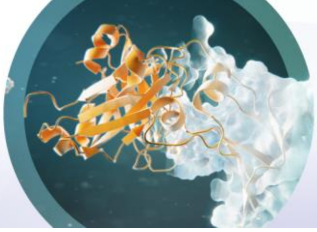

Rapid and reliable structural confirmation and comprehensive impurity analysis of cyclic peptides

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This technical note demonstrates the structural confirmation of cyclosporine A (CysA), a representative cyclic peptide drug, and the comprehensive CysA-related impurity analysis. MS-level identification and MS/MS-level structural confirmation, including unexpected impurities, were demonstrated at concentrations as low as 0.01% (w/w) and 0.04% (w/w), respectively. Impurities assigned as isocyclosporine A (IsoCysA) and cyclosporine C (CysC) based on MS and MS/MS fragments were by co-elution and MS/MS comparison with standards.

Development of cyclic peptides is rapidly gaining not only for new therapeutic modality but DDS systems. The sequence confirmation and in-depth impurity analysis are important due to their high specificity. But the analysis is complex because of the molecular size, the cyclic structures, composed isomeric amino acids and various modifications, and lack of processing software.

Here, a rapid and sensitive analysis of CysA and related impurities are demonstrated using ZenoTOF 7600+ system with Molecule Profiler software and SCIEX OS software. Sensitive, high-quality MS and MS/MS spectra and Molecule Profiler software enable comprehensive impurity identification and rapid and confident sequencing results.[3-6]

Key benefits for analysis of cyclic peptides using the ZenoTOF 7600+ system

- **Rapid and reliable sequence confirmation of target peptide using fragment assignment:** Fragment ions are automatically assigned to the sequence and sequence coverage is calculated. Target peptides can be confirmed not only by accurate mass but also by sequence confidence (e.g., achieving 100% sequence coverage for CysA).
- **Enhanced sensitivity and increased confidence in impurity identification:** Generate fragment-rich spectra, enabling more confident identification of low-abundant impurities using Zeno trap on the ZenoTOF 7600+ system
- **Rapid and comprehensive impurity identification:** Search for impurities using a variety of peak search strategies, including software-generated accurate mass lists of potential variations in different charge states, and common product ions and neutral losses with main components in molecule profiler software.

