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APPLICATION NOTE 65902

Improving targeted peptide quantification

Combining a TSQ Altis with a FAIMS Pro interface for peptides in complex matrices

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Goal

When quantifying peptides using LC-MS/MS, background interferences often negatively impact limit of quantification (LOQ). Here we show that by using the Thermo Scientific™ FAIMS Pro™ interface in combination with nanoflow chromatography and triple-stage quadrupole (TSQ) technology, the background can be reduced significantly, which leads to increased signal-to-noise (S/N) and improved dynamic range and LOQ.

Introduction

Characterization and accurate quantification of proteins in complex matrices is becoming increasingly important for a variety of applications in the pharma/biopharma sector. Targeted quantitative assays are used in PKPD, discovery, regulated bioanalysis, and therapeutic drug research. LC-MS/MS can provide quantitative information about proteins that is complementary to traditional techniques, such as

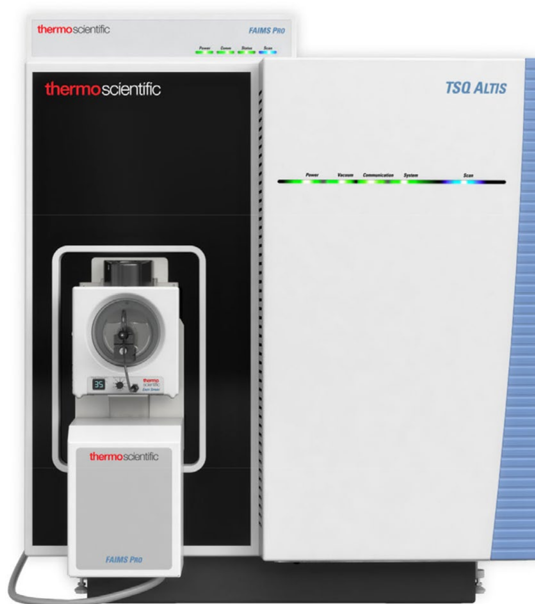

Figure 1. EASY-Spray ion source and FAIMS Pro interface mounted to TSQ Altis triple quadrupole mass spectrometer

ligand-binding assays (LBA). In some cases, proteins and native peptides, such as insulin, renin, and IGF-1, are analyzed directly; for instance, large therapeutic peptides are often quantified by LC-MS/MS without enzymatic digestion. But more often, protein quantification by LC-MS/MS is based on identification of signature peptides specific for the given protein of interest. After enzymatic digestion of the protein, the quantitative analysis of resulting peptides is performed, usually in

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the presence of spiked isotopically labeled peptides as internal standards. This approach, common for protein quantification by LC-MS/MS, is complicated by the fact that analyte peptides can be present across a wide concentration range and in complex matrices that contain many other coeluting peptides as well as other chemical background.

Triple quadrupole mass spectrometers operated in selected reaction monitoring (SRM) mode typically offer the most competitive LOQs for a large spectrum of analytes in a variety of matrices. The Thermo Scientific™ TSQ Altis™ triple quadrupole mass spectrometer (Figure 1) offers excellent sensitivity, speed, and dynamic range and is the platform of choice for the analysis of low abundant peptides. In a typical peptide quantification experiment by LC-MS/MS, the resolving power of reversed-phase chromatography is combined with selectivity and sensitivity of the mass spectrometer to extend the quantitative dynamic range. However, the LOQ is often negatively impacted by background chemical noise. These background interferences cannot be removed by LC separation alone and often overlap with the target analyte on multiple SRM transitions. This can limit the utility of an assay.

The FAIMS Pro interface is a differential ion mobility device, which can be used to selectively transmit ions of interest while suppressing transmission of interfering compounds. Selectivity is achieved by alternating between high and low electric field strengths applied to a set of cylindrical electrodes. Depending on the structural orientation of an ion and loosely on its mass and charge, ions experience different mobilities through the electrode gap while the electric field of alternating strength is applied. By applying an additional compensation voltage (CV), ions of specific mobility can be transmitted through the high field asymmetric waveform ion mobility spectrometry (FAIMS) device, while interfering ions are prevented from passing through. The respective CV value that allows transmission of a specific analyte of interest can be optimized for each analyte and becomes an additional parameter in the SRM table for each precursor ion. The additional separation provided by the FAIMS Pro interface is orthogonal to LC and MS, and results in improved S/N for analytes of interest and therefore better quantitative performance.

