Tunable electron activated dissociation (EAD) MS/MS to preserve particularly labile post-translational modifications

Site-localization of malonylated peptides using the SCIEX ZenoTOF 7600 system

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Post-translational modifications (PTMs) are important players in a diverse group of functions that include protein conformation and signaling. Lysine acylation, such as malonylation, is one such PTM, and is regulated in part by lysine deacylases, which are members of the sirtuin (SIRT) protein family. In a previous study investigating SIRT5-regulated lysine malonylome, it was shown that 183 malonyllysine sites (from 120 proteins) out of the 1,137 identified sites (from 430 proteins) were significantly increased in *Sirt5*$^{-/-}$ KO versus wild-type mice.[1] Specifically, it revealed that malonylation regulated GADPH activity. Malonylated peptides are however traditionally difficult to characterize using mass spectrometry and CID because the modification is extremely labile.

In this work, that involves using EAD technology with tunable kinetic energy, the effects of kinetic energy ramping on the preservation of highly labile PTMs (malonylation, for example) was studied, focusing on one previously identified malonyllysine site from GADPH (K-192). Two orthogonal fragmentation modes were compared (EAD vs. CID), to investigate the utility of each for PTM site localization. In addition, samples were measured using MRM^{HR} mode to investigate the use of EAD for quantitative PTM characterization of labile modifications.[2] MS/MS data were acquired with the Zeno trap activated, which provides significant sensitivity increases and enhances the quality of EAD MS/MS spectra.

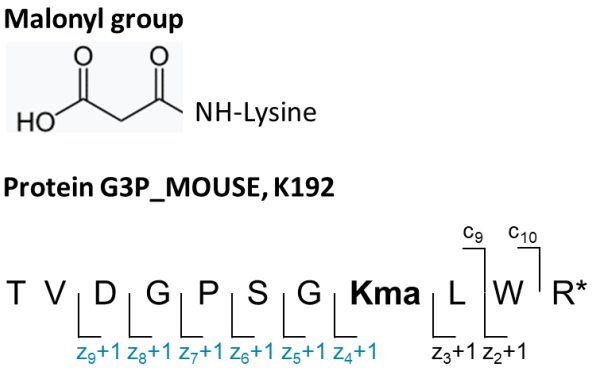


Figure 1. Sequence of the investigated malonylated peptide. Peptide at position 185-195 in mouse glyceraldehyde-3-phosphate dehydrogenase (P16858) and carrying a malonyl group on lysine **K-192** was investigated. PTM site-specific ions are highlighted in blue.

Key features of SCIEX ZenoTOF 7600 system for PTM characterization

- ZenoTOF 7600 system enables acquisition of high-resolution MS and MS/MS spectra at high acquisition rates (up to 130Hz)
- Multiple fragmentation capabilities for flexibility: collision induced dissociation (CID) and electron activated dissociation (EAD)[3]
- Use of the Zeno trap for 5-10x increase in signal intensity on MS/MS fragment ions[3]
- Prevention of neutral loss from labile PTMs (no CO_2-loss from malonyl modifications) using EAD MS/MS
- Tunable kinetic energy for EAD MS/MS using the EAD cell can be optimized and tailored for specific PTMs
- Efficient EAD product ion fragmentation generated strong ions for PTM site localization
- Preliminary assessment of EAD fragment ion quantification shows quantitative performance using high-resolution MRM (MRM^{HR}) workflows

Methods

Sample preparation: Malonylated synthetic peptides were obtained from Thermo Fisher Scientific and diluted in simple matrix for analysis.

Chromatography: A NanoLC 425 system plumbed for microflow chromatography (5 µL/min) was used and operated in direct inject mode. The analytical column used was a 0.3 mm x 150 mm (2.6 µm particle size) Phenomenex Omega Polar column. Column temperature was controlled at 30°C. Short gradients of 8 minutes were used.

