Application Note

Biologics

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Examining the Structural Influence of Site-Specific Phosphorylation by Ion Mobility Mass Spectrometry

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Abstract

This application note describes an automated workflow for the analysis of protein phosphorylation from sample preparation with phosphopeptide enrichment to analysis by ion mobility mass spectrometry (IMS-MS). Single-field collisional cross section (CCS) measurements were combined with 4D feature extraction to demonstrate that phosphopeptides are more compact than nonphosphorylated peptides of similar *m/z* values. Differences in CCS values were found with peptides with varying numbers and locations of phosphorylation sites, as well as peptides with varying sequences but equivalent numbers and positions of phosphorylation sites.

Introduction

Phosphorylation is a reversible post translational modification influencing protein folding and activity occurring on approximately one-third of eukaryotic proteins. A challenge is to determine the sites, abundances, and roles of these modifications in biological samples, often occurring at low abundance, with inefficient ionization and fragmentation. Using IMS facilitates improved peptide identification, where ions are separated on the size-to-charge ratio. The ability to distinguish conformations allows the separation of isobaric and isomeric species, such as phosphopeptide positional isomers, which are difficult to distinguish by MS alone. This allows exportation of conformation-specific fragmentation spectra with their CCS values. This workflow uses automated sample preparation from digestion to phosphopeptide enrichment for IMS analysis. This application note presents a workflow involving an automated single-field CCS measurement coupled with 4D feature extraction.

Experimental

Sample preparation

Bovine α and β-casein and commercial PhosphoMixes 1 to 3 Light Phosphopeptide Standards were obtained from Sigma-Aldrich (St. Louis, MO). Bovine α and β-casein were denatured, reduced, alkylated with iodoacetamide, digested with trypsin, and desalted with C18 cartridges in an automated fashion with the use of the Agilent AssayMAP Bravo in accordance with a previous protocol.[1] The resulting phosphopeptides were enriched with Fe(III)-NTA cartridges according to the Agilent AssayMAP phosphopeptide enrichment v2.0 application.

The individual PhoshoMixes were diluted to 6.66 pmol/µL in 20 % acetonitrile, 0.1 % formic acid. Approximately 1 µg of the digested α and β-casein (injection volumes of 11 and 10 µL, respectively), 1 µg of the flowthrough, and eluate from the phosphopeptide enrichment (injection volume of 5 µL), and 6.66 pmol of the individual PhosphoMixes (injection volume of 2 µL) were used for analysis.

Instrumental analysis

For sample analysis, the Agilent Infinity UHPLC Nanodapter (G1988A)[2] was placed onto the Agilent Infinity II 1290 binary pump to provide nanoflow rates to the Agilent nanospray ion source (G1992A) (shown as an inset in Figure 1, outlined in a red box) on the Agilent 6560 ion mobility LC/Q-TOF (Figure 1). The LC/Q-TOF was tuned in positive polarity low (*m/z* 1,700) mass range using the SWARM autotune for analysis in the mass range of *m/z* 100 to 1,700. On the IM-QTOF, the dual ion funnel interface and rear ion funnel are operated at 100 and 150 V peak-to-peak, respectively.

The 6560 ion mobility LC/Q-TOF system contains an ~80 cm long drift tube operated with a weak electric field applied across the drift tube that enables CCS measurements to be determined by the transient time of the ion through the drift cell. This allows the drift time to be a function of the following instrumental variables:

- Temperature
- Pressure
- Mass of the analyte and buffer gas
- Charge state of the analyte
- Electric field applied across the drift tube

and converted into a CCS value by the Mason-Schamp equation.[3] The single-field CCS[4] is obtained using a calibration equation to convert arrival times to a CCS value. This is accomplished with the generation of a linear regression using standardized CCS values for tune mix calibrant ions that generates a slope and intercept.

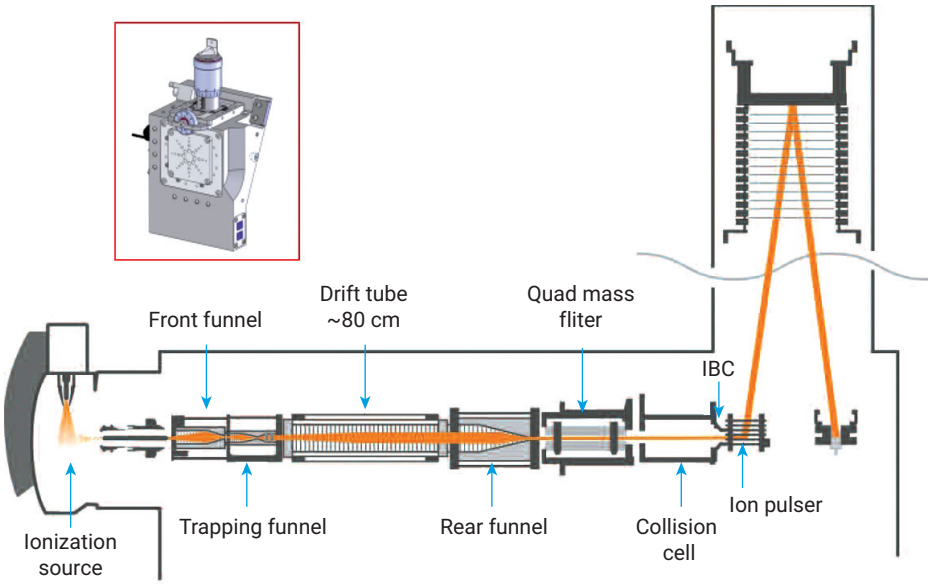

Figure 1. Schematic of the Agilent 6560 ion mobility LC/Q-TOF. The inset in the red box displays the schematic of the Agilent nanospray ion source (G1992A).

The terms in the linear regression can then be used to determine CCS values for unknown compounds measured at the same electric field applied across the drift tube and have reported *m/z* and charge state values.

Prior to analysis of the samples, Agilent ESI low concentration tune mix ions were infused at the same source and instrumental parameters used for the analysis of the bovine casein samples and PhosphoMixes with the use of a syringe and syringe pump at a flow rate of 18 µL/min. The instrument was operated in Alternating Frames, where MS and MS/MS analyses could be obtained in a single acquisition in ion mobility mode. For the MS/MS, a ramped collision energy as a function of drift time (Table 3) was applied to the collision cell in an All Ions approach, where all ions present are fragmented. Tables 1 to 3 consist of experimental and instrumental parameters.

Table 1. Nanosource parameters.

Parameter	Value
Sprayer Needle	New Objective noncoated needle (20 µm id, 10 µm tip id, 5 cm length) (p/n FS360-25-10-N-20-CT)
Gas Temperature	325 °C
Drying Gas Flow	5 L/min
Vcap	1,375 V
Fragmentor	175 V

Table 2. Liquid chromatography (LC) method setup.

Parameter	Value
Capillary Pump Flow Rate	4 µL/min (Agilent 1260 Infinity capillary pump)
Capillary Pump Mobile Phase	Water, 0.1 % formic acid
Trap Column	Thermo Acclaim PepMap, 75 µm × 2 cm (p/n 164535)
Analytical Column	Thermo Acclaim PepMap, 75 µm × 25 cm (p/n 164941)
Column Temperature	45 °C
Autosampler Temperature	4 °C
Binary Pump Flow Rate	0.11 mL/min primary flow ~300 nL/min on-column flow rate (Agilent 1290 Infinity II high speed pump)
Binary Pump Mobile Phase	Water, 0.1 % formic acid Acetonitrile, 0.1 % formic acid
Binary Pump Gradient	Time (min) % B 0 3 5 3 45 35 55 75 60 3
Stop Time	65 minutes
Post Time	7 minutes

Table 3. Agilent 6560 ion mobility LC/Q-TOF method setup.

MS Acquisition Parameters		
Instrument Mode	Positive, low (*m/z* 1700) mass range	
Ion Mobility Mode		
High Pressure Funnel RF	100 V	
Trap Funnel RF	100 V	
Drift Tube Entrance Voltage	1,500 V	
Drift Tube Exit Voltage	250 V	
Mass Range	100 to 1,700 *m/z*	
Alternating Frame Collision Energy	Drift time (ms)	Collision energy (V)
	0	0
	5	2
	10	5
	20	20
	30	30
	40	40

Auto MS/MS Parameters (Q-TOF Mode)		
	MS	MS/MS
Mass Range	*m/z* 100 to 1,700	*m/z* 50 to 1,700
Acquisition Rate/Time	10 spectra/s	5 spectra/s
Collision Energy	(Slope)*(*m/z*)/100+offset	
	Precursor Charge	Slope
	2	3.1
	3	3.6
	>3	3.6
Isotope Model	Peptides	
Sort Precursors	By abundance only; +2, +3, > +3	
Isolation Width	Medium, *m/z* 4	
Max Precursors/Cycle	3	
Threshold for MS/MS	1,000 counts	
Active Exclusion Enabled	Exclude after one spectrum, release after 0.15 minutes	
Precursor Abundance-Based Scan Speed	Yes	
Target	25,000 counts/spectrum	
MS/MS Accumulation Time Limit	Yes	

Data analysis

Data analysis was performed using Agilent MassHunter Qualitative Analysis 7 and BioConfirm 7. For IMS feature finding and CCS calculations, Agilent MassHunter IM-MS Browser 8 was used. The 6560 ion mobility LC/Q-TOF can also be used as a traditional Q-TOF. As a Q-TOF, auto MS/MS was performed on the digested and resulting eluate, and flowthrough α and β-casein phosphopeptide enriched samples for peptide identification. Features in the auto MS/MS dataset were determined with *Find Compounds by Auto MS/MS* to identify compounds in MS/MS data and create averaged MS and MS/MS spectra for each compound where compound-specific mass spectra and chromatograms can quickly be extracted. This was followed by targeted sequence matching for α- and β-casein in MassHunter BioConfirm. The matched peptides were used to help identify the same peptides in the IMS datasets.