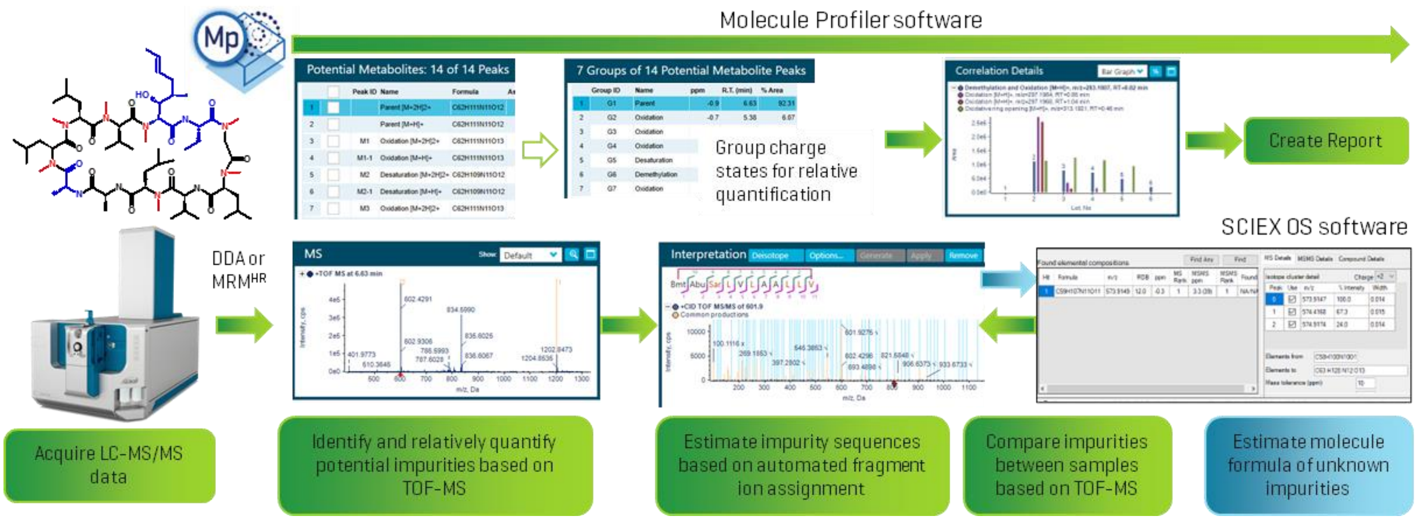


Figure 1. Cyclic peptide impurity analysis workflow using Molecule Profiler Software and SCIEX OS software.

Introduction

Cyclic peptides are more stable in structure than linear peptides and are rapidly becoming more common not only in new therapies but also in DDS systems.

Impurity analysis is important to ensure the safety of pharmaceutical products but are complicated for cyclic peptides due to the molecular size, cyclic structure, isomeric amino acids, presence of various modifications and lack of processing software and strong UV absorption.

MS/MS is a promising approach for analysing small molecule therapeutics, but the size and complex structure of cyclic peptides make it difficult to perform structural analysis.

MS/MS-based peptide sequencing is widely used for protein identification, but the cyclic structures, various synthetic amino acids, modifications and linkers, and lack of processing software make this technique difficult to apply.

Therefore, there is strong demand for methods that can quickly and easily identify impurities of cyclic peptides and confirm their structures. In this technical note, a method using the amino acid analysis mode of the software is demonstrated. By registering the main components as sequences, the detected impurities are also expressed as sequences. Being able to confirm MS/MS analysis from sequence notation rather than complex chemical structures also makes it easier to verify the structures.

Method

Samples: CysA and the 2 known impurities, the oxidized impurity CysC[2] and the isomeric impurity IsoCysA[1,2] (Figure 2), as standards for CysA impurities were prepared. The samples were dissolved in 2 mg/mL ethanol, and then 10 µg/mL CysA and 500 ng/mL CysC and IsoCysA were diluted in 50% (v/v) ethanol/water and used for impurity analysis and confirmation, respectively. Samples for co-elution testing of CysC and IsoCysA were prepared by spiking a 10 µg/mL CysA solution with 0.02–10% (w/w; corresponding to 2 ng/mL–1 µg/mL) of CysC and IsoCysA.

Chromatography: An ExionLC AE system was used with gradient elution using 0.1% (v/v) formic acid in water as mobile phase A and 0.1% (v/v) formic acid in acetonitrile as mobile phase B. Analysis was performed at a flow rate of 0.3 mL/min using a YMC Triart C18 metal-free column (2.1 × 150 mm, 3 µm) at 60°C. An injection volume of 2 µL was subjected to analysis. The gradient conditions are summarized in Table 1.

