Simultaneous Monitoring of Multiple Transitions in Mass Spectrometric Analysis Improves Limit of Detection for Low Abundance Substances in Complex Biological Samples

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Abstract
In mass-spectrometry, simultaneous monitoring of several transitions enables increased assay specificity, but also can be used as a tool to improve the lower limit of quantification. In this report, we propose a new approach to low abundant analyte detection in complex samples. The method consists of collecting two and potentially more, different mass spectrometry transitions with further analysis of a product function. For example, C-peptide detection was evaluated with two transitions monitored (1007.7-147.2 and 1007.7-927.8). Further extension of the method is theoretically analyzed and its potential limitations are discussed.

Keywords: Mass spectrometry; Limit of detection; SRM; C-peptide

Introduction
High sensitivity and specificity are crucially important in quantitative MS method development. Compared to Selected Ion Monitoring (SIM), Selected Reaction Monitoring (SRM) provides higher specificity. In analysis of large molecules, such as peptides and proteins, Collision Induced Dissociation (CID) can potentially result in many fragments, and monitoring a certain number of them is essential to have reliable sequence coverage. Additional transition reaction(s) can definitely increase the specificity of analysis for low abundant species detection. This becomes extremely important in quantifying endogenous or exogenous species in biological samples or cell extracts, containing many thousands of peptides and proteins [1-3]. Much less evident is the possibility of using several collision induced reactions for improving LOD and LLOQ, and in this communication we demonstrate such an example.

Insufficient chromatographic separation may result in co-elution of isobaric precursor species. Typically, when the standard deviation of the area ratio of the product ions (qualifier and quantifier) exceeds 20%, this is a flag for loss of specificity. Frequently, the most abundant product ions have only one or two amino acid residues, which are not highly specific. This situation is a potential reason for “false positive” signals [4]. When collision induced dissociation (CID) results in a number of fragments, frequently the detection method is based on the transition which provides the most intensive signal, although depending on the structure and physical properties of the compound analyzed, its LC/MS analysis can be done based in different transitions. Sometimes, there exist transitions of comparable efficiency that yield similar signal intensities that could be equally useful for method development. By utilizing both transitions, there is an opportunity for simultaneous multiple transition signal monitoring. One of the obvious advantages of such an approach is the detection method specificity increases [5].

At the same time the existence of multiple abundant product ions allows for introduction of a combined function. The new function represents the product of all counts corresponded to individual transitions monitored and this procedure essentially facilitates quantifying analytes at extremely low concentration, as we’ll show further.

In recent years, we have intensively investigated C-peptide and its behavior under different separation conditions. We have developed IDA reference methods for plasma and urinary C-peptide that applies isotope dilution electrospray ionization (ESI) and collision-induced dissociation (CID) of the triple charged peptide [6,7]. C-peptide is known as the definitive biomarker for insulin secretion. Being produced in equimolar concentration with insulin, C-peptide undergoes no metabolism by the liver, while the insulin is significantly cleared. Therefore, C-peptide concentration in plasma is three to five times higher compared to insulin levels for normal, obese and type 2 diabetic humans under fasting conditions. C-peptide undergoes partial urinary clearance resulting in considerably higher concentrations in urine (around one order of magnitude). Human C-peptide is a large hydrophobic 31-amino acid peptide with Mw 3020. The first report of serum quantification of C-peptide by LC/MS by Kippen et al. [8] in 1996 used single ion monitoring; subsequent publications by other groups have described different approaches for C-peptide quantitation [4,9].

In our work [5], we successfully used simultaneous monitoring of two mass transitions in order to provide higher specificity and to exclude false-positive signals. We monitored the two transitions 1007.7-142.7 and 1007.7-927.8. In the present communication we propose to use the data on two (or more) simultaneously obtained collision dissociation transitions for the purpose of improving the Lower Limit Of Quantification (LLOQ).