Single-field CCS calculations were performed as described previously using the Agilent ESI low concentration tune mix ions and applying the linear regression and calibration coefficients to the subsequent IMS files using MassHunter IM-MS Browser. In the MassHunter IM-MS Browser, iMFE was used for compound extraction. Each compound contained the *m/z* observed, retention time, drift time, CCS, and MS/MS spectra.

Results and discussion

Analysis of α and β-casein

Figure 2 displays a comparison of the MS total ion chromatograms (TICs) resulting from the phosphopeptide enrichment of α-casein. Figure 2A displays the chromatogram from the tryptic digestion without phosphopeptide enrichment, Figure 2B corresponds to the TIC profile from the enriched phosphopeptides, and Figure 2C is the TIC of the resulting peptides found in the flowthrough. Comparison of the unique TIC profiles in Figure 2 supports the assertion that the sample preparation conducted on the AssayMAP was successful both for the tryptic digestion as well as the phosphopeptide enrichment.

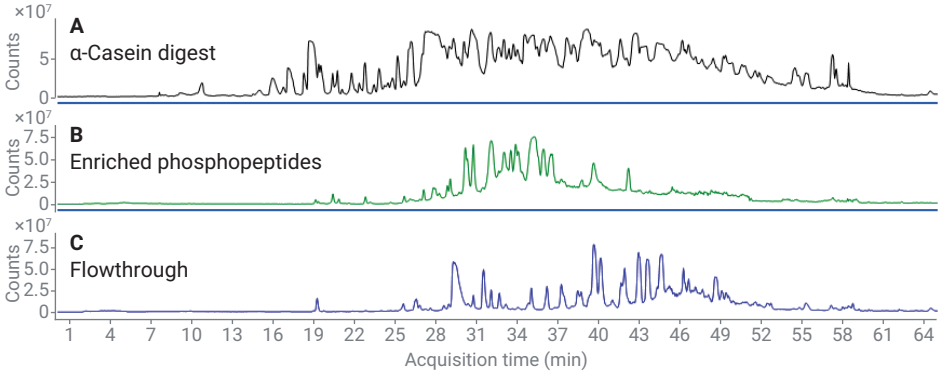

Figure 2. Total ion chromatograms (MS) of (A) α-casein digest, (B) enriched phosphopeptides, and (C) peptides found in the flowthrough resulting from the automated digestion using the Agilent AssayMAP Bravo and phosphorylation enrichment workflow.

One unique feature of ion mobility is the separation of isomeric structures that are not easily or readily obtained by LC/MS alone. An example extracted ion chromatogram (EIC) for the following doubly phosphorylated peptide from α-casein, ^{37}VNELSKDIGSESTEDQAMEDIK^{58}, with possible phosphorylated sites at amino residue positions 41, 46, and 48, is shown in Figure 3. Figure 3A displays the EIC for the $[M+3H]^{3+}$ ions of the phosphopeptide ^{37}VNELSKDIGSESTEDQAMEDIK^{58} with an *m/z* of 866.6892. Figure 3B shows the corresponding mass spectrum. When the drift time spectrum is extracted over the same retention times and *m/z* values in Figure 3C, two predominant peaks are observed. This suggests that there are multiple conformations for this phosphopeptide that are not distinguishable by LC/MS alone. The multiple conformations could be a result of isomeric structures or multiple sites of phosphorylation on the peptide ^{37}VNELSKDIGSESTEDQAMEDIK^{58}.

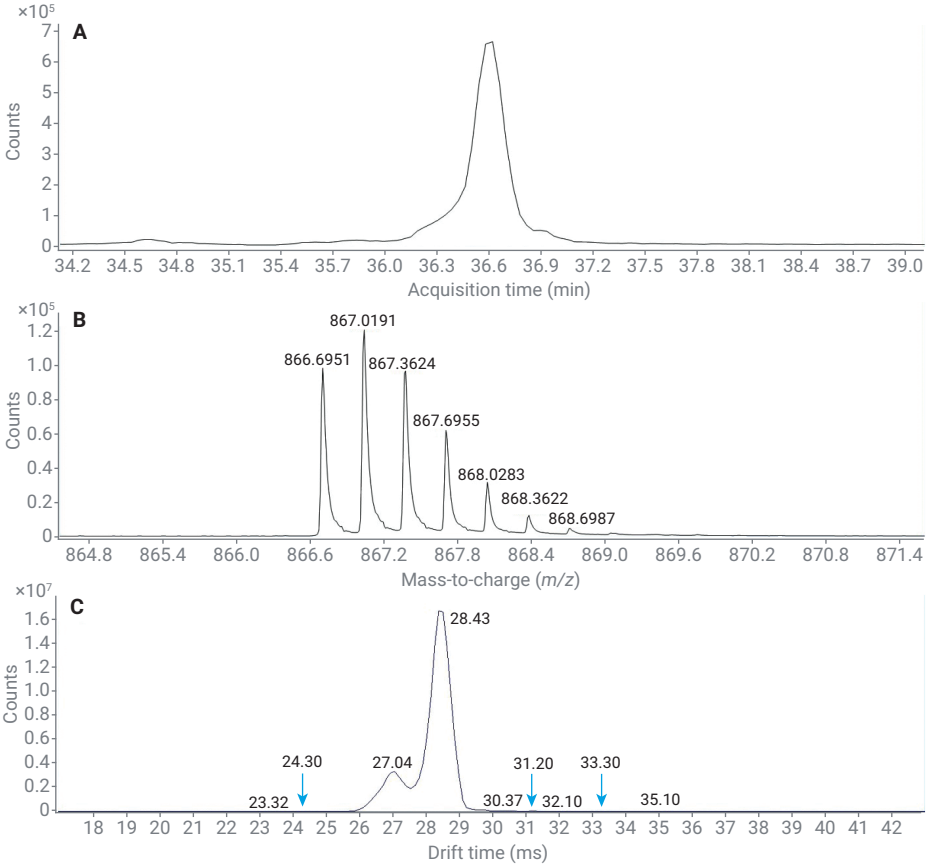

Figure 3. (A) EIC, (B) mass spectrum, and (C) drift time spectrum for the $[M+3H]^{3+}$ ions of the ^{37}VNELSKDIGSESTEDQAMEDIK^{58} phosphopeptide from α-casein, with an *m/z* of 866.6892.

Figure 4 displays a two-dimensional plot of the drift time as a function of *m/z* for the summation of the entire chromatogram, with intensity represented as a false color scale, reflecting the ion abundance with least intense features in blue and most intense in red. With the added dimension of separation provided by ion mobility separation, the different charge states fall along unique trendlines (labeled as +1, +2, +3, and +4) resulting from the increased force experienced by larger charge states as they travel though the drift tube, as shown in Figure 4.

Figure 5 displays a closer examination of one of the multiply phosphorylated peptides as a two-dimensional plot for the phosphopeptide ^{37}VNELSKDIGSESTEDQAMEDIK^{58}, from α-casein. For this peptide, multiple conformations are observed in the drift time distribution, a less abundant compact conformation and more abundant elongated conformations. The multiple conformations observed could be due to the multiple sites of phosphorylation possible within the peptide at residue positions 41, 46, and 48 or isomeric structures of the phosphopeptide, and would not be observed by LC/MS.

Table 4 lists the CCS values for the identified phosphopeptides and peptides and, for ease of visualization, plotted as a function of *m/z* for the $[M+2H]^{2+}$ (Figure 6A) and $[M+3H]^{3+}$ (Figure 6B) ions.