Experimental

A PRTC peptide standard (Thermo Scientific™ Pierce™ Peptide Retention Time Calibration Mixture, P/N 88321) was spiked into Thermo Scientific™ Pierce™ HeLa protein digest (Pierce catalog number 1862824) at concentrations ranging from 0.001 to 100 fmol/µL. The Thermo Scientific™ Pierce™ LC-MS/MS System Suitability Standard (7 x 5 Mix, P/N 88320) was prepared according to the manufacturer protocol to yield concentrations of 0.13–200 fmol/µL in 0.3 µg/µL digested plasma matrix. Angiotensin I standard (Sigma-Aldrich, St. Louis, MO) was prepared in 0.5 µg/µL digested plasma in concentrations ranging from 0.07 to 700 fmol/µL. For all analyses, 1 µL of each sample was injected onto a Thermo Scientific™ EASY-Spray™ column with integrated emitter (P/N ES800A) using a Thermo Scientific™ EASY-nLC™ 1200 system. A 40 min gradient was used at a flow rate of 200 nL/min. The column heater was set to 45 °C. The TSQ Altis instrument global settings are shown in Table 1. The peptides were detected as doubly charged ions and fragmented using optimized SRM transitions (4–5 per peptide). The FAIMS Pro interface was operated in normal resolution mode with no additional user gas. Data were analyzed using Thermo Scientific™ TraceFinder™ software.

Table 1. TSQ Altis triple quadrupole MS instrument settings

Parameter	Value
Resolution	0.7 (Q1 and Q3)
Cycle time	1 s
RF lens	Calibrated
CID gas pressure	1.5 mTorr
Ion transfer tube temperature	325 C
Spray voltage	2,100 V

FAIMS Pro compensation voltage optimization

FAIMS Pro compensation voltage (CV) values for each peptide were optimized by infusing the neat standard peptide solution and using the CV scan tool in the Tune interface (available in Tune 3.1 and above). Optimum CV values were transferred into the method file and each peptide was analyzed at its respective optimum CV value

to yield maximum transmission. If standards are not available, FAIMS CV values can be optimized by injection using either one of two strategies:

1. For assays involving only a small number of peptides, CV optimization can be done within 1–2 injections (depending on the number of precursors in the method). To achieve this, each precursor is entered into the SRM table multiple times, depending on how many different CV values the user wants to compare. In each row, the precursor is entered with a slightly different precursor *m/z* value (for example, *m/z* 433.34, 433.33, 433.32, etc.) For each of these "pseudo-precursors", one CV value is assigned. It is sufficient to monitor only one product ion for CV optimization because the FAIMS Pro interface mounts between the ion source and the mass spectrometer inlet; hence, the additional selectivity is applied at the precursor level. For coarse optimization, the CV values should be distributed in 10 V steps, usually between -80 and -20 V. After execution of the optimization run, the optimum CV value range for each precursor is determined by comparing peak areas for each CV value. The number of necessary coarse optimization injections depends on the LC peak width, size of the peptide panel and the elution overlaps, and thus on the available cycle time. Once the approximate optimal value range has been determined, the exact optimal value can be found by probing values in a narrower range around the coarse CV optimum, for example in steps of 2–3 V. An example of optimization by injection is shown in Table 2 and Figure 2.

Table 2. Example of compensation voltage (CV) optimization by injection in 10 V steps. Peak areas indicate that the optimum CV is around -65 V. In the next injection the value can be optimized in more narrow steps around -65 V

Precursor *m/z*	Adjusted precursor for CV optimization	CV value	Peak area
433.3	433.34	-45	973
	433.33	-55	9,765
	433.32	-65	47,470
	433.31	-75	386

