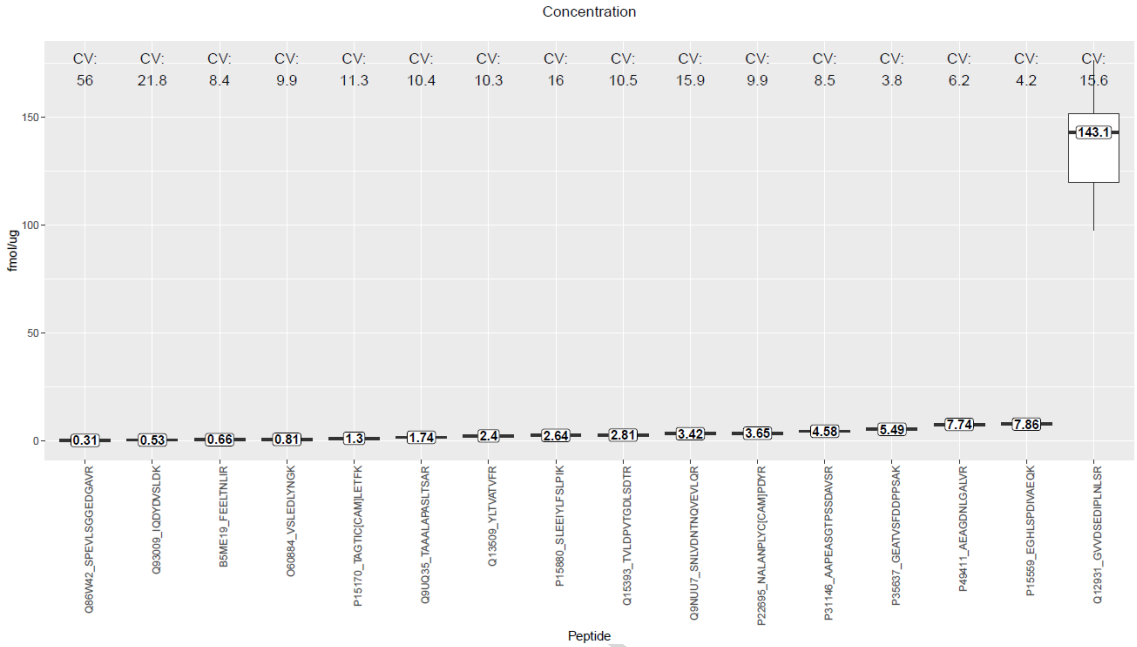


Figure 6a

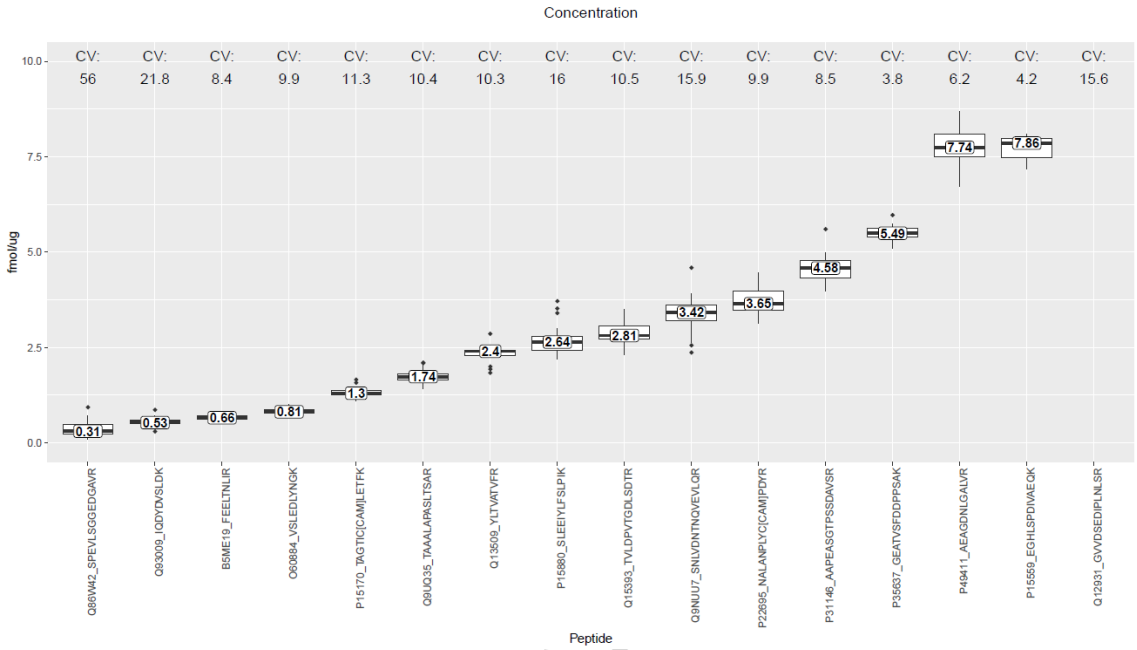
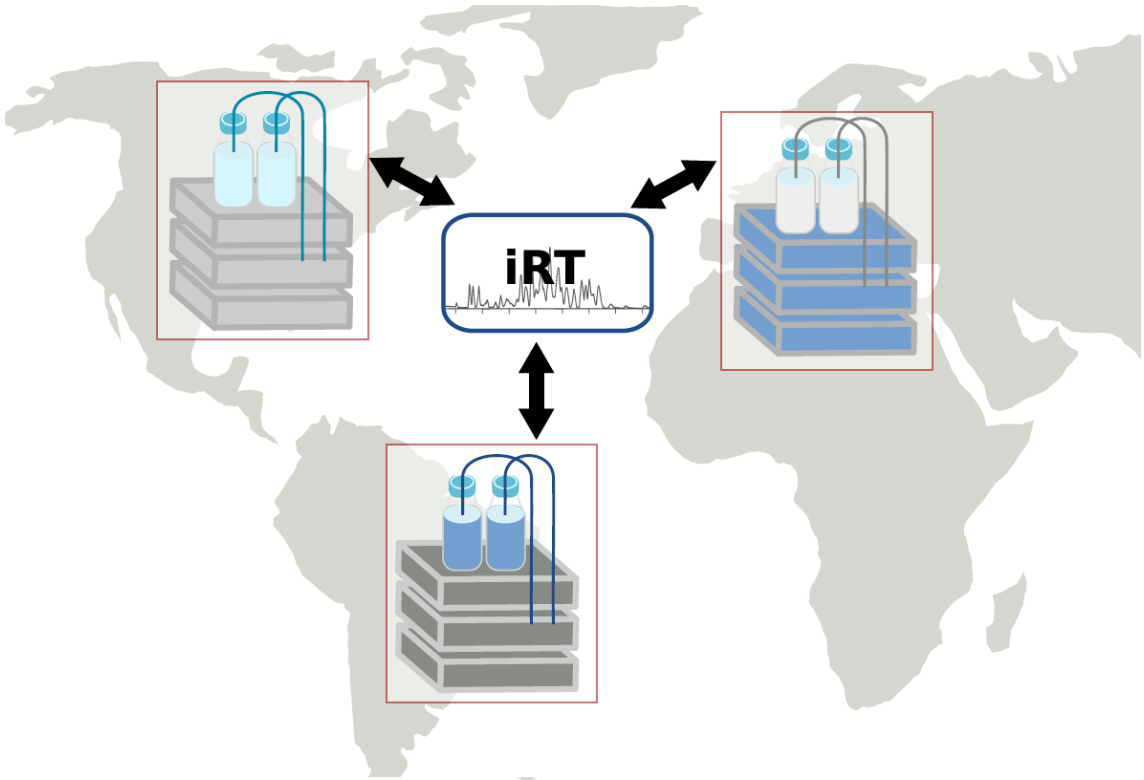


Figure 6b



Graphical abstract

Conflict of interest

The authors declare that there is no conflict of interest

Significance

From the very beginning of proteomics as an analytical science there has been a growing interest in developing standardized methods and experimental procedures in order to ensure the highest quality and reproducibility of the results. In this regard, the recent (2012) introduction of the dimensionless retention time concept has been a significant advance. In our multicentric (28 laboratories) study we explore the usefulness of this concept in the context of a targeted proteomics experiment, demonstrating that dimensionless retention time values is a useful tool for transferring and sharing peptide retention times across different chromatographic set-ups.

Highlights

- Dimensionless retention time (iRT) values for peptides have been proposed as a useful tool for transferring and sharing retention times across chromatographic set-ups both intra- and inter-laboratories.
- In a coordinated effort (28 laboratories) we have evaluated the suitability of retention time standards to improve targeted proteomic analyses.
- We have demonstrated that dimensionless retention time values (iRT) are a useful tool for transferring and sharing peptide retention times accross different chromatographic set-ups.
- Quantitative analyses showed a high reproducibility despite the variety of experimental strategies and analytical platforms employed.