

# Peptide Bioanalysis In Urine, A Case Study

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## Introduction

Bioanalysis of peptides has become increasingly important over the last decade because of the potential application of peptides as medicaments and disease biomarkers. Peptide and peptide-based compounds can be routinely analyzed by ligand-binding or LC-MS methods. LC-MS methods are particularly advantageous because they are selective and precise, offer a broad linear dynamic range, can be applied to quantify multiple peptides in the same run and, last but not least, because LC-MS method development is normally less time consuming. Nevertheless, LC-MS analysis of peptides remains challenging for various reasons related to their physico-chemical properties:

- Poor solubility
- Protein binding
- Non-specific binding
- Poor sensitivity
- Poor specificity
- Carry-over

A case study is presented concerning a peptide analogue whose bioanalysis is particularly challenging due to non-specific binding. The symptoms relating to non-specific binding and some solutions adopted to circumvent this issue are presented. This peptide will be referred to as "Peptide X" throughout the poster. Table 1 is showing the main characteristics of this compound.

Peptide X	
Application area	Diabetes
MW (Da)	~ 4,000
Analytical range (ng/mL)	3.00 - 250
Matrix	Human urine
Transition	1028.9 → 136.0

Table 1: Main characteristics of Peptide X.

## Results

In our laboratory, Peptide X has been analysed extensively in human plasma (K3EDTA). The bioanalytical approach used to extract this analyte from plasma followed the procedure outlined in Figure 1. The final extract was injected and separated on a UPLC system equipped with a BEH C18 300A column (50 x 2 mm). Detection is performed on a QTRAP 5500. A stable labelled Peptide X is used as internal standard.

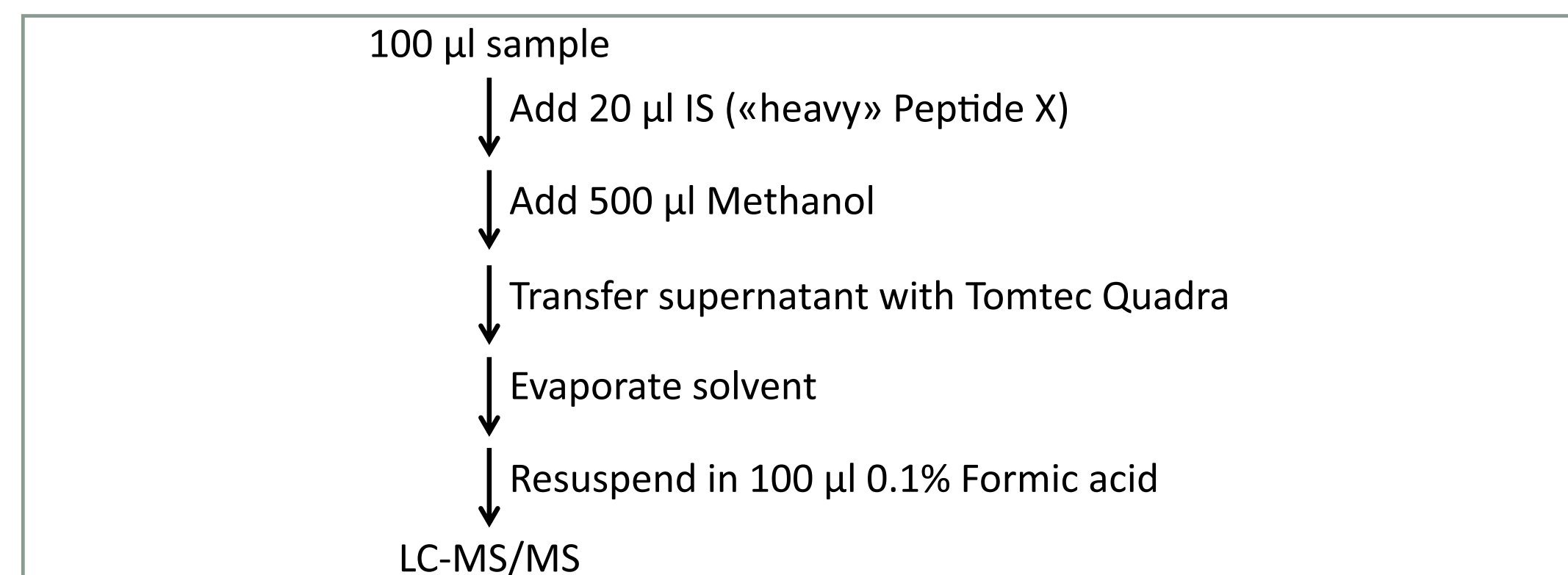


Figure 1: Procedure used to extract Peptide X from plasma or urine.