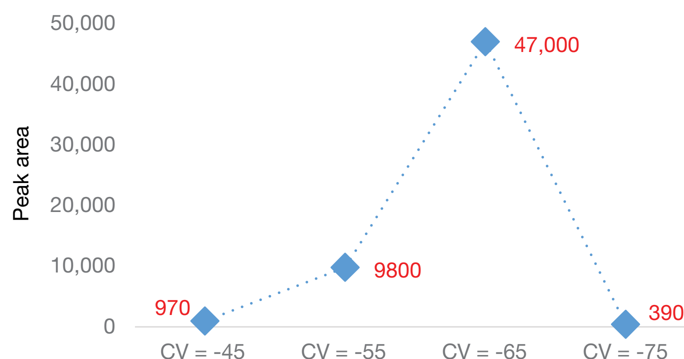

Figure 2. Integrated peak areas at different compensation voltage (CV) values

2. When assays involve a large number of precursors, not every precursor can be analyzed at multiple CV values within a few injections. In this case, multiple injections need to be performed, and each injection will be at one dedicated CV value global for all peptides. For example, injection 1 will be at a CV of -10 V, injection 2 will be at a CV of -15 V, etc. The CV optimum for each peptide will be determined based on peak area at each CV value.

Results and discussion

PRTC peptides

Figure 3a–d shows a comparison of peptide HVLTSIGEK at a level of 50 attomol on column in HeLa digest analyzed by the TSQ Altis mass spectrometer with and without the FAIMS Pro interface. Although the retention time can be determined by looking at the internal standard signal, at this concentration level the peak of the analyte cannot be properly integrated without use of the FAIMS Pro device. The additional level of selectivity that the FAIMS Pro interface provides removes the chemical background, which enables the detection and integration of the HVLTSIGEK peptide for quantitative determination. Without the FAIMS Pro device, HVLTSIGEK cannot be clearly distinguished from the background at levels below 100 attomol, while with the FAIMS Pro device, the peptide can be detected at 25 attomol.

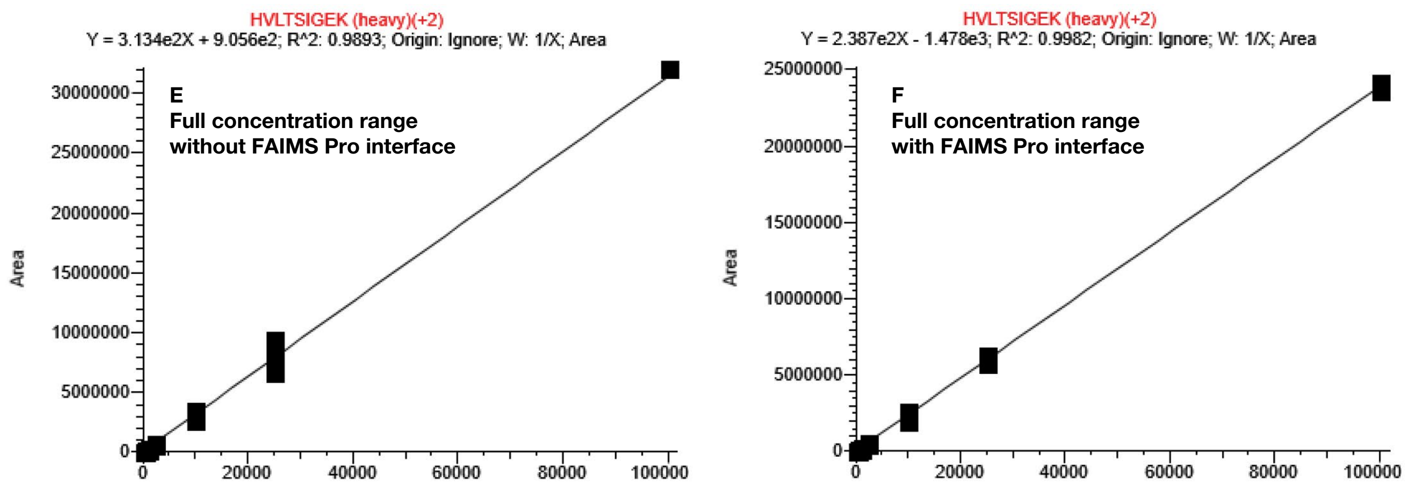

Figure 3. Suppression of background noise by FAIMS Pro interface improves S/N. Panel A shows a detectable peak of peptide HVLTSIGEK analyzed by TSQ Altis triple quadrupole mass spectrometer with the FAIMS Pro interface at 50 attomol on column. The retention time was confirmed by internal standard (Panel B). The same sample at the same concentration was analyzed without the FAIMS Pro interface. Strong background noise (Panel C) at the retention time determined by internal standard (Panel D) limits the S/N. Calibration curves are shown for the peptide HVLTSIGEK measured both with (Panel E), and without (Panel F), the FAIMS Pro interface.