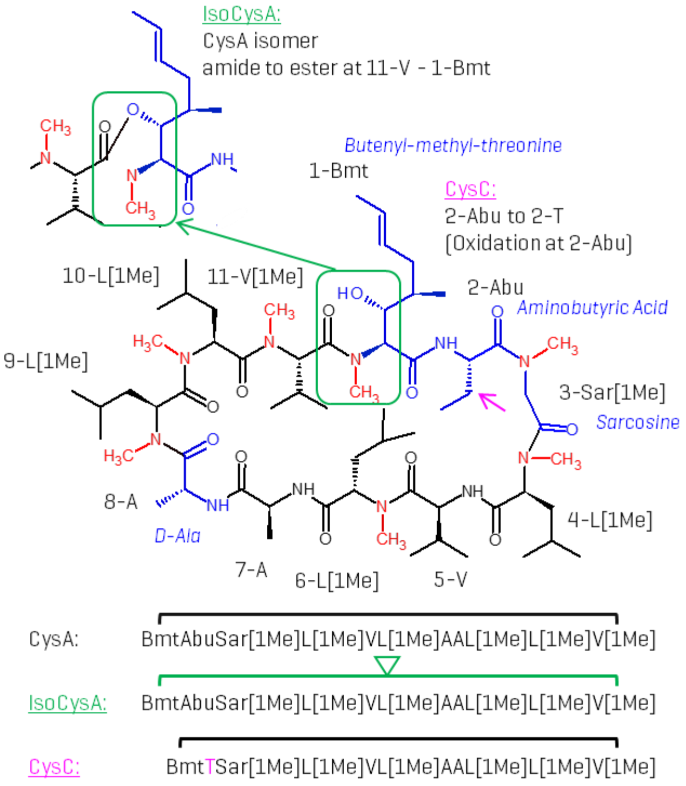


Figure 2. CysA, IsoCysA, and CysC structures and sequences were registered in the Molecule Profiler software.

Table 1. LC gradient condition.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	45	55
9.0	5	95
11.0	5	95
11.1	45	55
16.0	45	55

Mass spectrometry: ZenoTOF 7600+ system was used in positive polarity using Zeno CID DDA and Zeno MRM^{HR}. MS data were used to detect and quantify impurities, and MS/MS data were used to confirm the structures of CysA and impurities. The collision energy (CE) for CID was optimized to generate a fragment-rich MS/MS spectrum for doubly charged CysA (m/z601.92796), and the same CE was used for impurities. The detailed parameters are summarized in Table 2.

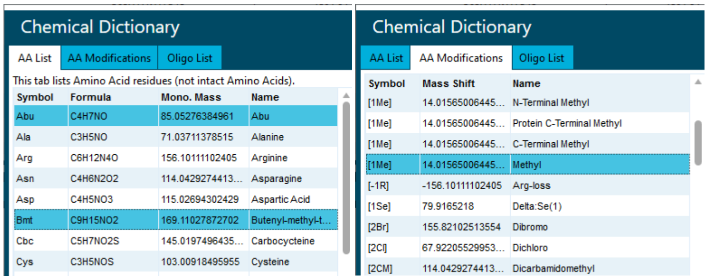
Table 2. MS parameters.

Parameters		Value	
		MS	MS/MS
Ion source gas 1 (psi)		50	
Ion source gas 2 (psi)		80	
Curtain gas (psi)		35	
CAD gas		7	
Source temperature (°C)		500	
Ion spray voltage (V)		4500	
Declustering potential (V)		80	
Precursor ion charge state		---	2+
Q1 resolution		---	Unit
Dissociation		---	CID
CE (V)		10	27
Mass range (m/z)		400-1500	100-1500
DDA	Accumulation time (sec)	0.1	0.05
	Cycle time (sec)	0.566 (MS & 8-MS/MS)	
MRM^{HR}	Accumulation time (sec)	0.1	0.2
	Cycle time (sec)	0.565 (MS & 2-MS/MS)	

Data processing: Structural confirmation and purity confirmation of CysA, as well as identification, structure assignment, and relative quantitation of impurities were performed using Molecule Profiler software and SCIEX OS software, version 1.3.4. The latest version of molecule profiler (version 1.3.4) officially supports data processing for cyclic peptide analysis.

MS and sequence confirmation and purity check of target compound, CysA

Therapeutic cyclic peptides generally contain modifications, such as N-alkylation, to improve their permeability and stability. The amino acids used for sequence registration, including common modifications, are pre-registered in Molecule Profiler software. User-specific amino acids and modifications can also be freely registered in the chemical dictionary of the software (Figure 3). The software combines the list of possible transformations with the catabolites to create a list of potential impurities for all the specified charge states and searches for the impurities within the list. Additionally, Molecule Profiler software can also search for impurities that produce product ions or neutral losses common to a predefined number of main components (5 in this technical note) as main component-related impurities. Peaks with different charge states from the same impurity can be grouped together and the total area of all detected charge states is calculated. The area % is the total area divided by the total area of all detected impurities in all charge states. By using the amino acid analysis mode of the software and registering the main components as sequences, all components are represented by sequences, making them easy to analyse without the need for structural elucidation from complex chemical structures.