Since the method used to quantify Peptide X in plasma was characterized by good precision, accuracy, recovery, selectivity, carry-over and robustness, a similar approach was used to measure this analyte in human urine. Unfortunately, in human urine poor recoveries for both analyte and IS were observed. Approximately 20 times less signal intensity could be detected in urine compared to plasma making the quantitation at LLOQ very challenging (Figure 2).

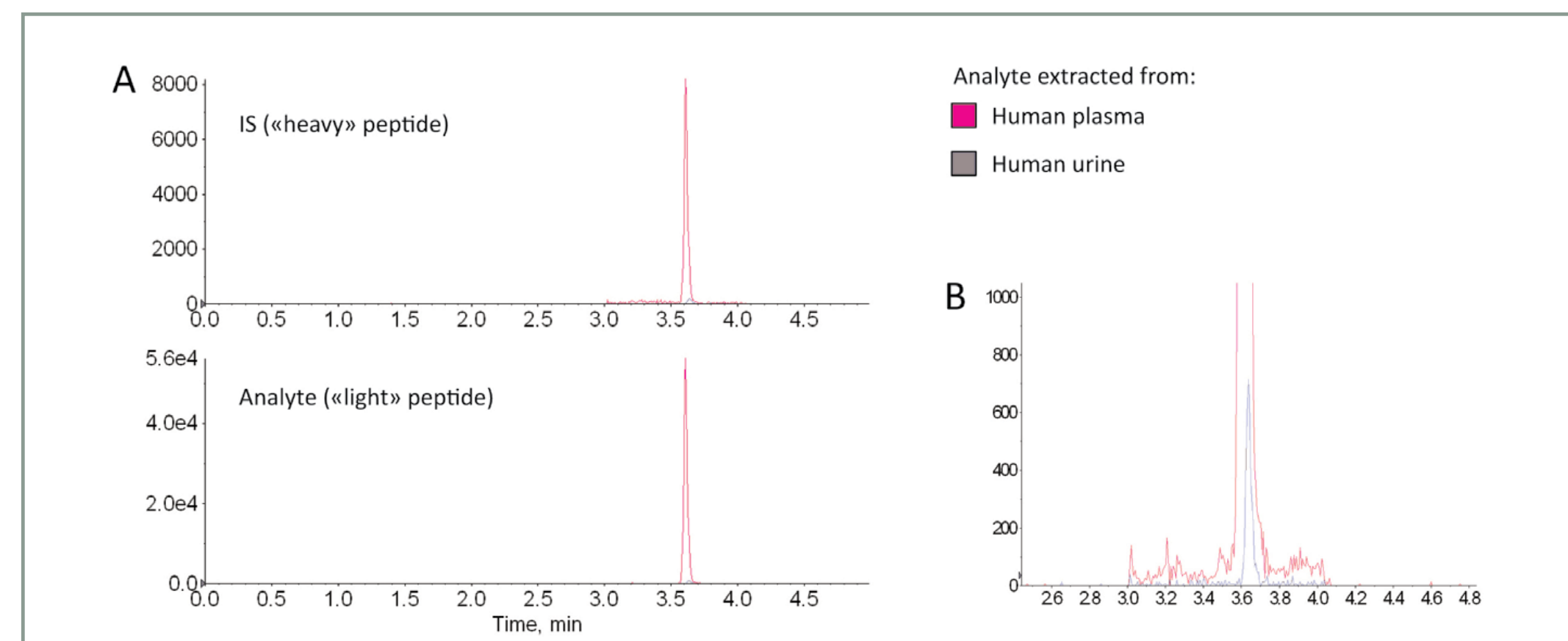


Figure 2A: Comparison between the recovery of Peptide X from plasma and from urine. 2B: Zoom where Peptide X is eluting.