Angiotensin I (non-tryptic peptide)

Peptide hormone angiotensin I (proangiotensin) was monitored by SRM assay in digested plasma as a doubly charged precursor ion at *m/z* 649.0 with five SRM transitions (*m/z* 583.5; 591.3; 784.4; 1000.6, and 1028.5). For the experiment with the FAIMS Pro interface, the device was optimized for maximum signal of the precursor ion by direct infusion. The optimum CV value for doubly charged angiotensin I was determined to be -56 V. Figure 4 shows angiotensin I chromatograms at various concentrations in plasma both with and without the FAIMS interface. At high on-column amounts (700 femtomol), there is no difference in the results obtained with and without the FAIMS Pro interface. This is because the absolute analyte

signal is high enough to make noise practically irrelevant for peak integration and S/N value. However, as the analyte on-column amount decreases, the background noise becomes an important factor for data quality. Eventually, at 70 attomol of angiotensin I on-column, the signal is indistinguishable from the background without the FAIMS Pro interface. Utilization of the FAIMS Pro device removes background noise sufficiently enough that the peak can be detected and integrated at a level as low as 70 attomol.

To demonstrate system robustness, the 700 femtomol on column sample in 0.5 μg/μL digested plasma was injected 40 times over a period of two full days. Figure 5 shows excellent reproducibility of the 40 injections with an RSD of 9.9%. Despite the concentrated matrix, area counts were consistent throughout the experiment. No cleaning of the FAIMS electrodes or of the TSQ Altis inlet was performed during the experiment. Solvent blank injections were run after every 10 plasma injections to keep the column and emitter in good condition.

