Automated MRM Method Optimizer for Peptides: Optimizing Mass Spectrometry Parameters for High-Throughput Protein Quantitation

Application Note

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Abstract

Multiple Reaction Monitoring (MRM) is commonly used in high-throughput protein quantitation experiments. Mass spectral parameters for MRM transitions must be optimized individually to enhance the sensitivity in the MRM studies. MassHunter Optimizer software provides a way to automatically optimize the data acquisition parameters on a triple-quadrupole instrument for MRM analysis. In this study, 40 MRM transitions were selected from 10 *Pyrococcus furiosus (*Pfu) proteins identified in a Q-TOF experiment and collision energies were optimized using the MassHunter Optimizer software for peptides. Five different collision energies (CE) were tested for each MRM transition. Up to a two-fold increase in sensitivity and signal-to-noise (S/N) ratio of MRM peaks were achieved using optimized collision energies as compared to collision energies derived from the Q-TOF experiment. Forty MRM transitions from 20 peptides could be optimized in a single LC/MS/MS analysis (45 minutes). Manual optimization of collision energies for 40 MRM transitions individually will require 200 LC/MS/MS analyses. The MassHunter Optimizer software helps reduce the time and amount of sample required for optimization by up to 200-fold.

Introduction

Multiple Reaction Monitoring is used for protein quantitation and biomarker validation processes. Hundreds of MRM transitions may have to be optimized for quantitation of multiple proteins to validate the putative biomarker candidates from a biomarker discovery study. Agilent's MassHunter Optimizer software provides a way to automatically optimize the data acquisition parameters on a triple-quadrupole instrument for each MRM transition analyzed on a chromatographic time scale. The MassHunter Optimizer software for peptides also helps to predict and select MRM transitions from peptide sequences when there is no MS/MS data available. In this study, 40 MRM transitions selected from 10 Pfu proteins were optimized in a single LC/MS/MS analysis using the MassHunter Optimizer software for peptides.

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Experimental

Sample preparation

Complex Proteomics Standard (Agilent Technologies, Inc., P/N 400510), composed of a complex mixture of proteins extracted from Pfu, was used in this study. The protein mixture was reduced, alkylated, and digested using trypsin (Agilent Technologies, Inc., P/N 204310) as described in the user manual. The digest was analyzed on an Agilent 6520 Accurate-Mass Q-TOF LC/MS System coupled to an Agilent 1200 Series HPLC-Chip/MS System for the initial identification of proteins/peptides. An Agilent 6410 Triple Quadrupole LC/MS System coupled to an Agilent 1200 Series HPLC-Chip/MS System was used for MRM experiments. An aliquot of 1 µg of Pfu digest was loaded on-column in each LC/MS analysis.

Selection of MRM transitions

MS/MS data from the Q-TOF analysis was searched against the NCBInr database using Spectrum Mill for MassHunter Workstation software. Forty MRM transitions were selected from the MS/MS spectra of 20 peptides from 10 proteins identified in the protein ID experiment. Precursor ion masses, product ion masses, and the retention time information for all the MRM transitions were obtained from the search results and used for MRM experiments.