Peptide X IS has been shown to be more stable in presence of BSA; therefore an experiment was performed where Peptide X was spiked in 0.5% BSA and in Urine / 0.5% BSA to assess whether the losses can be minimized. In presence of BSA alone the peptide was recovered to the same extent of plasma, however when BSA is added to urine poor recovery is obtained (Figure 3A). Moreover Peptide X was always lost independently of at which point in the procedure the peptide is spiked in (Figure 3B).

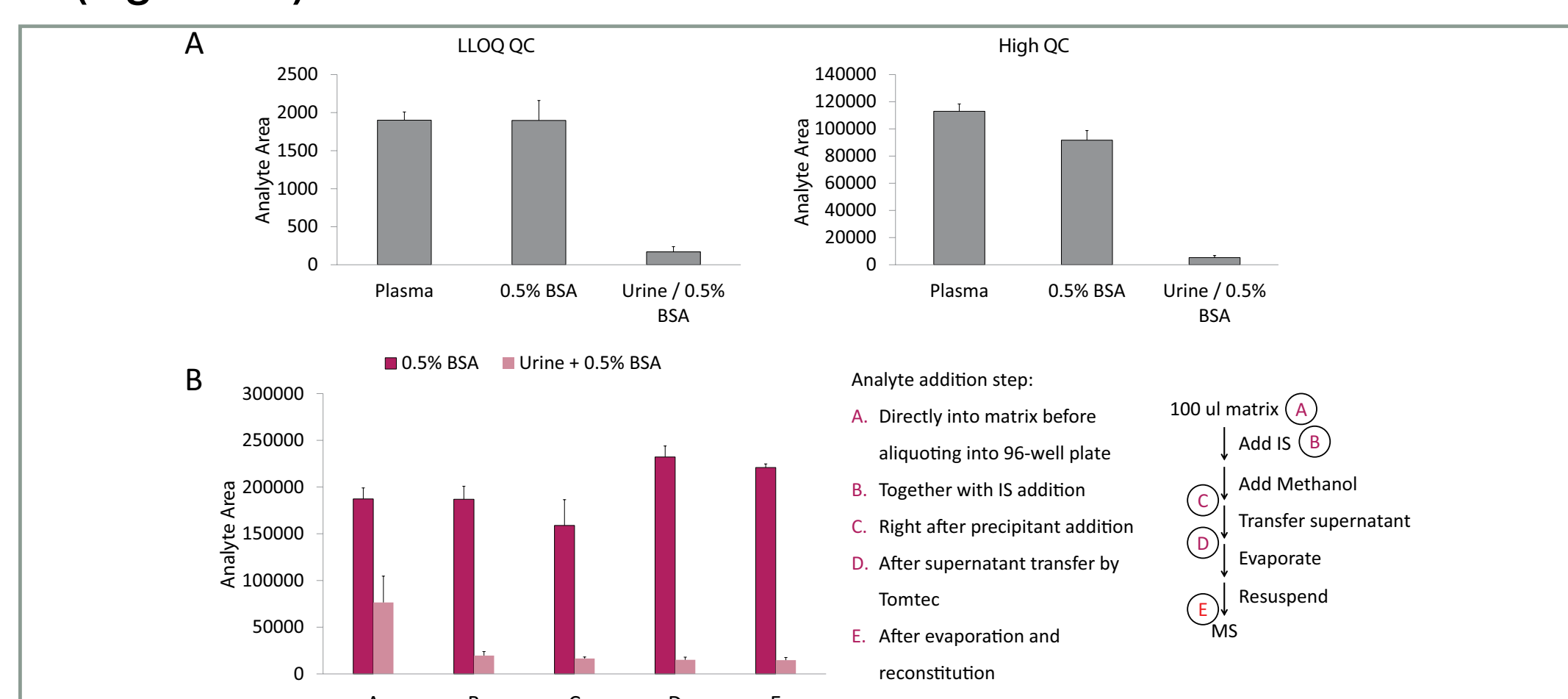


Figure 3A: Peptide X recovery from plasma, 0.5% BSA and Urine / 0.5% BSA. When peptide X is extracted from urine, a very low recovery is obtained and the addition of BSA to urine is unable to improve this low recovery. B: Peptide X is lost independently of where in the procedure it is spiked in.

The difference between the analyte recovery in 0.5% BSA and in Urine / 0.5% BSA could be explained by ion suppression occurring in urine but not in BSA alone or in human plasma. To check whether this hypothesis was correct, a post-column infusion experiment was performed (Figure 4A) together with an additional experiment where extracted plasma samples were mixed 1:1 with Recon solution (0.1% Formic acid), extracted blank plasma or extracted blank urine (Figure 4B). The results from both experiments clearly demonstrate that no significant ion suppression was occurring in urine.