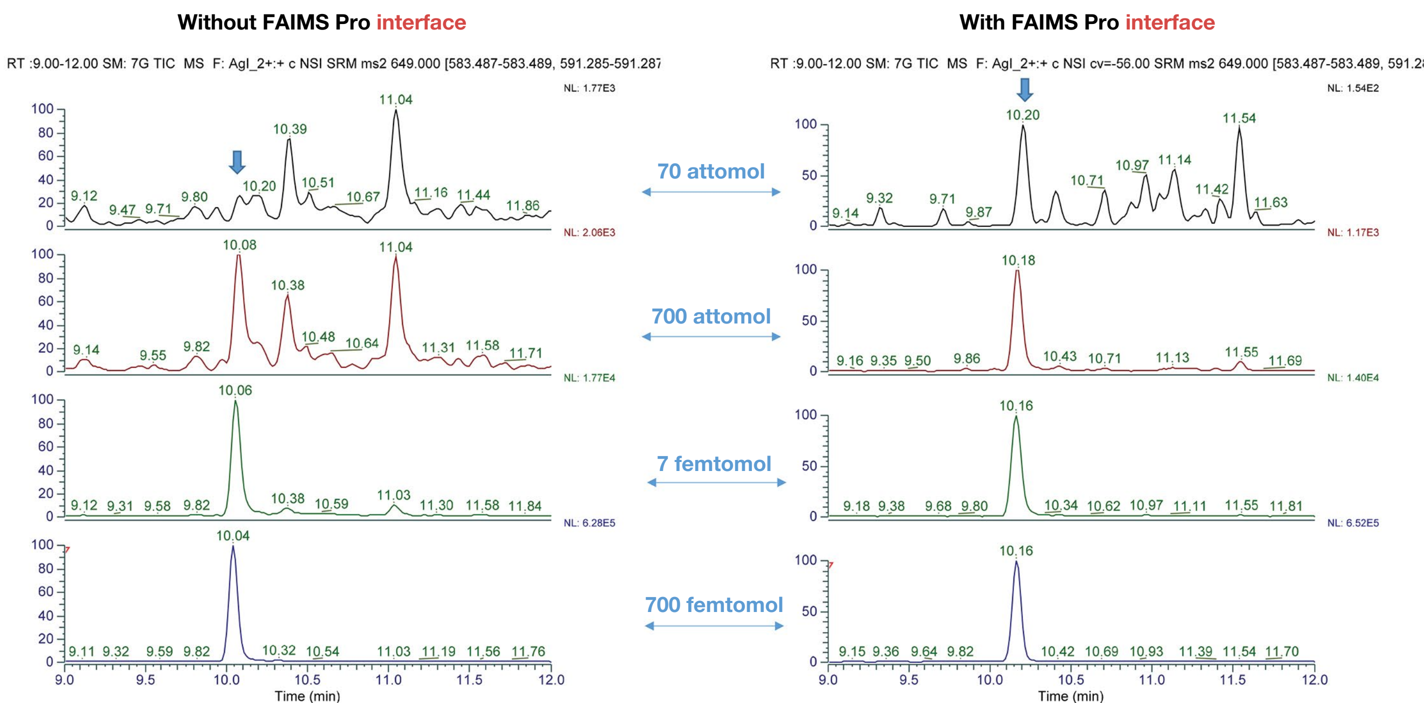

Figure 4. Quantification of angiotensin I in digested plasma. Combining the FAIMS Pro interface with an optimized CV value as part of the SRM table provides selective noise reduction, which results in a quantifiable signal (right panels) even at concentration levels where analyte signal is impacted by noise interference without FAIMS Pro interface (left panels).

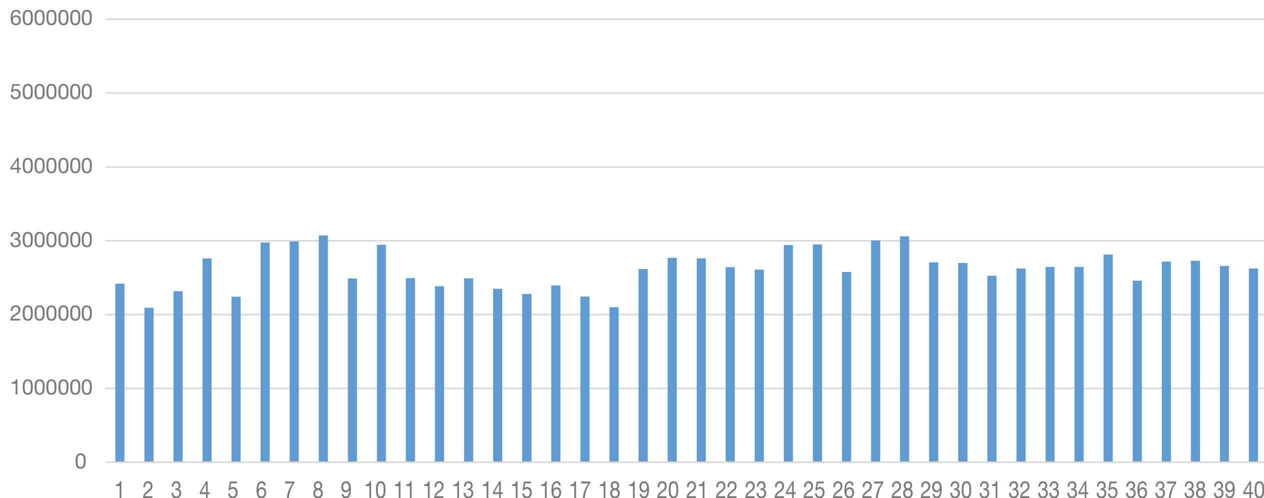

Figure 5. 40 consecutive injections of angiotensin I in 0.5 μg/μL digested plasma were performed with the FAIMS Pro device on the TSQ Altis triple quadrupole mass spectrometer. The overall % RSD was <10% across a 2-day experiment. (Thermo Fisher™ ChromaCare™ solution was injected every 10 analytical injections for column clean-up.)