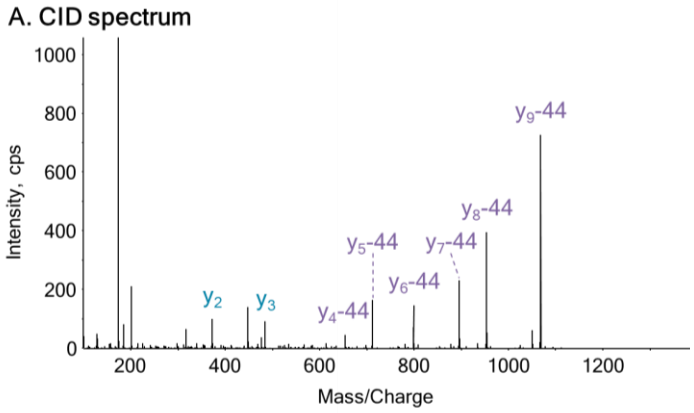
Mass spectrometry: All data were acquired using a SCIEX ZenoTOF 7600 system and an OptiFlow Turbo V ion source[4] equipped with the micro probe and 25 µm electrodes. The system is equipped with the electron activated dissociation (EAD) cell which enabled electron-based fragmentation to be performed in a targeted manner on the malonyl peptides. MRM^{HR} Acquisition data were collected using a TOF MS scan of 250 msec and MS/MS accumulation times of 150 msec both in CID and EAD mode. Kinetic energies were ramped and optimized per analyte. Electron current was also ramped to optimize and a final value of 5000 nA was used throughout study.

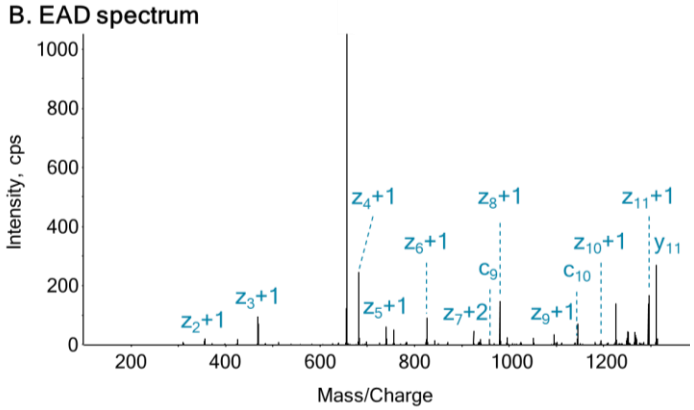
Data processing: The MRM^{HR} data was processed using SCIEX OS software 2.1 using both Explorer and Analytics modules.

EAD preserves labile PTMs preventing neutral losses

Malonylated peptide TVDGPSG**Kma**LWR from mouse glyceraldehyde-3-phosphate dehydrogenase (P16858) was synthetized with a malonylated lysine at position K-192 (Figure 1). MRM^{HR} analysis using CID fragmentation mode caused significant neutral loss of CO_2 (-44 m/z) from the labile malonyl group for the y-ion series (Figure 2A). However, EAD fragmentation generated intact z+1-ion and c-ion series, that were easily detectable in the MS/MS spectra (Figure 2B).[5] Specifically, fragment ions were observed, ranging from z_4+1 to z_{11}+1, or also c_9 and c_{10} that contained the labile PTM, that did not undergo a significant neutral loss, enabling confident PTM site localization.

Using the Peptide fragment pane in Bio Tool Kit, the fragment ion coverage is easily visualized between the two dissociation techniques (Figure 2C).





C. Fragment ion coverage for EAD spectrum

	Symbol	Res. Mass	# (N)	b	c	y	z	z + 1	z + 2	# (C)
▸	T	101.04768	1	102.05495	119.08150	*1311.65671*	1294.63016	1295.63799	1296.64581	11
	V	99.06841	2	201.12337	218.14992	1210.60903	1193.58248	1194.59031	1195.59813	10
	D	115.02694	3	316.15031	333.17686	1111.54062	1094.51407	1095.52189	1096.52972	9
	G	57.02146	4	373.17178	390.19832	996.51367	979.48713	980.49495	981.50278	8
	P	97.05276	5	470.22454	487.25109	*939.48221*	922.46566	923.47349	924.48131	7
	S	87.03203	6	557.25657	574.28312	842.43945	825.41290	826.42072	827.42855	6
	G	57.02146	7	614.27803	631.30458	755.40742	738.38087	739.38869	740.39652	5
	K[_Mal]	214.09536	8	828.37339	*845.39994*	698.38595	681.35941	682.36723	683.37506	4
	L	113.08406	9	941.45745	958.48400	484.29060	467.26405	468.27187	469.27970	3
	W	186.07931	10	1127.53677	1144.56331	371.20653	354.17998	355.18781	356.19563	2
	R[+10]	166.10938	11	1293.64615		185.12722	168.10067	169.10850	170.11632	1

Figure 2. MS/MS spectra of TVDGPSGKmaLWR peptide analyzed in CID and EAD modes. The malonylated peptide (m/z 656.3320) was analyzed in (A) CID mode and (B) EAD mode (KE = 5 eV). CID fragmentation resulted in neutral loss of -CO_2 (-44), and no "intact" PTM-specific differentiating ions were detectable. Comprehensive EAD fragmentation generated high-intensity fragment ions that provided evidence for straightforward PTM site localization. (C) Near complete sequence characterization was achieved with the EAD dissociation.