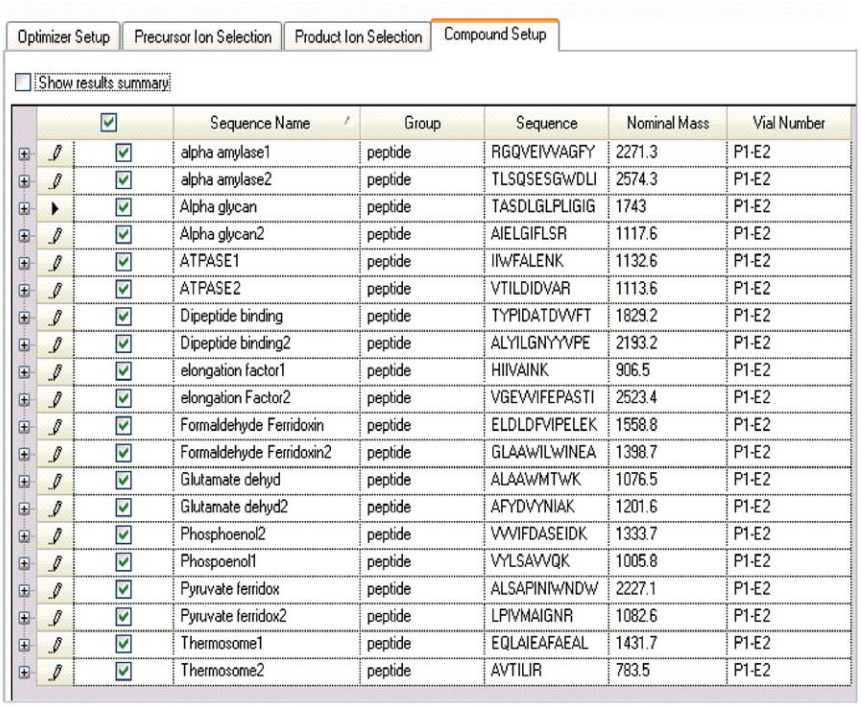
LC and MS conditions

LC and MS conditions used for the identification of the peptides/proteins were described previously.[1] MRM analysis was performed using an HPLC-Chip/MS System with a 40 nL enrichment column and a 75 mm x 43 mm analytical column packed with ZORBAX 300SB-C18 5 µm (300Å). The solvents were 0.1% formic acid in water (A) and 90% acetonitrile in water with 0.1% formic acid (B). The flow rates were 3 µL/min for loading the sample onto the enrichment column and 600 nL/min for the analytical column. Samples were loaded on the enrichment column using 3%B. The gradient used for the analytical column was as follows: 3%B at 0 min, 12%B at 3 min, 30%B at 37 min, 60%B at 40 min, 95%B at 42 min, and 3%B at 45 min.

Spectra were recorded in positive ion mode with a capillary voltage of 1950 V and drying gas flow rate of 5 L/min at 325ºC. Although MassHunter Optimizer can be used to optimize fragmentor voltage, there was no significant difference observed in the precursor ion abundance in the fragmentor voltage range tested between 100-150 V. Hence in this study, a constant fragmentor voltage of 135 V was used for all MRM transitions. Collision energies calculated with a proprietary algorithm were further optimized using MassHunter Optimizer. A dwell time of 5 ms was used for all MRM transitions. A cycle time of 1700 ms was automatically calculated by MassHunter Optimizer, which enabled acquisition of 10-15 data points across the chromatographic peaks.

Results and Discussion

Two peptides from each of the 10 Pfu proteins identified in the Q-TOF experiments were selected for optimization of collision energies. Two transitions from each peptide were selected, resulting in 40 MRM transitions for optimization. **Figure 1** shows a screenshot of the "compound set up" window in the MassHunter Optimizer software, which illustrates the peptide sequences, proteins from which the peptides were derived, and the calculated peptide masses.



Sequence Name	Group	Sequence	Nominal Mass	Vial Number
alpha amylase1	peptide	RGQVEIVVAGFY	2271.3	P1-E2
alpha amylase2	peptide	TLSQSESGWDLI	2574.3	P1-E2
Alpha glycan	peptide	TASDLGLPLIGIG	1743	P1-E2
Alpha glycan2	peptide	AIELGIFLSR	1117.6	P1-E2
ATPASE1	peptide	IIWFALENK	1132.6	P1-E2
ATPASE2	peptide	VTILDIDVAR	1113.6	P1-E2
Dipeptide binding	peptide	TYPIDATDVVFT	1829.2	P1-E2
Dipeptide binding2	peptide	ALYILGNYYVPE	2193.2	P1-E2
elongation factor1	peptide	HIIVAINK	906.5	P1-E2
elongation Factor2	peptide	VGEVVIFEPASTI	2523.4	P1-E2
Formaldehyde Ferridoxin	peptide	ELDLDFVIPELEK	1558.8	P1-E2
Formaldehyde Ferridoxin2	peptide	GLAAVILVINEA	1398.7	P1-E2
Glutamate dehyd	peptide	ALAAVMTVVK	1076.5	P1-E2
Glutamate dehyd2	peptide	AFYDVYNIAK	1201.6	P1-E2
Phosphoenol2	peptide	VVVIFDASEIDK	1333.7	P1-E2
Phospoenol1	peptide	VYLSAVVQK	1005.8	P1-E2
Pyruvate ferridox	peptide	ALSAPINIVVNDV	2227.1	P1-E2
Pyruvate ferridox2	peptide	LPIVMAIGNR	1082.6	P1-E2
Thermosome1	peptide	EQLAIEAFAEAL	1431.7	P1-E2
Thermosome2	peptide	AVTILIR	783.5	P1-E2