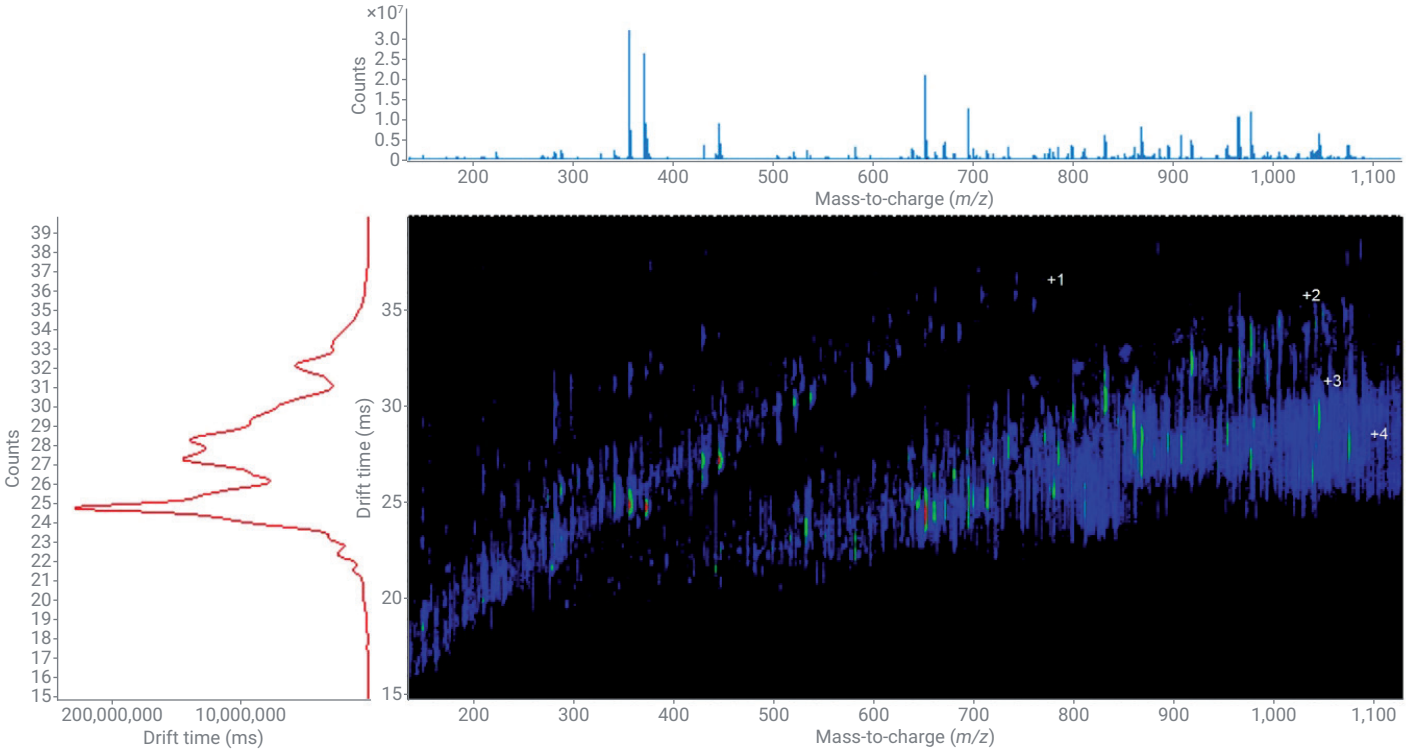

Figure 4. Two-dimensional plot displaying drift time as a function of *m/z* for the summation of all the frames with Agilent MassHunter IM-MS Browser, which corresponds to the collection of mass spectra observed at multiple drift times, from retention time 0 to 65 minutes, resulting from the α-casein digest phosphopeptide enrichment.

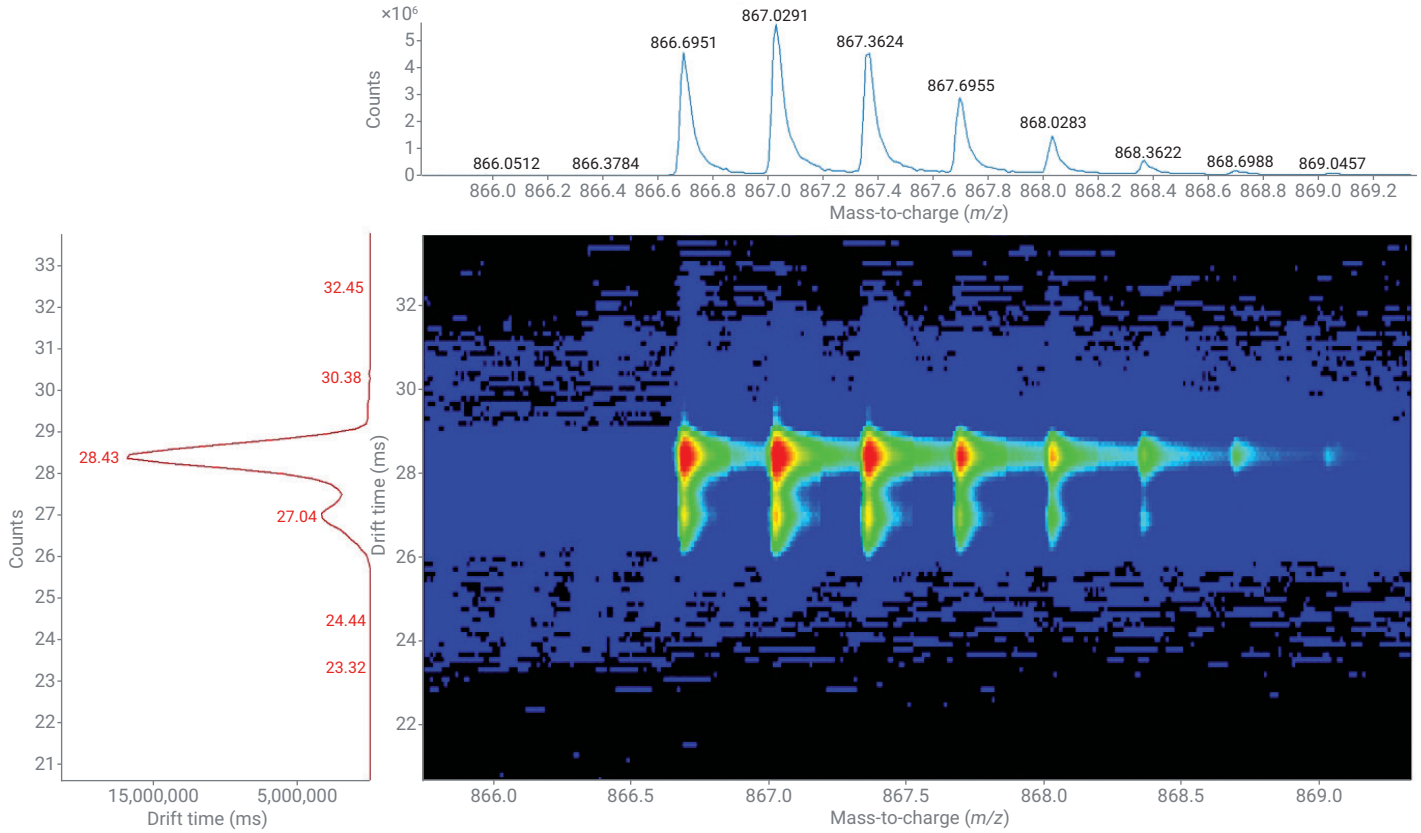

Figure 5. Two-dimensional plot displaying drift time as a function of *m/z* for the $[M+3H]^{3+}$ ions of the ^{37}VNELSKDIGSESTEDQAMEDIK^{58} phosphopeptide from α-casein, where there is a possibility of two phosphorylation sites at residue positions 41, 46, and 48.

Table 4. CCS values for identified peptides and phosphopeptides of α and β–casein.

Protein	Sequence Location	Sequence	Modification	Theoretical Mass (Da)	Observed Mass (Da)	RT (min)	Drift Time (ms)	*m/z*	Charge State	CCS (Å²)
α casein	A(161-165)	LNFLK		633.3850	633.3865	29.84	20.94	317.7005	2	294 ±0.6
α casein	A(200-205)	VIPYVR		745.4487	745.4493	27.55	22.26	373.7319	2	312 ±0.2
α casein	A(200-205)	VIPYVR		745.4487	745.4496	27.56	22.94	373.7321	2	321 ±0.2
α casein	A(161-166)	LNFLKK		761.4800	761.4805	25.00	21.69	381.7475	2	304 ±0.2
α casein	A(198-205)	TKVIPYVR		974.5913	974.5934	25.56	24.74	488.3040	2	345 ±0.3
α casein	A(174-181)	FALPQYLK		978.5539	978.5534	36.15	24.32	490.2840	2	339 ±0.3
α casein	A(174-181)	FALPQYLK	1*Deamidation(+0.984016)A178	979.5379	979.5385	36.93	24.71	490.7765	2	345 ±0.2
α casein	A(35-42)	EKVNELSK	1*Phosphorylation (S/T)(+79.966332)A41	1025.4794	1025.4784	19.17	23.95	513.7465	2	334 ±0.1
α casein	A(189-197)	AMKPWIQPK		1097.6056	1097.6056	26.42	22.19	366.8758	3	464 ±0.1
α casein	A(115-125)	NAVPITPTLNR		1194.6721	1194.6743	29.28	26.02	598.3444	2	361 ±1.7
α casein	A(71-80)	ITVDDKHYQK		1245.6354	1245.6368	19.70	21.02	416.2196	3	438 ±0.1
α casein	A(91-100)	YLGYLEQLLR		1266.6972	1266.6978	43.58	27.59	634.3562	2	384 ±0.2
α casein	A(80-90)	HIQKEDVPSER		1336.6735	1336.6741	19.22	22.65	446.5653	3	472 ±0.3
α casein	A(81-91)	ALNEINQFYQK		1366.6881	1366.6911	32.64	27.81	684.3528	2	387 ±0.4
α casein	A(81-91)	ALNEINQFYQK	1*Deamidation(+0.984016)A87	1367.6721	1367.6754	33.03	27.93	684.8450	2	389 ±0.1
α casein	A(23-34)	FFVAPFPEVFGK		1383.7227	1383.7248	44.58	27.93	692.8697	2	388 ±0.4