Chemical Dictionary

This tab lists Amino Acid residues (not intact Amino Acids).

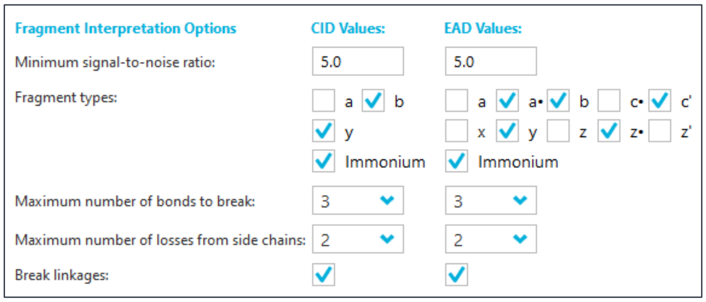
Symbol	Formula	Mono. Mass	Name
Abu	C4H7NO	85.05276384961	Abu
Ala	C3H5NO	71.03711378515	Alanine
Arg	C6H12N4O	156.10111102405	Arginine
Asn	C4H6N2O2	114.0429274413...	Asparagine
Asp	C4H5NO3	115.02694302429	Aspartic Acid
Bmt	C9H15NO2	169.11027872702	Butenyl-methyl-t...
Cbc	C5H7NO2S	145.0197496435...	Carbocycteine
Cys	C3H5NOS	103.00918495955	Cysteine

Chemical Dictionary

Symbol	Mass Shift	Name
[1Me]	14.01565006445...	N-Terminal Methyl
[1Me]	14.01565006445...	Protein C-Terminal Methyl
[1Me]	14.01565006445...	C-Terminal Methyl
[1Me]	14.01565006445...	Methyl
[-1R]	-156.10111102405	Arg-loss
[1Se]	79.9165218	Delta:Se(1)
[2Br]	155.82102513554	Dibromo
[2Cl]	67.92205529953...	Dichloro
[2CM]	114.0429274413...	Dicarbamidomethyl

Figure 3. Chemical dictionary. Abu, Bmt and Sar shown in Figure 2 were registered for CysA analysis. [1Me] and [Oxi] were pre-registered as methylation and oxidation in Molecule Profiler software.

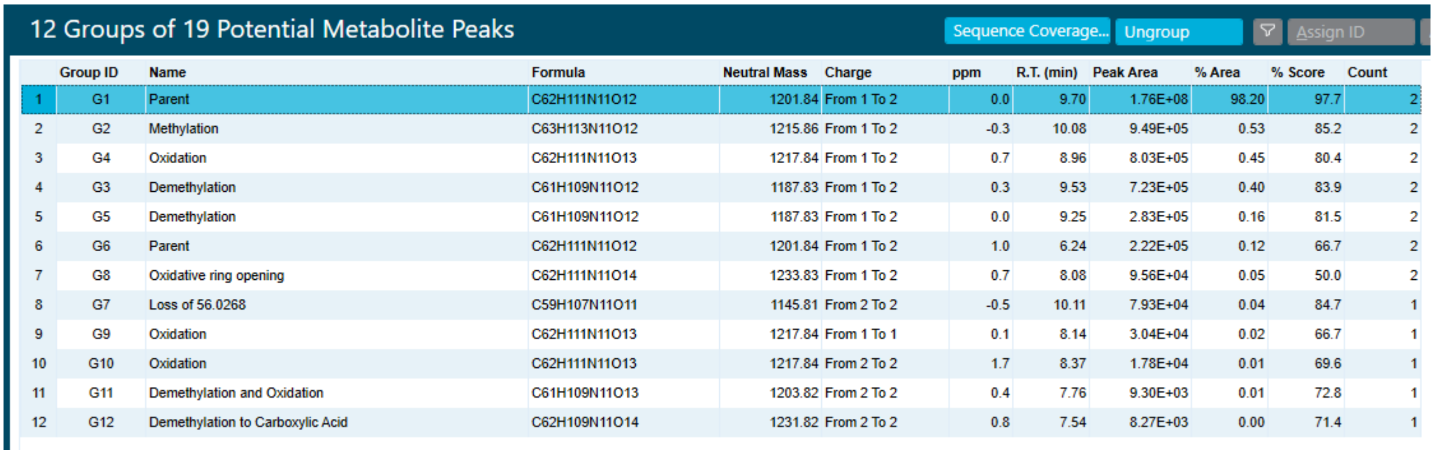
Based on the selected options (Figure 4) and the specified sequence, a list of theoretical fragment ions is generated, MS/MS peaks are assigned based on the list, and sequence coverage is calculated automatically. The assigned fragments are displayed in a table along with their corresponding sequence and ion type. The sequence confirmation of CysA was achieved with 100% (11/11) coverage in this case (Figure 6, bottom).