Pierce System Suitability Standard (7 x 5 peptide mix)

The Pierce LC-MS/MS System Suitability Standard (7 x 5 mixture) contains seven peptides provided at five dilution levels, distinguished by differential isotopic labelling. Each concentration level has different labelling for each of the seven peptides (no heavy, 1 heavy, 2 heavy, 3 heavy, and 4 heavy labelled amino acids). Data from only one injection allows construction of five-point calibration curves for all seven analytes at once. The 7 x 5 mixture is intended to be used for testing LC-MS/MS systems for sensitivity, reproducibility, and response linearity. The 7 x 5 mixture prepared in 0.3 µg/µL digested plasma was analyzed on the TSQ Altis mass spectrometer with the FAIMS Pro interface using an optimized SRM method and optimized CV values for each of the seven peptides. Figure 6 shows linearity across the entire concentration range of the assay. Five repeated injections of the 7 x 5 standard were performed to assess robustness. The calculated % RSD of absolute peak areas showed very good reproducibility for all peptides (Table 3). This demonstrates that when using the FAIMS Pro interface, data is consistent and reproducible.

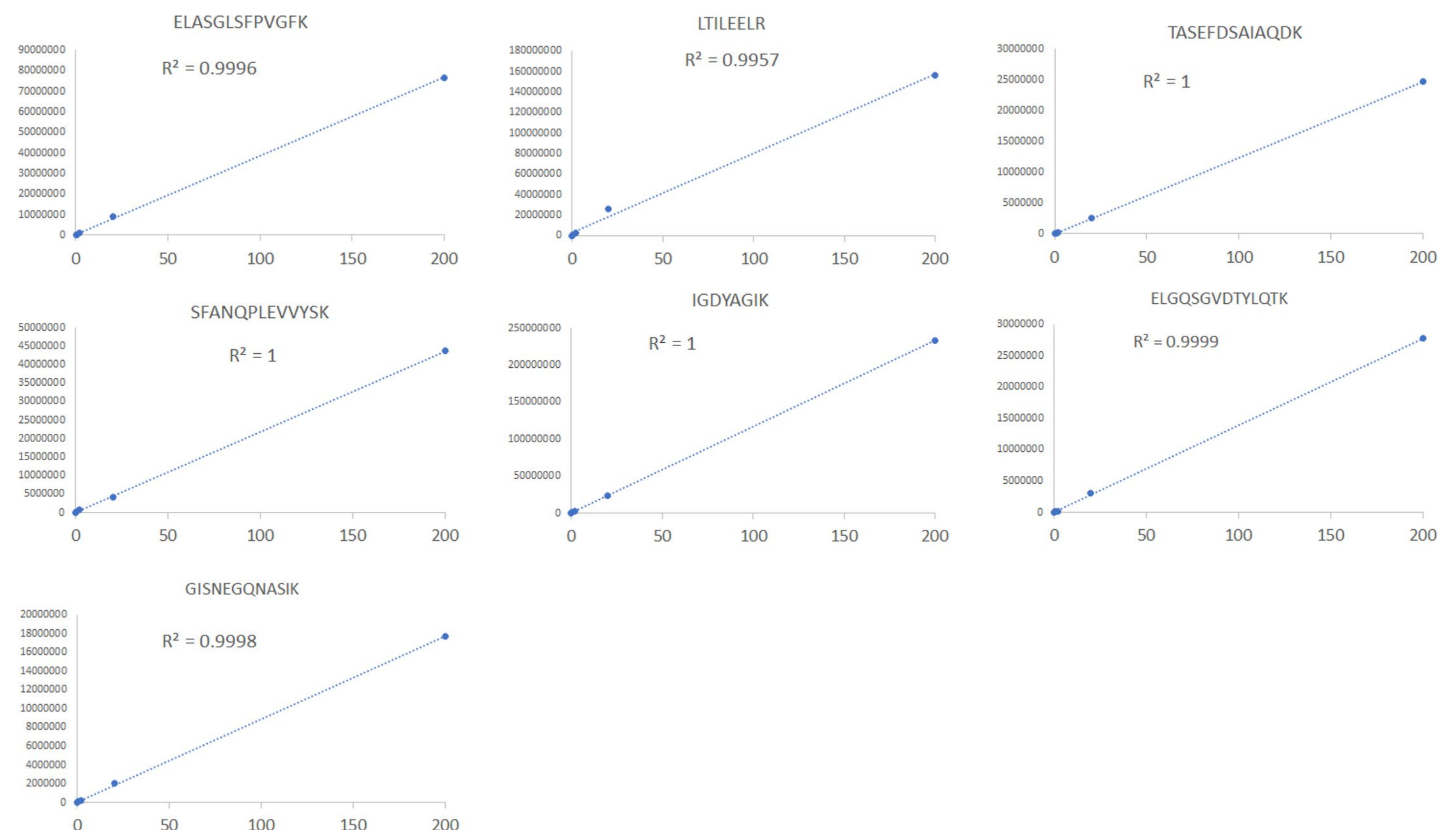

Figure 6. The Pierce 7 x 5 System Suitability Test was used to evaluate the performance of the FAIMS Pro interface with the TSQ Altis triple quadrupole mass spectrometer. The mixture was prepared according to manufacturer's protocol and 0.3 µg/µL digested plasma was used as a matrix.

Table 3. % RSDs (absolute peak areas) for five replicate injections of seven peptides at five concentration levels (isotopologues included in the Pierce System Suitability Test)