Protein	Sequence Location	Sequence	Modification	Theoretical Mass (Da)	Observed Mass (Da)	RT (min)	Drift Time (ms)	*m/z*	Charge State	CCS (Å^2)
α casein	A(80-90)	HIQKEDVPSER	1*Phosphorylation (S/T)(+79.966332)A88	1416.6399	1416.6380	20.17	22.80	473.2200	3	475 ±0.3
α casein	A(126-137)	EQLSTSEENSKK	1*Phosphorylation (S/T)(+79.966332)A129	1458.6239	1458.6249	19.10	27.92	730.3197	2	388 ±0.3
α casein	A(138-149)	TVDMESTEVFTK	1*Phosphorylation (S/T)(+79.966332)A143	1465.6048	1465.6032	34.12	27.85	733.8089	2	387 ±0.3
α casein	A(138-149)	TVDMESTEVFTK	1*Phosphorylation (S/T)(+79.966332)A143	1465.6048	1465.6046	34.12	30.21	733.8096	2	420 ±0.5
α casein	A(91-102)	YLGYLEQLLRLK		1507.8763	1507.8801	43.79	23.14	503.6340	3	482 ±0.4
α casein	A(137-149)	KTVDMESTEVFTK	1*Phosphorylation (S/T)(+79.966332)A138	1593.6997	1593.6977	30.10	22.98	532.2398	3	478 ±0.2
α casein	A(137-149)	KTVDMESTEVFTK	1*Phosphorylation (S/T)(+79.966332)A138	1593.6997	1593.6983	30.13	23.83	532.2400	3	496 ±0.2
α casein	A(137-149)	KTVDMESTEVFTK	1*Phosphorylation (S/T)(+79.966332)A138	1593.6997	1593.6998	30.14	25.35	532.2405	3	525 ±4.1
α casein	A(137-149)	KTVDMESTEVFTK	1*Phosphorylation (S/T)(+79.966332)A138	1593.6997	1593.7016	30.13	29.72	797.8581	2	412 ±0.7
α casein	A(138-150)	TVDMESTEVFTKK	1*Oxidation (M) (+15.994915);1*Phosphorylation (S/T) (+79.966332)A141A138	1609.6947	1609.6963	26.06	23.17	537.5727	3	482 ±0.2
α casein	A(153-165)	LTEEEKNRLNFLK		1632.8835	1632.8841	28.88	24.10	545.3020	3	502 ±0.1
α casein	A(23-36)	FFVAPFPEVFGKEK		1640.8603	1640.8625	40.11	26.63	547.9614	3	554 ±0.4
α casein	A(23-36)	FFVAPFPEVFGKEK		1640.8603	1640.8626	40.10	24.45	547.9615	3	509 ±0.1
α casein	A(106-119)	VPQLEIVPNSAEER	1*Phosphorylation (S/T)(+79.966332)A115	1659.7869	1659.7868	35.92	30.42	830.9007	2	422 ±0.5
α casein	A(106-119)	VPQLEIVPNSAEER	1*Phosphorylation (S/T)(+79.966332)A115	1659.7869	1659.7871	35.92	31.65	830.9008	2	440 ±0.4
α casein	A(106-119)	VPQLEIVPNSAEER	1*Deamidation(+0.984016); 1*Phosphorylation (S/T)(+79.966332) A108A115	1660.7709	1660.7720	37.21	30.30	831.3933	2	421 ±0.6
α casein	A(137-150)	KTVDMESTEVFTKK	1*Phosphorylation (S/T)(+79.966332)A143	1721.7947	1721.7946	27.07	24.08	574.9388	3	501 ±0.1
α casein	A(8-22)	HQGLPQEVLNENLLR		1758.9377	1758.9406	35.00	24.72	587.3208	3	514 ±0.6
α casein	A(8-22)	HQGLPQEVLNENLLR	1*Deamidation(+0.984016)A13	1759.9217	1759.9233	36.01	25.65	587.6484	3	534 ±0.9
α casein	A(8-22)	HQGLPQEVLNENLLR	1*Deamidation(+0.984016)A14	1759.9217	1759.9249	35.95	24.62	587.6489	3	514 ±1.6
α casein	A(43-58)	DIGSESTEDQAMEDIK	1*Phosphorylation (S/T)(+79.966332)A46	1846.7180	1846.7191	35.73	31.98	924.3668	2	445 ±0.4
α casein	A(104-119)	YKVPQLEIVPNSAEER		1870.9789	1870.9836	33.12	24.05	624.6685	3	500 ±0.1
α casein	A(104-119)	YKVPQLEIVPNSAEER		1870.9789	1870.9836	33.12	24.74	624.6685	3	514 ±0.2
α casein	A(104-119)	YKVPQLEIVPNSAEER	1*Phosphorylation (S/T)(+79.966332)A115	1950.9452	1950.9476	35.13	33.52	976.4811	2	465 ±0.2
α casein	A(104-119)	YKVPQLEIVPNSAEER	1*Phosphorylation (Y)(+79.966332); 1*Deamidation(+0.984016)A104A108	1951.9292	1951.9329	36.35	24.67	651.6516	3	513 ±0.5
α casein	A(25-41)	NMAINPSKENLCSTFCK	2*Alkylation (iodoacetamide)(+57.021464) A40A36	2012.9118	2012.9130	30.72	25.47	671.9783	3	529 ±1
α casein	A(25-41)	NMAINPSKENLCSTFCK	2*Alkylation (iodoacetamide)(+57.021464) A40A36	2012.9118	2012.9140	30.72	26.40	671.9786	3	549 ±0.7
α casein	A(182-197)	TVYQHQKAMKPWIQPK	1*Phosphorylation (Y)(+79.966332); 3*Deamidation(+0.984016) A184A195A185A187	2064.9744	2064.9902	35.50	25.00	689.3373	3	519 ±1
α casein	A(103-119)	KYKVPQLEIVPNSAEER	1*Phosphorylation (Y)(+79.966332); 1*Deamidation(+0.984016)A104A108	2080.0242	2080.0269	33.17	25.40	694.3496	3	528 ±0.2
α casein	A(103-119)	KYKVPQLEIVPNSAEER	1*Deamidation(+0.984016); 1*Phosphorylation (S/T)(+79.966332) A108A115	2080.0242	2080.0296	32.78	25.22	694.3505	3	525 ±0.5
α casein	A(25-41)	NMAINPSKENLCSTFCK	2*Alkylation (iodoacetamide)(+57.021464); 1*Phosphorylation (S/T)(+79.966332) A40A36A31	2092.8781	2092.8790	32.95	25.24	698.6336	3	525 ±0.3
α casein	A(25-41)	NMAINPSKENLCSTFCK	1*Oxidation (M)(+15.994915); 2*Alkylation (iodoacetamide)(+57.021464); 1*Phosphorylation (S/T)(+79.966332) A26A40A36A31	2108.8731	2108.8733	30.66	25.20	703.9651	3	524 ±0.3
α casein	A(106-124)	VPQLEIVPNSAEERLHSMK	1*Phosphorylation (S/T)(+79.966332)A115	2256.0974	2256.0992	33.98	26.93	753.0403	3	559 ±0.5
α casein	A(133-151)	EPMIGVNQELAYFYPELFR		2315.1296	2315.1358	49.04	36.77	1158.5752	2	511 ±0.1
α casein	A(133-151)	EPMIGVNQELAYFYPELFR	1*Oxidation (M)(+15.994915)A135	2331.1246	2331.1296	47.66	29.22	778.0505	3	608 ±0.6
α casein	A(133-151)	EPMIGVNQELAYFYPELFR	1*Oxidation (M)(+15.994915)A135	2331.1246	2331.1299	47.66	28.52	778.0506	3	594 ±0.4