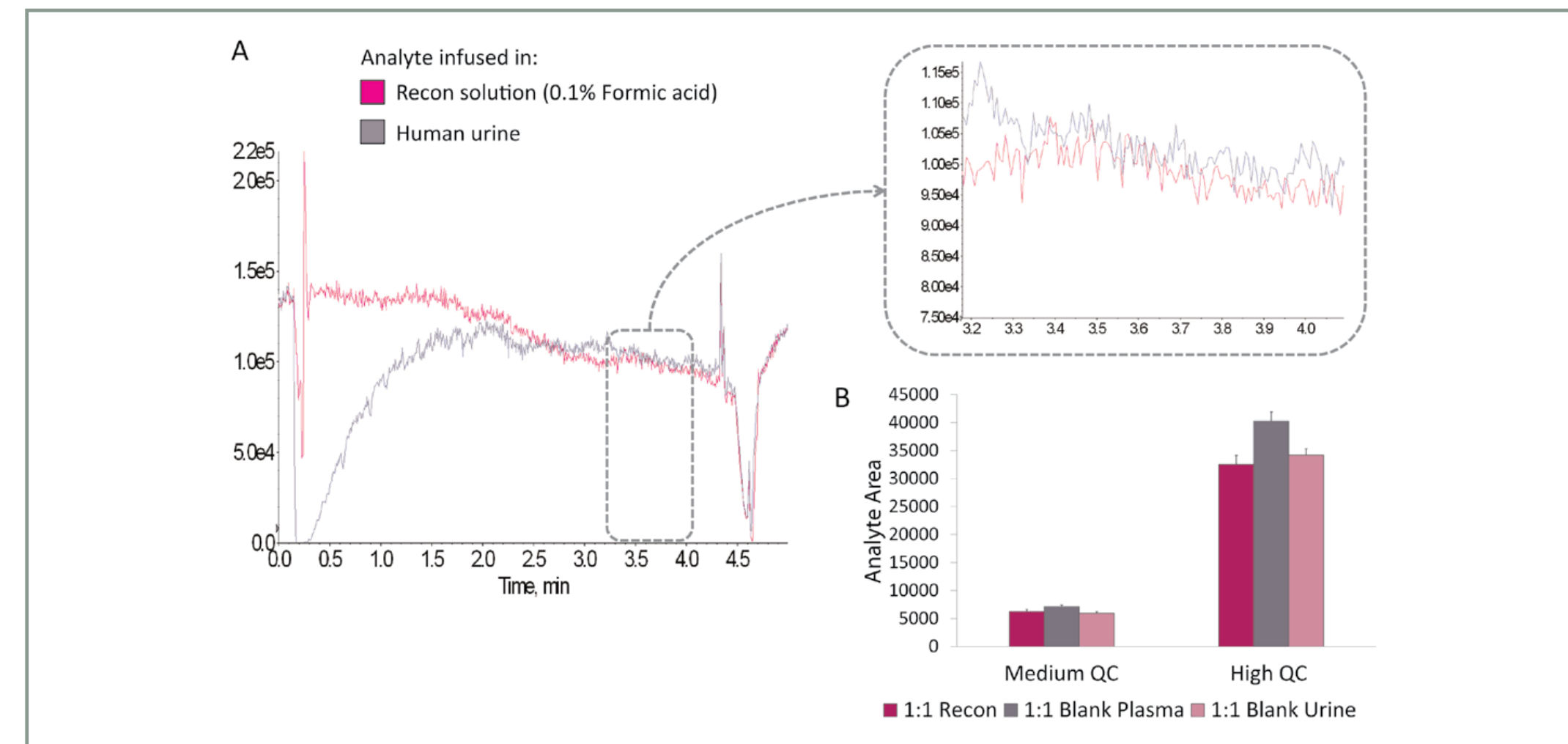


Figure 4A: Assessment of ion suppression by post-column infusion of analyte. B: Assessment of ion suppression by mixing plasma-extracted Peptide X with Recon solution (0.1% Formic acid), extracted blank plasma and extracted blank urine.