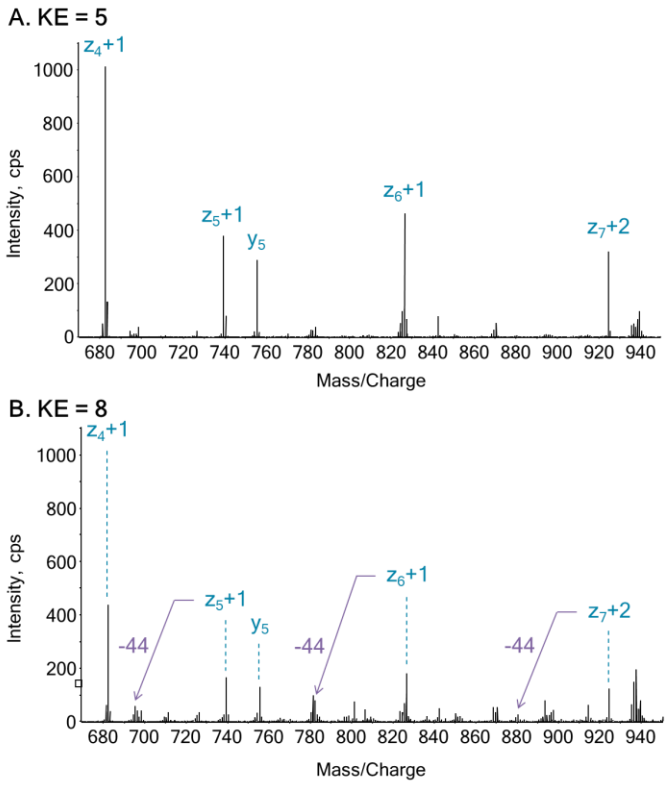


Figure 3. Impact of the kinetic energy (KE) value in EAD on fragmentation pattern. MS/MS spectra of malonylated peptide TVDGPSG**Kma**LWR (m/z 656.3320) analyzed in EAD mode with a kinetic energy value of (A) 5 eV and (B) 8 eV.

Kinetic energy ramping for EAD MS/MS

EAD fragmentation was performed across a range of kinetic energies, taking advantage of the tunable nature of EAD on the ZenoTOF 7600 system. This allowed determination of the optimal fragmentation parameters to preserve labile modifications on PTM modified peptides, while achieving best sensitivity. Kinetic energies were ramped from 0 eV to 11 eV. In this study, it was observed that a KE value of 5 eV generated high intensity, intact PTM site-specific fragment ions with very little background noise. When increasing KE to 8 eV or higher, MS/MS spectra became more complex, and neutral losses from fragment ions containing the PTM modification emerged (Figure 3).

Interestingly, different dissociation patterns were observed for fragment ions across the different kinetic energies during EAD MS/MS fragmentation (Figure 4). The peak area for most fragment ions containing the modified residue and the labile malonyl modification was highest at a KE value of 5 eV. With higher KEs some neutral PTM losses were observed, but a KE value of 5 eV maintained the integrity of the post-translational modification and maximized peak area intensity for site specific fragment ions.