Protein	Sequence Location	Sequence	Modification	Theoretical Mass (Da)	Observed Mass (Da)	RT (min)	Drift Time (ms)	*m/z*	Charge State	CCS (Å^2)
α casein	A(115-136)	NAVPITPTLNREQLSTSEENSK	1*Phosphorylation (S/T)(+79.966332)A129	2507.1905	2507.1933	31.17	26.90	836.7384	3	559 ±0.3
α casein	A(37-58)	VNELSKDIGSESTEDQAMEDIK	1*Phosphorylation (S/T)(+79.966332)A46	2517.0830	2517.0869	34.16	27.55	840.0362	3	572 ±0.4
α casein	A(37-58)	VNELSKDIGSESTEDQAMEDIK	1*Phosphorylation (S/T)(+79.966332)A46	2517.0830	2517.0875	34.41	28.17	840.0365	3	585 ±0.5
α casein	A(25-45)	NMAINPSKENLCSTFCKEVVR	2*Alkylation (iodoacetamide)(+57.021464); 1*Phosphorylation (S/T)(+79.966332) A40A36A37	2576.1587	2576.1626	33.84	28.32	859.7281	3	587 ±1.1
α casein	A(115-136)	NAVPITPTLNREQLSTSEENSK	2*Phosphorylation (S/T)(+79.966332) A129A120	2587.1568	2587.1595	33.27	27.19	863.3938	3	564 ±0.6
α casein	A(25-45)	NMAINPSKENLCSTFCKEVVR	1*Oxidation (M)(+15.994915); 2*Alkylation (iodoacetamide)(+57.021464); 1*Phosphorylation (S/T)(+79.966332) A26A40A36A31	2592.1536	2592.1583	32.28	28.13	865.0600	3	584 ±0.9
α casein	A(37-58)	VNELSKDIGSESTEDQAMEDIK	2*Phosphorylation (S/T)(+79.966332) A48A46	2597.0493	2597.0459	36.53	27.03	866.6892	3	561 ±0.3
α casein	A(37-58)	VNELSKDIGSESTEDQAMEDIK	2*Phosphorylation (S/T)(+79.966332) A48A47	2597.0493	2597.0498	36.53	28.50	866.6906	3	592 ±0.6
α casein	A(37-58)	VNELSKDIGSESTEDQAMEDIK	2*Phosphorylation (S/T)(+79.966332); 1*Oxidation (M)(+15.994915)A41A46A54	2613.0442	2613.0483	32.63	28.30	872.0234	3	588 ±0.2
α casein	A(37-58)	VNELSKDIGSESTEDQAMEDIK	2*Phosphorylation (S/T)(+79.966332); 1*Oxidation (M)(+15.994915)A41A46A54	2613.0442	2613.0488	32.62	26.99	872.0235	3	561 ±0.9
α casein	A(115-137)	NAVPITPTLNREQLSTSEENSKK	1*Phosphorylation (S/T)(+79.966332)A122	2635.2855	2635.2893	28.81	29.80	879.4370	3	618 ±0.8
α casein	A(115-137)	NAVPITPTLNREQLSTSEENSKK	1*Phosphorylation (S/T)(+79.966332)A122	2635.2855	2635.2894	28.81	27.72	879.4371	3	575 ±0.9
α casein	A(37-58)	VNELSKDIGSESTEDQAMEDIK	3*Phosphorylation (S/T)(+79.966332) A48A46A41	2677.0156	2677.0191	39.53	26.93	893.3470	3	559 ±0.2
α casein	A(37-58)	VNELSKDIGSESTEDQAMEDIK	3*Phosphorylation (S/T)(+79.966332) A48A46A41	2677.0156	2677.0200	39.53	28.24	893.3473	3	586 ±0.3
α casein	A(92-113)	FPQYLQYLYQGPIVLNPWDQVK		2708.4003	2708.4092	49.34	32.16	903.8103	3	668 ±0.3
α casein	A(92-113)	FPQYLQYLYQGPIVLNPWDQVK		2708.4003	2708.4097	49.34	29.05	903.8105	3	604 ±0.2
α casein	A(115-137)	NAVPITPTLNREQLSTSEENSKK	2*Phosphorylation (S/T)(+79.966332) A122A120	2715.2518	2715.2542	30.29	30.61	906.0920	3	635 ±1.5
α casein	A(115-137)	NAVPITPTLNREQLSTSEENSKK	2*Phosphorylation (S/T)(+79.966332) A122A121	2715.2518	2715.2548	30.29	27.80	906.0922	3	576 ±1.5
α casein	A(115-137)	NAVPITPTLNREQLSTSEENSKK	2*Phosphorylation (S/T)(+79.966332) A122A122	2715.2518	2715.2557	30.29	29.70	906.0925	3	616 ±1.1
α casein	A(115-137)	NAVPITPTLNREQLSTSEENSKK	1*Deamidation(+0.984016); 2*Phosphorylation (S/T)(+79.966332) A127A129A122	2716.2358	2716.2403	30.97	27.70	906.4207	3	576 ±1.9
α casein	A(35-58)	EKVNELSKDIGSESTEDQAMEDIK	1*Phosphorylation (S/T)(+79.966332)A41	2774.2205	2774.2261	32.79	28.34	925.7493	3	588 ±0.4
α casein	A(59-83)	QMEAESISSSEEIVPNSVEQKHIQK	3*Deamidation(+0.984016); 1*Oxidation (M)(+15.994915) A82A78A59A60	2845.3175	2845.2996	43.91	29.16	949.4405	3	606 ±0.3
α casein	A(35-58)	EKVNELSKDIGSESTEDQAMEDIK	2*Phosphorylation (S/T)(+79.966332) A46A41	2854.1869	2854.1912	34.02	28.68	952.4043	3	595 ±0.9
α casein	A(35-58)	EKVNELSKDIGSESTEDQAMEDIK	2*Phosphorylation (S/T)(+79.966332); 1*Deamidation(+0.984016)A41A46A52	2855.1709	2855.1768	34.76	28.45	952.7329	3	591 ±1.1
α casein	A(92-114)	FPQYLQYLYQGPIVLNPWDQVKR		2864.5014	2864.5121	46.21	33.16	955.8446	3	642 ±41.5
α casein	A(92-114)	FPQYLQYLYQGPIVLNPWDQVKR		2864.5014	2864.5121	46.20	29.76	955.8447	3	665 ±40.3
α casein	A(35-58)	EKVNELSKDIGSESTEDQAMEDIK	3*Phosphorylation (S/T)(+79.966332) A49A48A46	2934.1532	2934.1566	35.89	29.11	979.0595	3	604 ±0.3
α casein	A(35-58)	EKVNELSKDIGSESTEDQAMEDIK	3*Phosphorylation (S/T)(+79.966332) A49A48A46	2934.1532	2934.1570	35.89	27.48	979.0596	3	570 ±0.9
α casein	A(126-149)	EQLSTSEENSKKTVDMESTEVFTK	3*Phosphorylation (S/T)(+79.966332) A131A130A129	2986.1845	2986.1889	33.75	28.90	996.4036	3	598 ±1.6
α casein	A(1-24)	KNTMEHVSSSEESIISQETYKQEK	3*Phosphorylation (S/T)(+79.966332) A13A9A3	3051.2223	3051.2263	31.77	29.42	1018.0827	3	610 ±0.9