It seems that in urine there is one or more components which are destabilizing the analyte. Since the main 3 components of urine are water, urea and salts, 0.5% BSA was supplemented with 15 g/L urea and/or 5 g/L NaCl. Interestingly both components were demonstrated to destabilize Peptide X in urine and their action is synergistic (Figure 5A). Unfortunately it is very difficult to remove both urea and salts from urine samples in a robust and high-throughput way, therefore the attention was drawn to finding an urine additive, which is able to counteract the negative impact of urea and salts. Previously, it was demonstrated that BSA is not a good additive for Peptide X at a concentration below 5%. Also, pH modifiers did not seem to play an important role in stabilizing Peptide X. The addition of 0.1% Triton X-100, 0.05% Tween-20 or 0.1% CHAPS to stabilize Peptide X in urine were evaluated (Figure 5B). Both 0.1% Triton X-100 and 0.05% Tween-20 are able to stabilize Peptide X in urine.

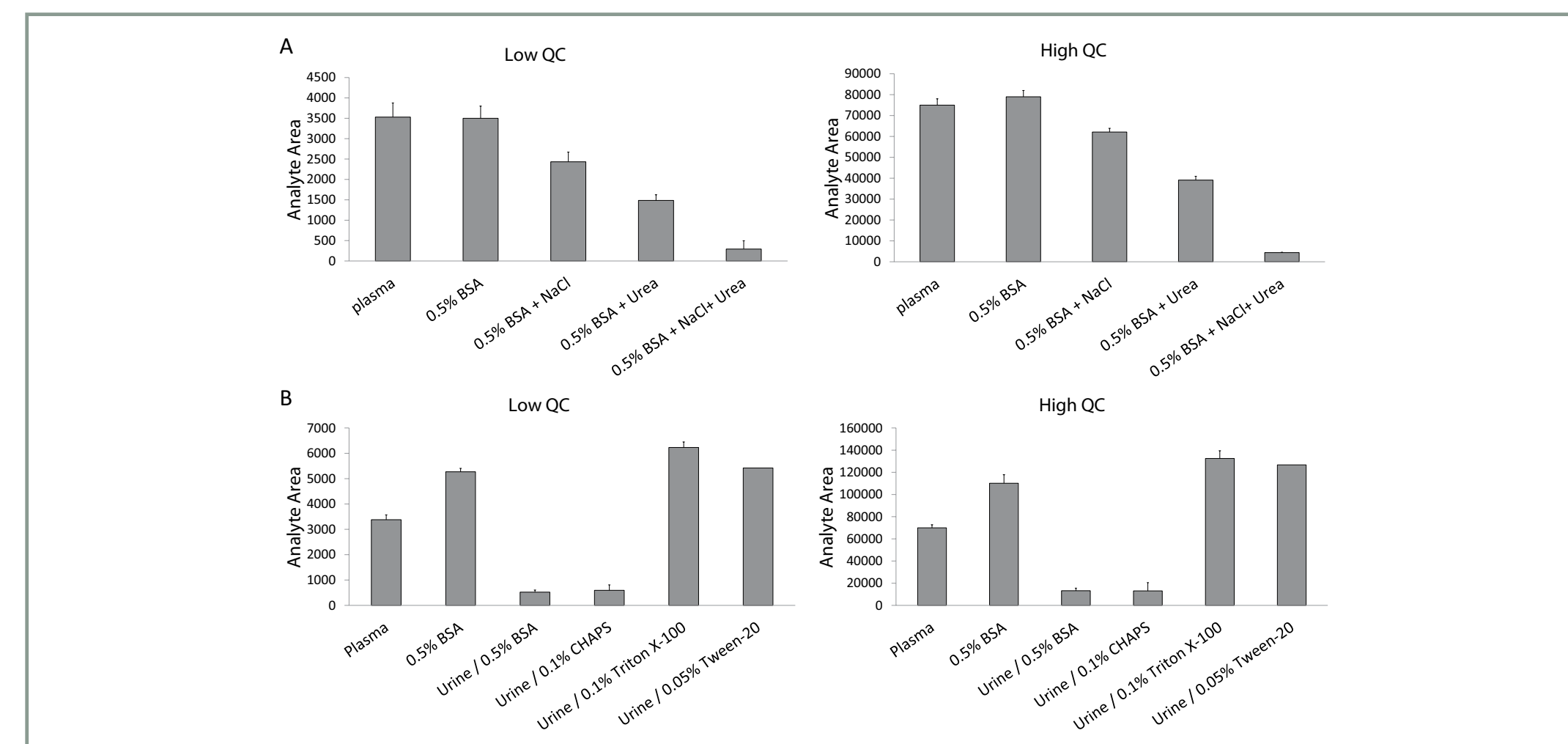


Figure 5A: Identification of the urine component(s) causing the loss of Peptide X. B: Screening of urine additives able to rescue the loss of Peptide X in urine.