Fragment Interpretation Options	CID Values:	EAD Values:
Minimum signal-to-noise ratio:	5.0	5.0
Fragment types:	☐ a ☑ b ☑ y ☑ Immonium	☐ a ☑ a• ☑ b ☐ c• ☑ c' ☐ x ☑ y ☐ z ☑ z• ☐ z' ☑ Immonium
Maximum number of bonds to break:	3	3
Maximum number of losses from side chains:	2	2
Break linkages:	☑	☑

Figure 4. Fragment interpretation options. The ZenoTOF 7600+ system offers 2 types of MS/MS, CID and EAD, each with their own unique fragmentation patterns, allowing customers to configure the appropriate settings for each separately within a single processing parameter file.

Figure 5 shows the target compound CysA and the related impurities detected along with their respective MS area % after grouping. The purity of this sample was calculated to be 98.20%. Figure 6 (top) shows the MS and MS/MS confirmation of CysA. The calculated confidence score using MS accuracy, isotopic pattern, and MS/MS shows 97.3%.



12 Groups of 19 Potential Metabolite Peaks

	Group ID	Name	Formula	Neutral Mass	Charge	ppm	R.T. (min)	Peak Area	% Area	% Score	Count
1	G1	Parent	C62H111N11O12	1201.84	From 1 To 2	0.0	9.70	1.76E+08	98.20	97.7	2
2	G2	Methylation	C63H113N11O12	1215.86	From 1 To 2	-0.3	10.08	9.49E+05	0.53	85.2	2
3	G4	Oxidation	C62H111N11O13	1217.84	From 1 To 2	0.7	8.96	8.03E+05	0.45	80.4	2
4	G3	Demethylation	C61H109N11O12	1187.83	From 1 To 2	0.3	9.53	7.23E+05	0.40	83.9	2
5	G5	Demethylation	C61H109N11O12	1187.83	From 1 To 2	0.0	9.25	2.83E+05	0.16	81.5	2
6	G6	Parent	C62H111N11O12	1201.84	From 1 To 2	1.0	6.24	2.22E+05	0.12	66.7	2
7	G8	Oxidative ring opening	C62H111N11O14	1233.83	From 1 To 2	0.7	8.08	9.56E+04	0.05	50.0	2
8	G7	Loss of 56.0268	C59H107N11O11	1145.81	From 2 To 2	-0.5	10.11	7.93E+04	0.04	84.7	1
9	G9	Oxidation	C62H111N11O13	1217.84	From 1 To 1	0.1	8.14	3.04E+04	0.02	66.7	1
10	G10	Oxidation	C62H111N11O13	1217.84	From 2 To 2	1.7	8.37	1.78E+04	0.01	69.6	1
11	G11	Demethylation and Oxidation	C61H109N11O13	1203.82	From 2 To 2	0.4	7.76	9.30E+03	0.01	72.8	1
12	G12	Demethylation to Carboxylic Acid	C62H109N11O14	1231.82	From 2 To 2	0.8	7.54	8.27E+03	0.00	71.4	1

Figure 5. Parent and detected potential impurities sorted by peak area. G7 was detected as a CysA-related impurity with common fragment ions as CysA.