Protein	Sequence Location	Sequence	Modification	Theoretical Mass (Da)	Observed Mass (Da)	RT (min)	Drift Time (ms)	*m/z*	Charge State	CCS (Å²)
α casein	A(152-193)	QFYQLDAYPSGAWYYVPLGT QYTDAPSFSDIPNPIGSENSEK	1*Deamidation(+0.984016)A172	4716.1497	4716.1552	49.52	37.05	1573.0590	3	769 ±0.6
β casein	A(29-32)	KIEK		516.3271	516.3293	7.59	19.41	259.1719	2	276 ±2.1
β casein	A(108-113)	EMPFPK		747.3625	747.3625	28.16	23.03	374.6885	2	322 ±0.3
β casein	A(108-113)	EMPFPK		747.3625	747.3631	28.15	21.81	374.6888	2	305 ±0.1
β casein	A(170-176)	VLPVPQK		779.4905	779.4910	23.81	22.94	390.7528	2	321 ±0.3
β casein	A(170-176)	VLPVPQK		779.4905	779.4912	23.81	21.59	390.7529	2	302 ±0.3
β casein	A(177-183)	AVPYPQR		829.4446	829.4460	22.76	22.52	415.7303	2	314 ±0.6
β casein	A(26-32)	INKKIEK		871.5491	871.5529	9.18	19.90	291.5249	3	417 ±0.2
β casein	A(98-105)	VKEAMAPK		872.4790	872.4798	14.12	23.48	437.2472	2	328 ±0.6
β casein	A(98-105)	VKEAMAPK		872.4790	872.4801	14.11	22.88	437.2473	2	319 ±0.3
β casein	A(106-113)	HKEMPFPK		1012.5164	1012.5169	21.58	21.14	338.5129	3	442 ±0.2
β casein	A(106-113)	HKEMPFPK		1012.5164	1012.5177	21.55	20.34	338.5132	3	425 ±0.3
β casein	A(106-113)	HKEMPFPK	1*Oxidation (M)(+15.994915)A109	1028.5113	1028.5128	18.16	20.97	343.8449	3	438 ±0.1
β casein	A(170-183)	VLPVPQKAVPYPQR		1590.9246	1590.9254	28.73	23.87	531.3157	3	497 ±0.2
β casein	A(170-183)	VLPVPQKAVPYPQR		1590.9246	1590.9256	28.73	23.11	531.3158	3	482 ±0.8
β casein	A(170-183)	VLPVPQKAVPYPQR		1590.9246	1590.9263	28.73	25.80	531.3161	3	537 ±0.5
β casein	A(170-183)	VLPVPQKAVPYPQR		1590.9246	1590.9276	28.73	24.68	531.3165	3	513 ±0.7
β casein	A(33-48)	FQSEEQQQTEDELQDK		1980.8549	1980.8587	27.67	33.13	991.4366	2	460 ±0.3
β casein	A(33-48)	FQSEEQQQTEDELQDK	1*Phosphorylation (S/T)(+79.966332)A35	2060.8212	2060.8229	30.39	24.93	687.9482	3	518 ±0.1
β casein	A(33-48)	FQSEEQQQTEDELQDK	1*Phosphorylation (S/T)(+79.966332)A41	2060.8212	2060.8240	27.42	26.06	687.9486	3	542 ±0.3
β casein	A(33-48)	FQSEEQQQTEDELQDK	1*Phosphorylation (S/T)(+79.966332)A41	2060.8212	2060.8247	27.42	33.01	1031.4196	2	458 ±0.1
β casein	A(33-48)	FQSEEQQQTEDELQDK	1*Phosphorylation (S/T)(+79.966332)A35	2060.8212	2060.8248	30.40	33.72	1031.4197	2	468 ±0.1
β casein	A(33-48)	FQSEEQQQTEDELQDK	1*Phosphorylation (S/T)(+79.966332)A41	2060.8212	2060.8250	27.42	24.74	687.9489	3	514 ±0.5
β casein	A(33-48)	FQSEEQQQTEDELQDK	1*Phosphorylation (S/T)(+79.966332)A35	2060.8212	2060.8258	30.39	26.69	1031.4202	2	370 ±0.1
β casein	A(33-48)	FQSEEQQQTEDELQDK	1*Phosphorylation (S/T)(+79.966332)A35	2060.8212	2060.8259	30.38	28.24	1031.4202	2	391 ±0.4
β casein	A(184-202)	DMPIQAFLLYQEPVLGPVR		2185.1606	2185.1655	49.30	35.91	1093.5900	2	499 ±0.1
β casein	A(184-202)	DMPIQAFLLYQEPVLGPVR		2185.1606	2185.1669	49.31	30.22	1093.5907	2	418 ±2.6
β casein	A(184-202)	DMPIQAFLLYQEPVLGPVR	1*Oxidation (M)(+15.994915)A185	2201.1555	2201.1630	46.31	28.60	734.7283	3	594 ±0.3
β casein	A(30-48)	IEKFQSEEQQQTEDELQDK	1*Phosphorylation (S/T)(+79.966332)A35	2431.0428	2431.0454	29.93	26.30	811.3557	3	546 ±0.2
β casein	A(30-48)	IEKFQSEEQQQTEDELQDK	1*Phosphorylation (S/T)(+79.966332)A35	2431.0428	2431.0468	32.43	25.41	811.3562	3	528 ±0.3
β casein	A(30-48)	IEKFQSEEQQQTEDELQDK	1*Phosphorylation (S/T)(+79.966332)A36	2431.0428	2431.0483	32.42	26.77	811.3567	3	557 ±0.4
β casein	A(29-48)	KIEKFQSEEQQQTEDELQDK	1*Phosphorylation (S/T)(+79.966332)A35	2559.1378	2559.1403	28.13	27.11	854.0540	3	563 ±0.3
β casein	A(29-48)	KIEKFQSEEQQQTEDELQDK	1*Phosphorylation (S/T)(+79.966332)A41	2559.1378	2559.1409	26.16	26.86	854.0542	3	558 ±0.2
β casein	A(29-48)	KIEKFQSEEQQQTEDELQDK	1*Phosphorylation (S/T)(+79.966332)A35	2559.1378	2559.1427	27.69	26.99	854.0548	3	561 ±0.4
β casein	A(184-209)	DMPIQAFLLYQEPVLGPVRGPFPIIV		2908.5925	2908.6028	57.18	30.40	970.5416	3	632 ±0.1
β casein	A(184-209)	DMPIQAFLLYQEPVLGPVRGPFPIIV	1*Oxidation (M)(+15.994915)A185	2924.5874	2924.5979	54.57	30.52	975.8733	3	634 ±0.4
β casein	A(1-25)	RELEELNVPGEIVESLSSSEESITR	2*Phosphorylation (S/T)(+79.966332) A18A15	2961.3257	2961.3342	42.46	29.19	988.1187	3	607 ±0.4
β casein	A(1-25)	RELEELNVPGEIVESLSSSEESITR	2*Phosphorylation (S/T)(+79.966332) A18A16	2961.3257	2961.3374	42.77	29.83	988.1198	3	619 ±0.9
β casein	A(1-25)	RELEELNVPGEIVESLSSSEESITR	2*Phosphorylation (S/T)(+79.966332) A18A17	2961.3257	2961.3388	44.16	29.93	988.1202	3	622 ±0.2
β casein	A(177-202)	AVPYPQRDMPIQAFLLYQEPVL GPVR	1*Oxidation (M)(+15.994915)A185	3012.5895	3012.5938	42.51	30.41	1005.2052	3	630 ±2
β casein	A(1-25)	RELEELNVPGEIVESLSSSEESITR	3*Phosphorylation (S/T)(+79.966332) A18A17A15	3041.2921	3041.3042	46.24	29.66	1014.7753	3	616 ±0.3

Analysis of PhosphoMixes

With the instrument operating in alternating frames, the system is oscillating between MS and MS/MS analysis throughout the experiment. For the MS/MS analysis, quadrupole isolation does not occur—instead, an all-ions approach, where all ions are passed through to the collision cell based on drift separation, is used. Since the collision cell is positioned after the drift tube as shown in Figure 1, the fragments will have the same drift time as the parent ions, as shown in Figure 7.

Figure 7 displays a two-dimensional plot of the $[M+2H]^{2+}$ ion, *m/z* 872.3480, for the phosphopeptide ADEPSSEEpSDLEIDK, where p corresponds to the site of phosphorylation on the subsequent serine residue. The heat map displays the difference view of the low energy channel (MS) in green and the high energy channel (MS/MS) in red. The collision energy is defined by the ramp used in Table 3. The fragments in red align with the drift time of the parent ion (*m/z* 872.3480), with the extracted fragmentation spectra displayed in Figure 8. The resulting sequence ladder displayed in Figure 8 shows nearly complete sequence coverage of the phosphopeptide with the operation of the instrument in alternating frames. Not only can MS and MS/MS data be obtained in a single acquisition, but CCS values can also be determined to provide information in regard to the structure of the phosphopeptides, as shown in Table 5.

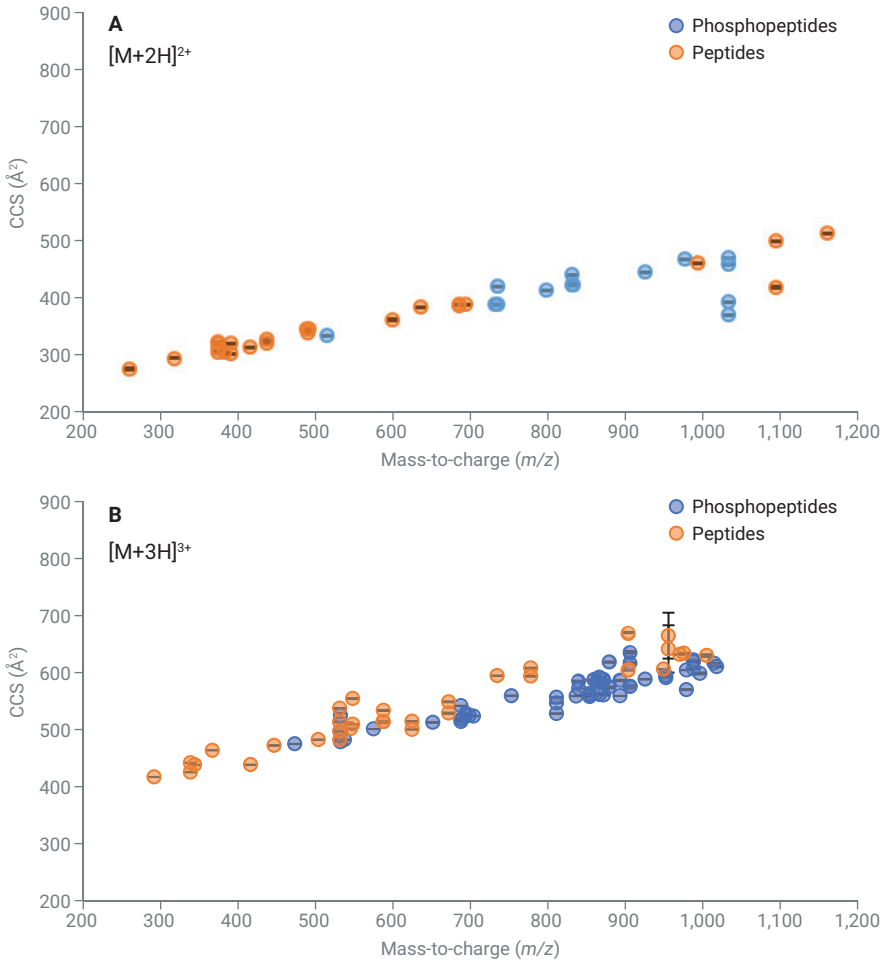

Figure 6. CCS as a function of *m/z* for (A) $[M+2H]^{2+}$ and (B) $[M+3H]^{3+}$ charge states of peptides and phosphopeptides resulting from the tryptic digestion and phosphopeptide enrichment of α and β-casein. Error bars correspond to the standard deviation obtained from triplicate measurements.