Since using Tween-20 gave less background compared to Triton X-100, it was decided to use the former as urine additive and to screen several urine samples. Interestingly, all but 5 individual urine samples did result in good accuracy after addition of Tween-20 prior to spiking with analyte. It is important to underline that this problem was only observed by screening a high number of samples (up to 23 different urine samples) and that a screening of only a few urines may not have shown this issue. Since Tween-20 at a final concentration of 0.05% was not applicable in all cases, it was tested at an increased concentration. Also, the addition of 0.1% Triton X-100 was tested in the problematic individuals. Only 0.1% Triton X-100 gave acceptable recoveries in all individuals tested.

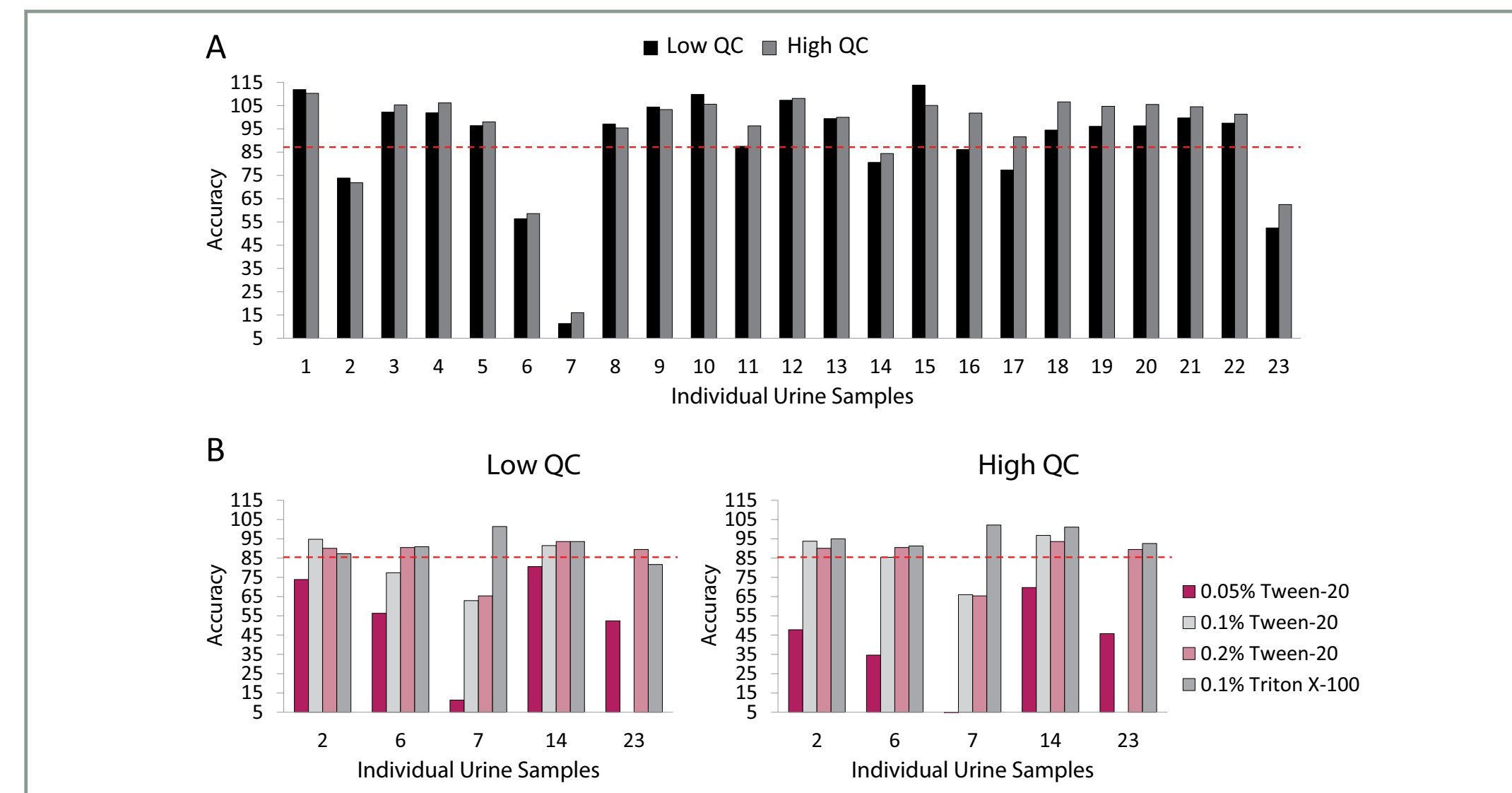


Figure 6A: Screening of individual urine samples to assess the efficacy of the urine additive Tween-20 (final concentration: 0.05%). B: Screening of urine additives able to rescue those 5 urine samples, which were failing with 0.05% Tween-20 as an additive.

Once Peptide X was demonstrated to be stable in urine already containing 0.1% Triton X-100, it remained to investigate whether the same effect was true by adding Triton X-100 after spiking Peptide X in urine. This is an important experiment because its outcome has a significant impact on sample collection at the clinical site. If Triton X-100 is able to stabilize the compound in urine when added retrospectively, it is possible to collect urine over a certain time interval, measure its weight and then add the corresponding amount of detergent to reach a final concentration of 0.1%. If this strategy is not applicable because

Peptide X loss is irreversible, then the detergent must be added to the container prior urine collection. This latter approach would require testing several final Triton X-100 concentrations, as final urine volumes are likely to vary.

Triton X-100 was added to urine at t0 (i.e. before analyte addition) and at t12 and t24 hours after analyte addition to urine. Typical containers used at the clinical site were used for this experiment and two extreme container-to-sample volume ratios are tested: 50 mL urine in a 2 L container and 1.8 L urine in a 2 L container (Figure 7). Addition of Triton X-100 after 12 hours from analyte addition to urine was able to recover the analyte losses and to stabilize Peptide X in urine. This enables the accurate measurement of urine and the corresponding detergent volume to be added to reach the target concentration of 0.1%.