Potential Metabolites: 19 of 19 Peaks

	☐	Peak ID	Name	Formula	Assigned	Neutral Mass	Average Mass	m/z	Charge	ppm	R.T. (min)	Peak Area	% Area	% Score	MS/MS Spectra
1	☑		Parent [M+2H]2+	C62H111N11O12	✓	1201.84	1202.47	601.9280	2	0.0	9.71	1.35E+08	75.77	97.7	1
2	☐		Parent [M+H]+	C62H111N11O12	✓	1201.84	1202.45	1202.8499	1	1.0	9.70	4.01E+07	22.43	66.7	0

Score: 97.7%

Property	Assigned Score	Maximum Score
Mass defect	0.0	0
Isotope pattern	100.0	100
MS/MS*	93.2	100
Mass accuracy	100.0	100
Total confirmation score	293.2	300

*MS/MS(CID):		*MS/MS(EAD):	
Quality:	1.00	Quality:	0.00
Similarity:	0.94	Similarity:	0.00

Source of MS/MS

D:\SCIEX OS Data\KK_Peptides_01_CysA\Data\007_Run3_Imp\202_331_A_10u_Imp_0p_MRMHR_MS031_LC016.wiff2

MS Sample, Sum XIC of M0 (from 601.9093 to 601.9495), M+1 (from 602.4093 to 602.4495), M+2 (from 602.9093 to 602.9495)

AA Index: 1:1-11, Mono. Mass: 1201.8414, m/z: 601.928 (2), Composition: C62H111N11O12

~~Bmt[*1]AbuSar[1Me]L[1Me]VL[1Me]AAL[1Me]L[1Me]~~ V[1Me][*1,(OH)-1]

CID Assigned: 69 of 7797 peaks, MSMS Peak Area Assigned: 44.1%, Sequence Coverage: 11 of 11 amino acids (100.0%)

Fragments: 69 of 320 Proposed Formulae

	Use	Mass (m/z)	Sequence	Ion	Charge	Error (ppm)	Intensity (cps)
13	☑	368.2897	L[1Me]L[1Me]V[1Me]	8|b3	1	-2.9	41675.2
14	☑	524.3798	AbuSar[1Me]L[1Me]VL[1Me]	1|b5	1	-1.5	37696.9
15	☑	806.5749	V[1Me]BmtAbuSar[1Me]L[1Me]VL[1Me]	10|b7	1	-0.2	35475.6
16	☑	114.0903	V[1Me]	4|b1	1	-9.1	33694.2

Figure 6. Results (top) and fragment interpretation (bottom) of CysA. The result view shows chromatograms, MS, and CID-MS/MS and the interpretation view allows sequence analysis using CID-MS/MS. Common product ion peaks matched to the selected parent MS/MS spectrum in the processing parameters are shown in orange. Blue lines indicate peaks assigned to the sequence. The fragment ions are assigned as cyclic structures (bottom right). Annotation details are shown in Figure 8.

Impurity profiling

The target compound CysA and the 11 detected impurities are shown in Figure 5 and Figure 7 (bottom). These impurities were detected with high mass accuracy within 2 ppm and high scores above 50%, including a low-level impurity (G12) with a relative intensity of less than 0.01%. These impurities were not detected by UV or TIC (Figure 7, top). MS/MS spectra of the impurities were automatically acquired by DDA, where the fragmentation patterns confirmed that the impurities were related to CysA. Additionally, an unexpected impurity (G7) was detected, resulting in 5 fragments identical to CysA. These results demonstrate that the ZenoTOF 7600+ system can detect and confirm trace-level impurities with high sensitivity and accuracy, enabling comprehensive identification of unexpected impurities. Methylated, demethylated, oxidative, and isomeric impurities were detected as high-abundance impurities, which were in good agreement with the literature information[1,2].

Determining modification site of impurities based on CID-MS/MS data

As a further investigation, structural elucidation of methylated (G2), the most intense and oxidized (G4), isomer (G6, detected as parent), and unexpected impurity (G7) were performed using MS/MS data.