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Results and Discussion

The main idea of the current approach is that the product function of several different transitions (two or more) can provide a higher signal, which allows more reliable compound identification, even in the case when each signal for a single transition is comparable to the noise level. Indeed, let us consider a simple idealized situation with $n$ is the number of transitions monitored. Each corresponding graph has approximately the same low signal to noise ratio $R_0$, and $S/N$ can beat the standard cut off threshold, usually taken as three or close to that value. By multiplying all the $n$ functions, one obtains for the signal to noise ratio of the product function, $R_n$:

$$ R_n = (R_0)^n $$  \hspace{1cm} (1)

This idealized consideration does not take into account many real technical and instrumental aspects, but still clearly shows the potential of the approach for lowering the LLOQ, since the resulting product function always possesses considerably improved signal to noise level. The latter exponentially grows with the number of individual transition considered ($n$).
In Figures 1-3, we illustrate the improvement in LOD for C-peptide analysis when simultaneous monitoring two transitions. Each figure contains three panels. The two upper panels represent mass-spectrograms for each single reaction (1007.7-147.2) – Y1 and (1007.7-927.8) – Y9 ion [panels A and B, respectively]. The lower panel (C) corresponds to the product of the two upper transitions. The product graph is normalized to give a unit value of maximal intensity to the peak of interest. The figure clearly shows that product function of the two transitions provides considerably higher signal compared to the background that makes peak easily quantifiable, while no one single MRM transition does provide a detectable signal.

In the analyzed example for the pairs of MRM, we considered the multiplication product function of the two different daughter ions having the same precursor parent ion, but technically this multiplication approach could potentially be applied wherever multiple simultaneous signals are obtained from the same analyte. What is critically important is the fact that the two signals (in this case MRM transitions) occur exactly at the same time. In particular, one can imagine methods like chromatography or electrophoresis where the same analyte is monitored at different wavelength (although more potential interferences could be expected, in contrast to MS-MS example we analyzed). Additional applicability of this theoretical approach in mass spectrometry would be the monitoring of multiple charged species (different m/z).

Figure 2: A moderate sample load (8 pg) was specially selected to illustrate the intermediate situation. One transition, 1007.7-147.2, gives a quite weak signal (but still would be considered reliable), while the second reaction doesn’t provide a signal that can be differentiated from background. The product function represents a good signal.
The proposed example is a demonstration of how to reconstruct a readily quantifiable signal based on two ones of relatively low quality. To a certain extent, what makes the demonstration so effective is the nature of the two initial signals considered. They are quasi-discrete low density signals that result in almost complete background elimination. In the case of smooth functions the background will not disappear that effectively, by still will be subjected to considerable reduction. In general case the efficiency of the procedure can be essentially influenced by a particular type of individual signals and the method of detection.

Our approach reported here could be compared to the other one reported recently [10] and already followed by other communications that used the concept of arithmetically summing two or more transitions in order to improve LLOQ in detecting different substances. The authors reported a two-fold S/N increase by summation of two signals corresponding to the two transitions with roughly same intensity [10]. In general their approach potentially provides just a moderate increase in sensitivity. The advantage of the approach with a product function, where the increase in sensitivity is given by formula (1) is clearly visible. As an illustration we give here a comparison of the two approaches applied under the same conditions (Figures 3 and 4).

Figure 3: Low sample load (3pg) results in a situation where no one single transition reaction possesses a detectable signal (panels A and B). The product function, nevertheless, allows for C-peptide detection easily (panel C).

![Graph](image-url)
Figure 4: Summation of the same transition pair signals from Figure 3 results in just moderate quality improvement for the final signal. Low sample load; under the same conditions as in Figure 3, the sum of signal 4A and 4B is shown in panel 4C. There is no significant improvement for the combined signal, compared to individual signals. In contrast, the multiplication product function clearly demonstrates a significantly increased S/N ratio (Figure 3C and Figure 4C).

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