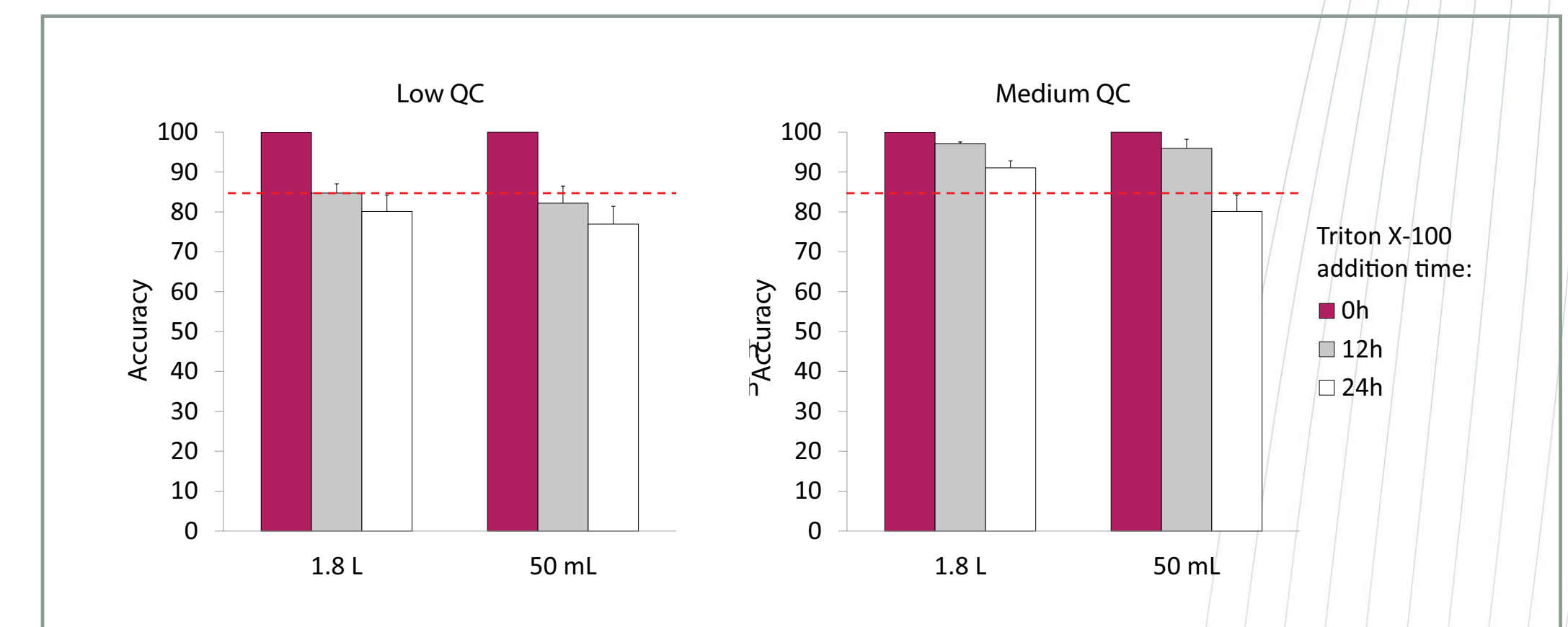


Figure 7: Evaluation of the appropriate Triton X-100 addition time to urine.

Once all necessary conditions to stabilize Peptide X in urine were evaluated, the final method was tested for the following parameters:

- Precision/Accuracy
- Carry-over
- Matrix effect (16 individuals)
- Selectivity of blanks and STD 0 (16 individuals)
- Dilution integrity
- Stability (Freeze/Thaw; bench-top)
- Reinjection reproducibility

The results are presented in Figure 8. These results clearly show that by adding Triton X-100 to urine at a final concentration of 0.1%, it is possible to stabilize Peptide X enabling its reproducible and accurate quantitation.

Run	Accuracy (%)	CV (%)	N	LLOQ QC	Low QC	Medium QC	High QC	Carry-over (%)	
				3 ng/mL	9 ng/mL	40 ng/mL	200 ng/mL	Min.	Max.
Run 1	104.4	6.8	6	93.6	4.7	97.1	92.8	1.7	19.0
Run 2	94.7	3.2	6	89.3	4.0	92.3	89.8	5.1	9.3
Run 3	96.6	5.0	6	95.3	3.2	99.9	96.3	4.8	12.3
Interbatch	98.6	6.7	18	92.7	4.7	96.4	93.0		

Ind.	Low QC 9 ng/mL		High QC 200 ng/mL		Stability accuracy (%)	
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Low QC 9 ng/mL	High QC 200 ng/mL
Ind. 1	92.2	-	101.0	-	91.2	89.5
Ind. 2	87.3	-	95.0	-	88.9	89.7
Ind. 3	95.6	-	105.0	-	89.9	90.6