The results of structural analysis of the isomeric impurity (G6) are shown in Figure 9. Comparing the MS/MS spectra of CysA and G6, the strong 0 | b ion series observed in CysA—such as 0 | b9 and 0 | b10—which are derived from cleavage of bond 0 (i.e., the 11-V(1Me)–1-Bmt bond), either disappeared or showed a significant decrease in sensitivity in G6. This suggested that the bond between 11-V(1Me) and 1-Bmt had changed to a different bond that was less susceptible to cleavage. Because the known impurity IsoCysA matched these characteristics, the MS/MS spectrum of impurity G6 was compared with that of IsoCysA. Furthermore, a coelution test was performed by spiking CysA with IsoCysA. Based on the MS/MS spectral match and the coelution test results, G6 was identified as IsoCysA.

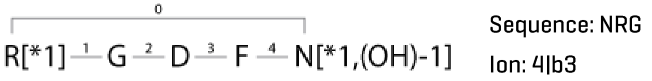


Figure 8. Annotation of fragment ions of cyclic peptides in Molecule Profiler software. RIng-opening is a y-type fragment, and the ion name indicates the position of ring opening | type of N-terminal fragment and number of amino acids in the fragment. In this example, NRG is annotated as 4 | b3.

The identification of the oxidation site in G4, the most strongly detected oxidized impurity, is shown in Figure 10. Comparing the MS/MS of CysA and G4, the strong 1 | b3 ion at *m/z* 284.1954 of CysA disappears in G4, while an ion at *m/z* 300.1903 appears. This suggests that the oxidation site may be within the AbuSar[1Me]L[1Me] subsequence. Sequence analysis using Molecule Profiler software indicated that the oxidation site is most likely Sar[1Me], and the b ion series supports this. The impurity in which Sar[1Me] is oxidized is known as CysC. Based on the MS/MS spectral match and the results of the coelution test (data not shown), G4 was identified as CysC.

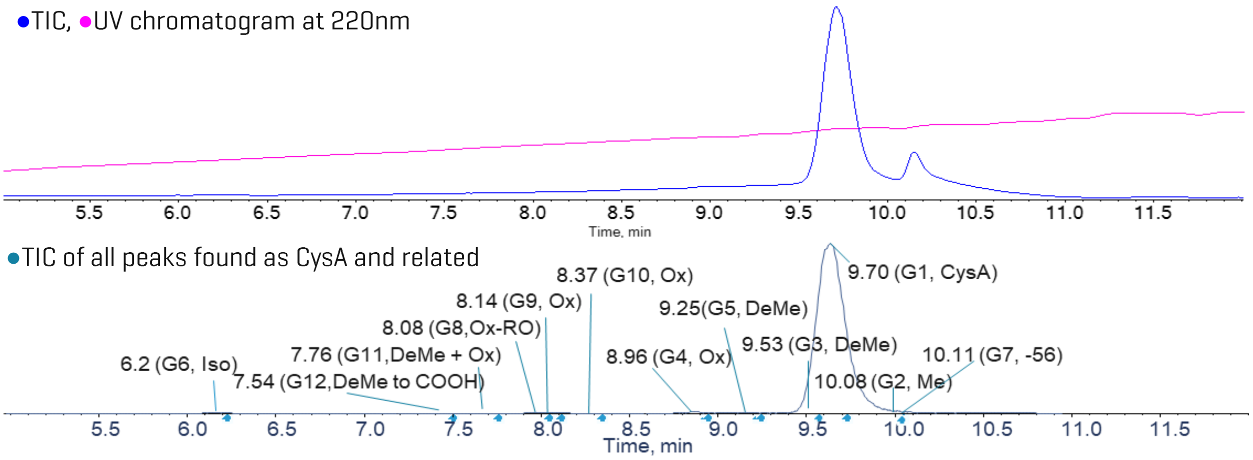


Figure 7. Overlay of total ion chromatogram (TIC) and UV 220 nm chromatogram, and TIC of CysA and detected CysA-related impurities with label (bottom). Iso, DeMe, Ox, RO and -56 stands for isomer of CysA, demethylation, oxidation, ring opening and loss of 56Da from CysA.