Peptide	0.13 fmol [%RSD]	0.5 fmol [%RSD]	2 fmol [%RSD]	20 fmol [%RSD]	200 fmol [%RSD]
ELASGLSFPVGFK	6.1	8.9	10.6	10.8	7.4
ELGQSGVDTYLQTK	6.6	4.1	8.9	6.1	5.9
GISNEGQNASIK	6.1	8.1	8.7	5.6	5.9
IGDYAGIK	2.9	2.1	2.9	3.1	1.9
LTILEER	8.2	8.8	12.6	4.3	11.7
SFANQPLEVVYS	9.6	8.4	11.8	15.1	15.1
TASEFDSAIAQDK	4.8	3.8	3.8	2.9	3.5

Isomeric interference

The FAIMS Pro interface is a differential ion mobility device that utilizes separation principles orthogonal to both LC and MS. The main benefit of using the FAIMS Pro interface for targeted assays is the removal of background interferences and therefore an increase in S/N and improvement of LOQ. However, depending on the assay, isomeric species can exhibit different behavior inside the FAIMS electrodes. Differences in CV values can then be used to remove isobaric interference. An example is shown in Figure 7. Two isomeric peptides, one analyte and one unknown interference from the sample matrix, co-elute at very close retention times and have identical SRM transitions. For demonstration purposes, the analyte peptide was replaced by its labelled internal standard. In the standard assay without the FAIMS Pro interface, the unknown interfering peptide makes quantification impossible because its signal fully overlaps with the analyte peak. The FAIMS Pro device separates the isobaric peptides based on different CV values. At a CV value of -45 V, the analyte is transmitted with the same efficiency as without the FAIMS Pro interface. The background isobaric interference, however, is significantly suppressed (roughly by 20x, from 7000 to 300 intensity counts (Figure 7). This enables accurate quantification of the analyte of interest.