Figure 1. Screenshot from the MassHunter Optimizer software for the peptides "compound setup" window showing the peptide sequences and their calculated masses of 20 peptides from which 40 MRM transitions were optimized.

Five different collision energies in steps of 4 V were tested for each MRM transition. Collision energy values above and below the reference value were tested. To optimize the collision energy manually for one MRM transition, five LC/MS/MS analyses must be performed to test five different collision energies. Using the MassHunter Optimizer software for peptides, all five collision energy values can be tested in a single analysis. Furthermore, multiple MRM transitions can be optimized in a single LC/MS/MS analysis. In this study, 40 MRM transitions selected from the Q-TOF experiment were optimized in a single LC/MS/MS analysis. Manual optimization of collision energies for 40 MRM transitions will require 200 LC/MS/MS analyses. The software summarizes the optimized collision energies and abundance values for each of the 40 transitions as shown in **Figure 2**. (A partial list is shown in this figure). These results can be directly imported into MassHunter Acquisition software for MRM-based quantitation.

Figure 3 shows a comparison of two of the MRM transitions studied using the calculated collision energy and optimized collision energy. **Table 1** shows the peak area and S/N ratio observed in the MRM transitions in this figure. Up to a two-fold increase in the S/N ratio is observed in MRM transitions using optimized collision energies.

Show results summary

Sequence Name	Sequence	Nominal Mass	Method	Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance
Thermosome2	AVTILIR	783.5	D:\MassHunter\m	393.3	135	514.3	13	1296
						615.4	5	6556
elongation factor1	HIIVAINK	906.5	D:\MassHunter\m	454.3	135	223.2	20	20621
						657.4	16	9673
Phospoenol1	VYLSAVVQK	1005.8	D:\MassHunter\m	503.8	135	544.4	13	1091
						631.4	13	4994
Glutamate dehyd	ALAAWMTWK	1076.5	D:\MassHunter\m	539.3	135	256.2	15	220
						893.4	15	476
Pyruvate ferridox2	LPIVMAIGNR	1082.6	D:\MassHunter\m	542.3	135	873.5	23	568
						661.3	19	1042
ATPASE2	VTILDIDVAR	1113.6	D:\MassHunter\m	557.8	135	801.4	15	8763
						688.4	15	9782
Alpha glycan2	AIELGIFLSR	1117.6	D:\MassHunter\m	559.8	135	805.4	23	305
						692.4	19	225
ATPASE1	IIWFALENK	1132.6	D:\MassHunter\m	567.3	135	574.3	20	2648
						721.4	16	3047
Glutamate dehyd2	AFYDVYNIAK	1201.6	D:\MassHunter\m	602.3	135	608.3	17	2035
						822.4	17	2514
Phosphoenol2	VVVIFDASEIDK	1333.7	D:\MassHunter\m	667.9	135	1136.6	15	623
						924.4	19	755
Formaldehyde Ferr	GLAAWILWINEA	1398.7	D:\MassHunter\m	700.4	135	675.3	21	390
						788.4	25	408
Thermosome1	EQLAIEAFAEAL	1431.7	D:\MassHunter\m	716.9	135	331.2	29	812
						749.4	21	1978

Figure 2. A partial list of results obtained in the collision energy optimization for 40 MRM transitions.