Ind. 4	102.3	-	105.0	-	89.8	90.0
Ind. 5	96.9	-	102.5	-	81.8	83.7
Ind. 6	90.9	-	91.5	-		
Ind. 7	101.4	-	102.0	-		
Ind. 8	105.3	-	104.5	-		
Ind. 9	107.3	-	105.0	-		
Ind. 10	101.3	-	104.5	-		
Ind. 11	93.6	-	101.0	-		
Ind. 12	97.6	-	107.0	-		
Ind. 13	93.2	-	101.0	-		
Ind. 14	99.1	-	101.5	-		
Ind. 15	81.8	-	92.5	-		
Ind. 16	91.6	-	104.0	-		
All	96.1	7.0	101.4	4.5		

Figure 8: Summary of the main prevalidation results.

## Conclusions

Peptide analysis in urine can be hampered by non-specific binding. This is the case for Peptide X, whose recovery was demonstrated to be about 20-fold less in urine than in plasma. To alleviate this problem, several urine additives were screened and Tween-20 and Triton X-100 were found to be the best in stabilizing the compound in urine. However, when urine samples from different donors were screened, it was demonstrated that, at the same final concentration, Triton X-100 was slightly better than Tween-20. Notably, non-specific binding of Peptide X in urine was partially reversible, enabling to add the detergent retrospectively. This expedient enabled the accurate and precise quantitation of Peptide X in human urine from 3 to 250 ng/mL. This assay was validated and successfully applied to clinical samples.