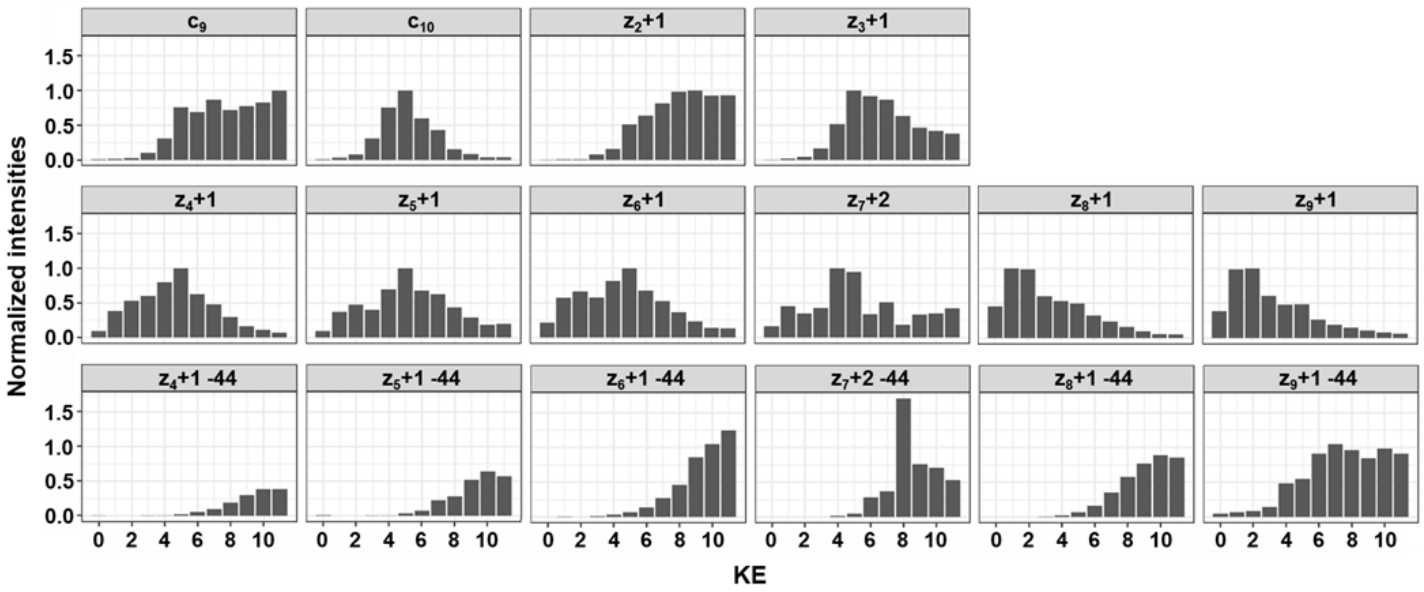


Figure 4. Kinetic energy ramping for EAD MS/MS. Malonylated peptide TVDGPSG**Kma**LWR was analyzed in EAD mode with kinetic energy (KE) values varying from 0 eV to 11 eV. Chromatographic peaks were extracted for 16 ions. For the intact ions (two first lines), peak area values were normalized on the highest area. For the ions with a neutral loss (third line), area values were normalized to their respective intact ion.

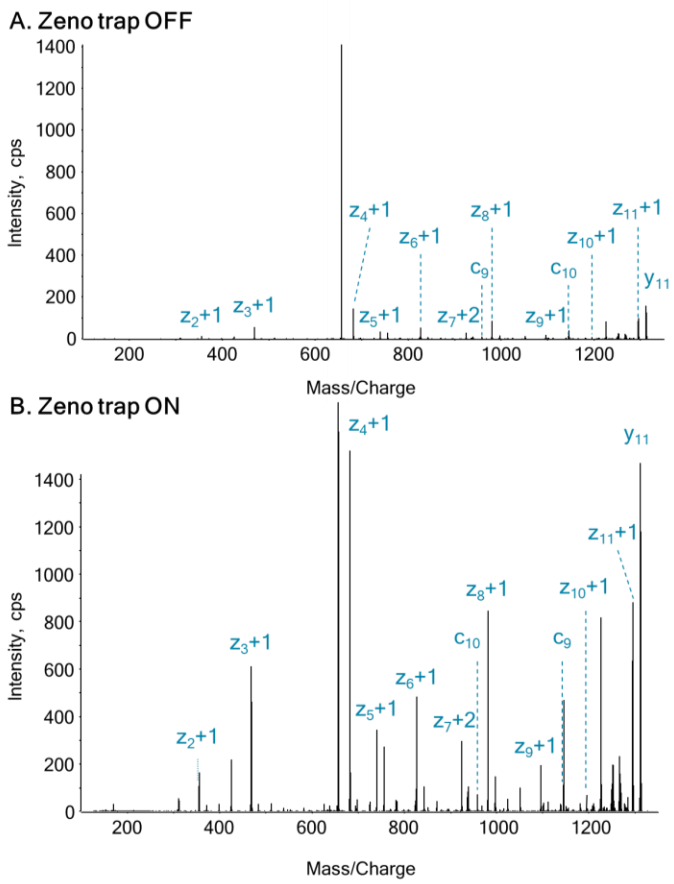


Figure 5. Increased sensitivity when the Zeno trap is activated. MS/MS spectra of TVDGPSG**Kma**LWR peptide (m/z 656.3320) analyzed in EAD mode (KE = 5 eV) (A) without and (B) with Zeno trap activated. Significant improvements in signal intensity and thus spectral quality was observed while using the Zeno trap in combination with EAD, enabling more confident PTM localization.