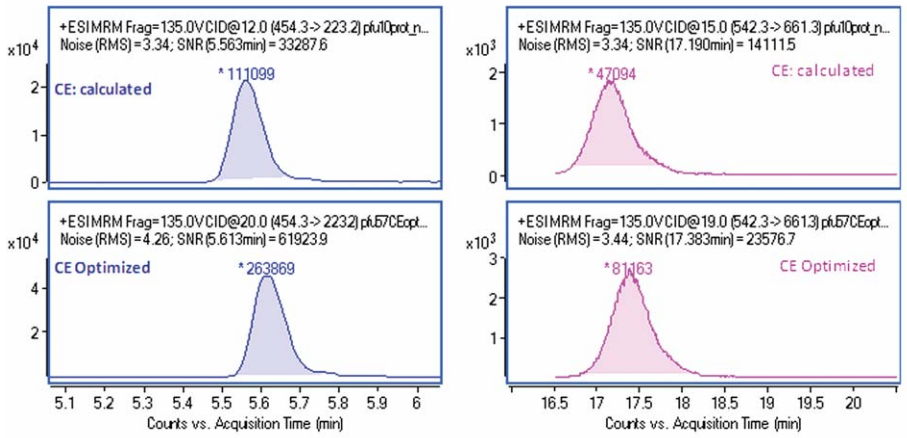


Figure 3. Comparison of calculated and optimized collision energies in MRM transitions listed in Table 1.

Transition	454.3 → 223.2		542.3 → 661.3	
	Calculated CE 12 V	Optimized CE 20 V	Calculated CE 15 V	Optimized CE 19 V
Area	111099	263869	47094	81163
S/N ratio	33287.6	61923.9	14111.5	23576.7

Table 1. Peak area and S/N ratio in the MRM transitions shown in Figure 3.

When Q-TOF data is not available, MassHunter Optimizer can be used to select the precursor ion and product ions that will provide more sensitive MRM transitions for a peptide. **Figure 4** shows the results obtained in optimizing collision energies for various MRM transitions for the 2+ and 3+ charge states of the peptide **GFYFNKPTGYGSSSR.** The most abundant transition arising from the 3+ charge state of the peptide, 556.6 ⟶ 1153.5, can be selected as the quantifier for this peptide. MRM transitions arising from the same precursor ion giving different product ions have varied optimum collision energies. This indicates that collision energies must be optimized for individual MRM transitions to improve the sensitivity in protein quantitation using MRM analysis.

Conclusions

- The MassHunter Optimizer software for peptides helps to select sensitive MRM transitions that can be used for protein quantitation.
- 40 MRM transitions could be optimized in a single LC/MS/MS run (45 minutes) using MassHunter Optimizer software.
- Number of sample injections required for the optimization is reduced by 200-fold.
- Amount of sample required for optimization is also reduced by 200-fold.
- Up to a two-fold increase in sensitivity and S/N ratio of MRM peaks is achieved using optimized collision energy values.

Optimizer Setup | Precursor Ion Selection | Product Ion Selection | Compound Setup

Show results summary

Sequence Name	Sequence	Nominal Mass	Method	Precursor Ion	Fragmentor	Product Ion	Collision Energy	Abundance	Product Ion Name
IGFpeptide1	GFYFNKPTGYG	1666.77	D:\MassHunter\m	556.6	135	656.3	49	1274	y6
						713.32	17	601	y7
						814.37	9	726	y8
						911.42	9	979	y9
						1039.52	9	899	y10
						1153.56	41	3963	y11
						577.28	21	1272	y11
						1300.63	21	1374	y12
						650.82	21	1048	y12
						1463.69	9	1436	y13
						732.35	9	1130	y13
						1610.76	50	1578	y14
						805.88	9	444	y14
				834.39	135	911.42	49	1024	y9
						1039.52	25	1387	y10
						1153.56	50	1283	y11
						1300.63	25	1272	y12
						1463.69	21	1638	y13
						1610.76	29	2187	y14

*Figure 4. Collision energy optimization for MRM transitions from 2+ and 3+ charge states of the peptide **GFYFNKPTGYGSSSR**.*

Reference

S. Rajagopalan, R. Gudihal, and K. Waddell, "Dynamic MRM: A Clear Advantage for High-throughput Protein Quantitation," Agilent publication number 5990-5092EN, **2010**.

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