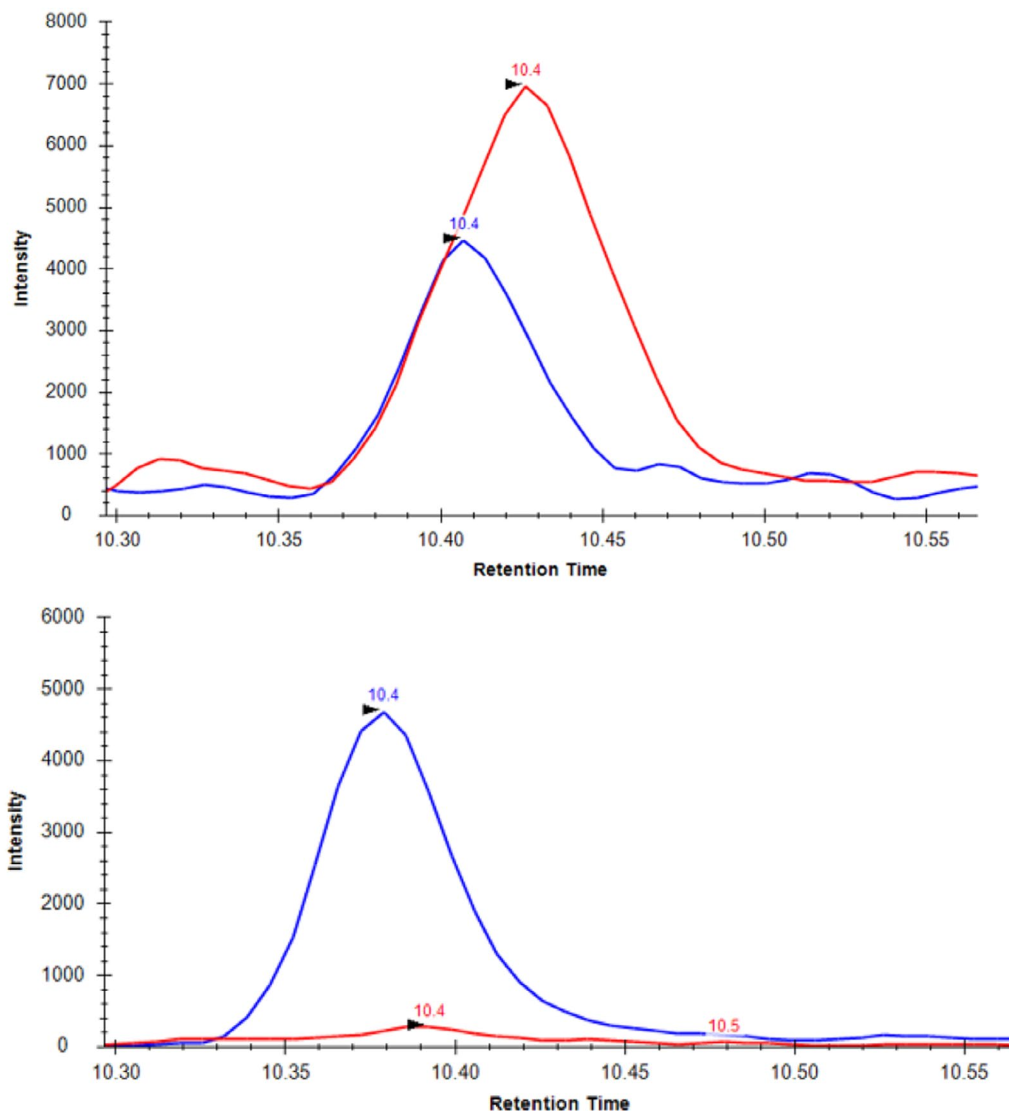

Figure 7. FAIMS Pro interface suppresses interfering isomeric peptide species. Two isomeric peptides, one analyte and one unknown interference from the sample matrix, co-elute at very close retention times and have identical SRM transitions. For demonstration purposes, the analyte peptide was replaced by its labelled internal standard to be distinguishable. Top: Standard assay without the FAIMS Pro interface. The interfering peptide (red) makes quantification impossible because its signal overlaps with the analyte peak (blue). Bottom: Using the FAIMS Pro interface at the optimized CV value of the analyte (blue), the interference (red) is sufficiently suppressed to enable quantification. Data processed in Skyline (University of Washington).

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Conclusion

The FAIMS Pro interface offers orthogonal precursor ion selectivity based on differential gas phase mobility. The compensation voltage (CV) setting determines which groups of ions pass the FAIMS Pro interface to the mass spectrometer. In targeted workflows on triple quadrupole mass spectrometers, the CV value is used as an additional parameter in the SRM table to selectively transmit precursor ions, while suppressing background interferences. The FAIMS Pro interface coupled to the TSQ Altis triple quadrupole mass spectrometer improves peptide quantification due to reduced noise and removal of co-eluting interferences. Utilization of the FAIMS Pro interface is robust and analyte signals are reproducible. FAIMS technology can significantly improve LOQs for assays where noise is the limiting factor. Because of its capabilities to remove background ions and reduce the level of interferences, combining the FAIMS Pro interface with the TSQ Altis triple quadrupole mass spectrometer increases the dynamic range and enables accurate quantification of peptides at low levels even from complex matrices.

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