Zeno trap boosts MS/MS sensitivity for fragment ions

The impact on MS/MS spectral quality and sensitivity when using the Zeno trap was also investigated. The Zeno trap is located in the back half of the collision cell and is used to reduce ion losses between the collision cell and the accelerator. Ions are captured in the Zeno trap and then ejected in order of high *m/z* to low *m/z,* such that each ion reaches the center of the TOF accelerator simultaneously. This increases the duty cycle to greater than 90% through this region and greatly increases the sensitivity for MS/MS acquisition.[3]

EAD MS/MS spectra, collected with and without the Zeno trap activated, illustrate the clear gain in sensitivity provided by this feature (Figure 5). The ratios of extracted fragment ion peak areas, obtained with and without the Zeno trap activated, were determined for PTM site-specific ions at two different amounts loaded (Table 1). The sensitivity gain ranged between 3.5 and 9.6-fold for the various fragment ions, with an average value of 6.4. This highlights the added value of the Zeno trap for investigating very low-abundance ions.

Table 1. Gain of MS/MS sensitivity using the Zeno trap. TVDGPSG**Kma**LWR peptide was analyzed in EAD mode (kinetic energy = 5 eV) with and without using the Zeno trap at various amounts (16 and 80 fmol) on column. Chromatographic peak areas were extracted and sensitivity changes between having the Zeno trap on and off were determined.

Ions	m/z	Zeno trap on/off 16 fmol	Zeno trap on/off 80 fmol
z_4+1	682.3672	7.5	7.8
z_5+1	739.3887	9.6*	5.6
z_6+1	826.4207	8.0	8.0
z_7+2	924.4813	5.2	4.0
z_8+1	980.4950	7.0	6.8
z_9+1	1095.5219	4.0	6.2
c_9	958.4840	9.2*	4.1
c_{10}	1144.5633	3.5	6.8

** Weak signal without using the Zeno trap*

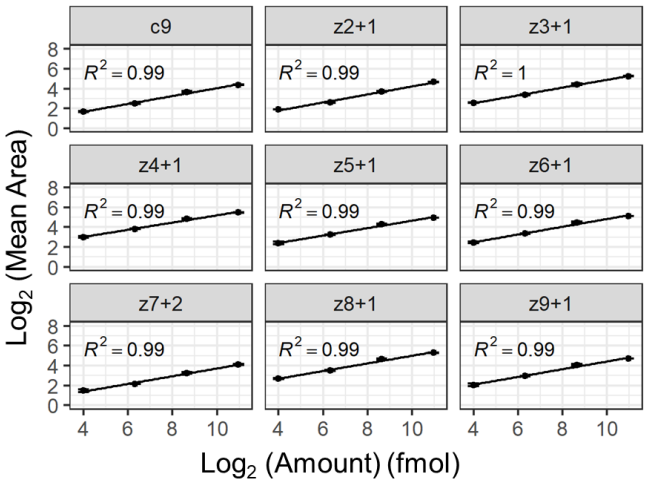


Figure 6. Linear response of fragment ions during EAD MRM-HR. Peptide TVDGPSG**Kma**LWR was analyzed in EAD mode (KE = 5 eV), in triplicate, loading various amounts (16, 80, 400 and 2000 fmol). 9 fragment ions are displayed. The R^2 coefficient for determination of the linear regression is displayed for each ion.

Preliminary assessment of quantitative response using targeted MRM^{HR}

To gain first insights into the quantitative performances of EAD MS/MS, initial dilution curves were generated by performing triplicate injections of 4 different peptide concentrations (16, 80, 400 and 2000 fmol on column) (Figure 6).

Good linearity performances were achieved for 9 investigated fragment ions ($R^2 \geq 0.99$) in these initial assessments.

Conclusions

In this work, the utility of electron activated dissociation on the SCIEX ZenoTOF 7600 system was investigated for the characterization and quantification of labile post translational protein modifications.

- Kinetic energy for EAD can be adjusted and exactly tailored to the different analyte necessities
- For this extremely labile PTM, malonyl, it appeared that a KE value of 5 eV was a good kinetic energy impact to preserve the PTM and to achieve optimal sensitivity
- The ability to optimize the kinetic energy for a specific PTM should enable accurate site-localization of labile PTM-containing peptides and maximize/improve quantification accuracy
- In addition, use of the Zeno trap provides significant gains in sensitivity, further improving the utility of this approach for characterizing PTMs
- Preliminary results using MRM^{HR} with EAD fragmentation suggests this approach should provide robust quantification of labile PTMs, but work is planned to explore this further.

References

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