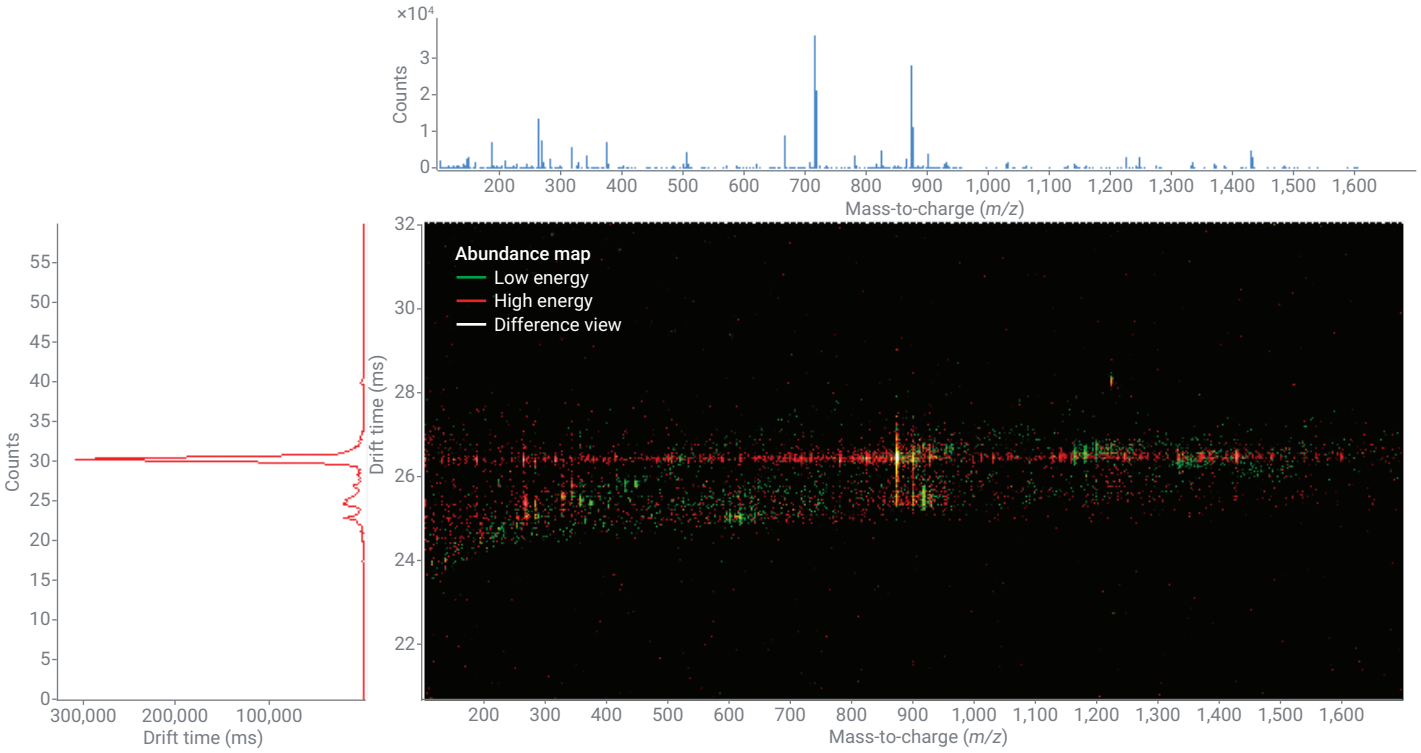

Figure 7. Two-dimensional plot displaying an overlay of the Alternating Frames acquisition, low (green) and high (red) energy fragmentation channels, with the drift time versus *m/z* plotted for the $[M+2H]^{2+}$ ions of the phosphopeptide ADEPSSEEpSDLEIDK with a measured *m/z* value of 872.3480, where p corresponds to the site of phosphorylation on the subsequent serine residue.

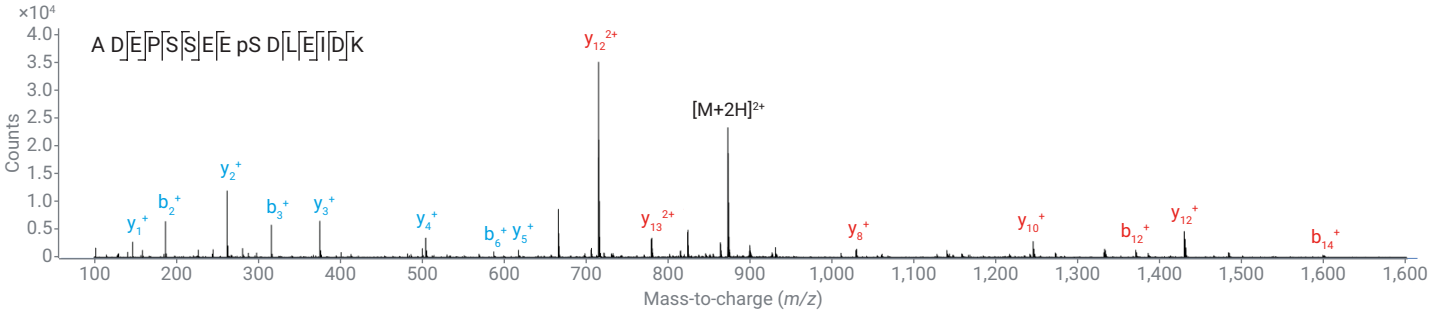

Figure 8. Extracted fragmentation mass spectrum for the $[M+2H]^{2+}$ ion, *m/z* 872.348, of the phosphopeptide ADEPSSEEpSDLEIDK, where p corresponds to site of phosphorylation on the following serine residue. Phosphorylated residues are shown in red, while nonphosphorylated residues are displayed in blue.

Table 5. CCS values for identified phosphopeptides from PhosphoMixes 1–3, with p denoting the site of phosphorylation on the following amino acid.

Sequence	Theoretical Mass (Da)	Measured Mass (Da)	RT (min)	Drift Time (ms)	*m/z*	Charge State	CCS (Å²)
VLHSGpSR	834.3749	834.3759	7.83	22.36	418.1952	2	313 ±0.1
RSpYpSRSR	1070.4060	1070.4080	7.45	24.31	536.2113	2	339 ±0.3
RSpYpSRSR	1070.4060	1070.4088	7.45	19.66	357.8102	3	410 ±0.2
RDSLGpTYSSR	1220.5187	1220.5193	22.90	26.09	611.2669	2	363 ±0.1
RDSLGpTYSSR	1220.5187	1220.5197	22.90	21.69	407.8472	3	452 ±0
pTKLIpTQLRDAK	1445.7044	1445.7081	29.64	22.49	482.9100	3	469 ±0.1
pTKLIpTQLRDAK	1445.7044	1445.7121	29.64	29.37	723.8633	2	409 ±0.1
EVQAEQPSSpSSPR	1480.6195	1480.6200	21.77	22.48	494.5473	3	468 ±0.3
EVQAEQPSSpSSPR	1480.6195	1480.6220	21.77	28.65	741.3183	2	398 ±0.2
EVQAEQPSSpSSPR	1480.6195	1480.6224	21.77	27.97	741.3185	2	389 ±0.1
EVQAEQPSSpSSPR	1480.6195	1480.6229	21.77	29.59	741.3187	2	412 ±0.1
ADEPpSSEESDLEIDK	1742.6772	1742.6818	31.50	30.99	872.3482	2	431 ±0
ADEPSpSEEpSDLEIDK	1822.6435	1822.6462	34.59	31.08	912.3304	2	431 ±0.5
FEDEGAGFEESpSETGDYEEK	2333.8373	2333.8426	33.84	34.56	1167.9286	2	480 ±0.1
FEDEGAGFEESpSETGDYEEK	2333.8373	2333.8429	33.83	27.32	778.9549	3	568 ±0.1
ELSNpSPLRENSFGpSPLEFR	2338.0032	2338.0080	41.07	28.78	1170.0113	2	399 ±0.2
ELSNpSPLRENSFGpSPLEFR	2338.0032	2338.0097	41.08	35.76	1170.0122	2	496 ±0.2
ELSNpSPLRENSFGpSPLEFR	2338.0032	2338.0104	41.08	26.07	780.3441	3	542 ±0.1
SPTEYHEPVpYANPFYRPTpTPQR	2809.1939	2809.1951	34.50	39.78	1405.6048	2	552 ±0.3
SPTEYHEPVpYANPFYRPTpTPQR	2809.1939	2809.2000	34.51	27.17	703.3073	4	752 ±0.1
SPTEYHEPVpYANPFYRPTpTPQR	2809.1939	2809.2008	34.51	28.63	937.4075	3	595 ±0.2
LPQEpTAR	893.4008	893.4017	21.27	39.7	894.4090	1	278 ±0.2
LPQEpTAR	893.4008	893.4022	21.28	23.06	447.7084	2	322 ±0.1
LPQEpTAR	893.4008	893.4022	21.27	30.99	894.4095	1	217 ±0.3
RYpSpSRSR	1070.4060	1070.4062	7.53	19.81	357.8093	3	413 ±0.5
RYpSpSRSR	1070.4060	1070.4067	7.52	24.41	536.2106	2	340 ±0.3
EpTQSPEQVK	1124.4751	1124.4771	19.84	24.78	563.2458	2	345 ±0
VIEDNEpYTAR	1288.5337	1288.5346	23.33	26.87	645.2746	2	374 ±0.1
pSRSPpSSPELNNK	1474.5855	1474.5883	22.55	22.11	492.5367	3	460 ±0.1
pSRSPpSSPELNNK	1474.5855	1474.5886	22.54	27.45	738.3016	2	382 ±0.1
ADEPSSEEpSDLEIDK	1742.6772	1742.6814	31.43	30.57	872.3480	2	425 ±0.1
HQYSDYDpYHSSpSEK	1904.6292	1904.6314	22.70	28.63	953.3230	2	396 ±1.4
HQYSDYDpYHSSpSEK	1904.6292	1904.6341	22.70	25.45	635.8853	3	529 ±0.2
HQYSDYDpYHSSpSEK	1904.6292	1904.6349	22.70	32.13	953.3247	2	446 ±0.3
HQYSDYDpYHSSpSEK	1904.6292	1904.6350	22.70	30.11	953.3248	2	418 ±0.2
NTPpSQHSHpSIQHSPER	2000.7891	2000.7909	18.88	26.45	1001.4027	2	367 ±1
NTPpSQHSHpSIQHSPER	2000.7891	2000.7936	18.90	23.75	667.9385	3	493 ±0.7
NTPpSQHSHpSIQHSPER	2000.7891	2000.7941	18.89	25.66	667.9386	3	534 ±0
NTPpSQHSHpSIQHSPER	2000.7891	2000.7948	18.89	22.42	501.2060	4	621 ±0.1
NTPpSQHSHpSIQHSPER	2000.7891	2000.7949	18.88	32.04	1001.4047	2	445 ±0.1
ELpSNpSPLRENSFGSPLEFR	2338.0032	2338.0057	44.57	25.91	780.3425	3	538 ±0.1
LGPGRPLPTFPpTSE(CAM)TSDVEPDTR	2708.2153	2708.2175	35.24	39.11	1355.1160	2	542 ±0.3
LGPGRPLPTFPpTSE(CAM)TSDVEPDTR	2708.2153	2708.2184	35.27	31.25	1355.1165	2	433 ±0.6
LGPGRPLPTFPpTSE(CAM)TSDVEPDTR	2708.2153	2708.2229	35.25	28.02	903.7483	3	582 ±0.2

Sequence	Theoretical Mass (Da)	Measured Mass (Da)	RT (min)	Drift Time (ms)	*m/z*	Charge State	CCS (Å²)
LGPGRPLPTFPpTSE(CAM)TSDVEPDTR	2708.2153	2708.2241	35.25	25.88	678.0633	4	717 ±0.3
LQGpSGVpSLApSK	1285.4758	1285.4772	26.85	26.38	643.7459	2	367 ±0.6
PPpYpSRVIpTQR	1455.5714	1455.5718	28.51	27.62	728.7932	2	384 ±0.3
PPpYpSRVIpTQR	1455.5714	1455.5740	28.51	22.21	486.1986	3	463 ±0.1
PPpYpSRVIpTQR	1455.5714	1455.5748	28.51	28.52	728.7947	2	397 ±0.1

With the PhosphoMixes, we were able to examine site-specific effects of phosphorylation of a given peptide sequence. Figures 9 to 11 display the drift time distributions and CCS values for a specific peptide sequence with differing sites and amounts of phosphorylation. Figure 9 displays the resulting drift time distributions and CCS values for the $[M+2H]^{2+}$ ions of ADEPpSSEEpSDLEIDK (*m/z* 912.3304), ADEPSSEEpSDLEIDK (*m/z* 872.3480), and ADEPpSSEESDLEIDK (*m/z* 872.3482), where p corresponds to the site of phosphorylation on the subsequent serine residue. The EICs (not shown here) for the two singly phosphorylated peptides are not fully resolved, but with ion mobility, we determined that there is a difference in conformation of the singly phosphorylated peptides with the same peptide sequence but differing in the site of phosphorylation. In Figure 9, the first site of phosphorylation has a larger effect on the resulting CCS of the peptide ADEPpSSEEpSDLEIDK. Figures 10 and 11 display the drift time distributions for the $[M+3H]^{3+}$ and $[M+2H]^{2+}$ ions of RSpYpSRSR and RYpSpSRSR, where p corresponds to the site of phosphorylation on the subsequent residue, respectively. This enables the determination of how the CCS changes when the number and sites of phosphorylation remain the same but vary in terms of peptide sequence.

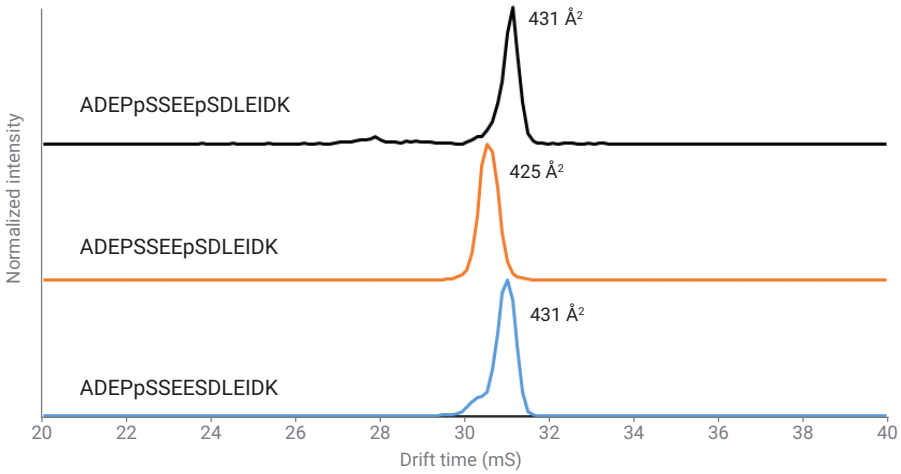

Figure 9. Drift time distributions and corresponding CCS values for three $[M+2H]^{2+}$ phosphopeptides with the same peptide sequences but varying numbers and locations of phosphorylation sites.

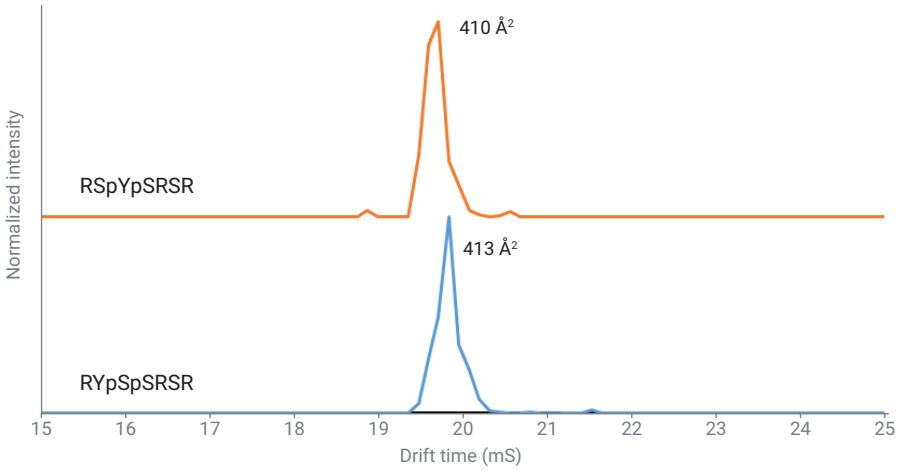

Figure 10. Drift time distributions and corresponding CCS values for two $[M+3H]^{3+}$ phosphopeptides with the same number and position of phosphorylation sites but different peptide sequences.

From Figures 10 and 11, we can determine that RSpYpSRSR is more compact than RYpSpSRSR with a larger difference in CCS observed for the $[M+3H]^{3+}$ ions. This suggests that swapping the order of the second and third residues in this phosphopeptide causes a conformational change that would not easily be observed by LC/MS.

Conclusion

This Application Note presents an automated workflow from sample preparation and phosphopeptide enrichment to analysis by IMS-MS. We determined that phosphopeptides are more compact than nonphosphorylated peptides of similar *m/z* values. Differences in CCS values were found with peptides with varying numbers and locations of phosphorylation sites, as well as peptides with varying sequences with the same number and position of phosphorylation sites. With the combination of offline phosphopeptide enrichment and analysis using the Agilent 6560 ion mobility LC/Q-TOF, site localization of phosphorylated peptides is readily characterized by CCS values and MS/MS data.

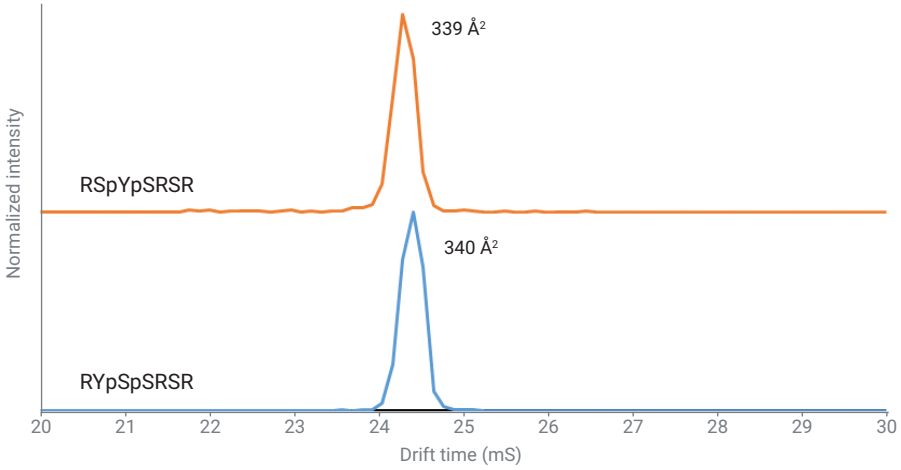

Figure 11. Drift time distributions and corresponding CCS values for two $[M+2H]^{2+}$ phosphopeptides with the same number and position of phosphorylation sites but different peptide sequences.

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