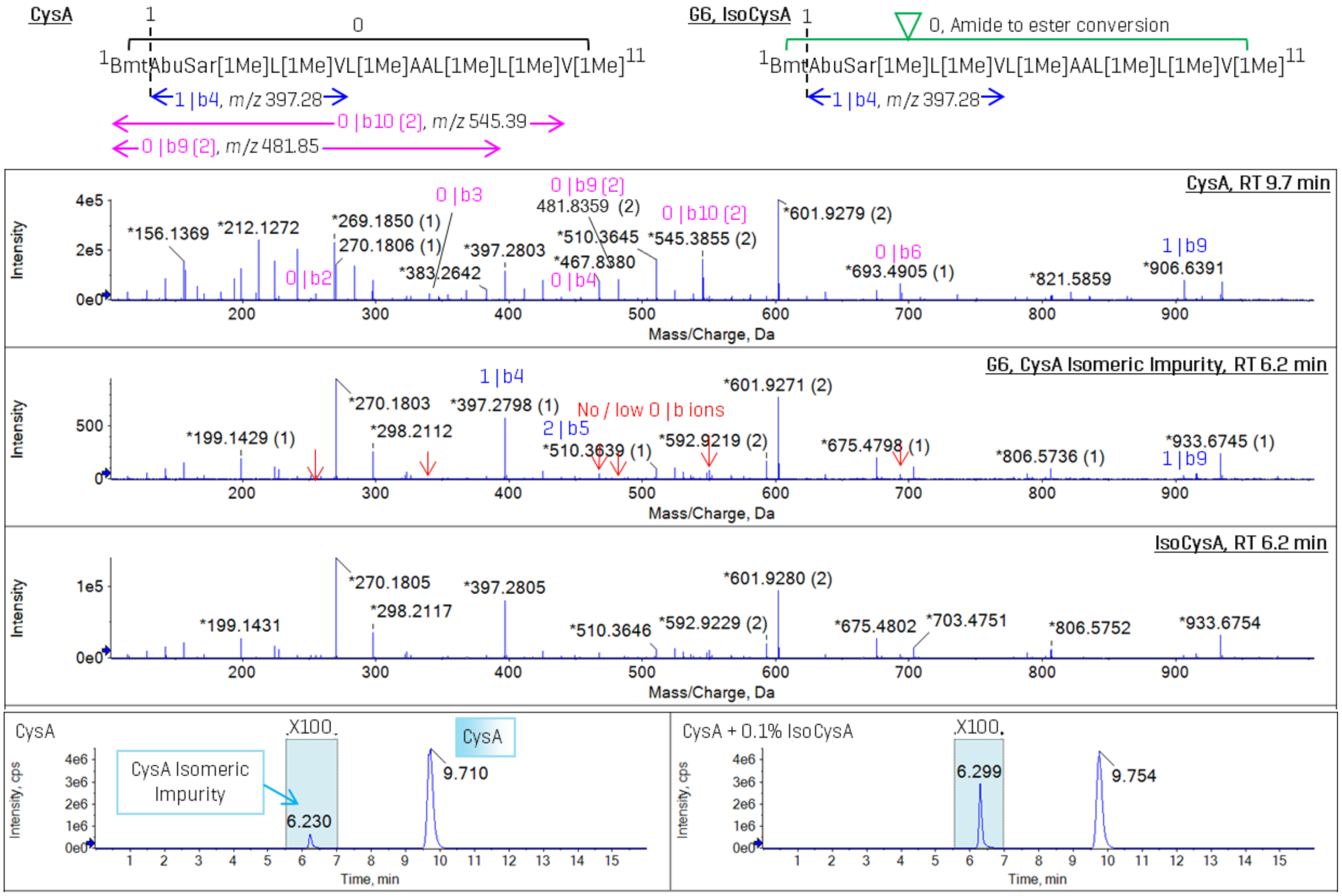


Figure 9. Structure confirmation of isomeric impurity G6. Sequence assignments of significant fragment ions (top), MS/MS spectra of CysA, G6 and IsoCysA (middle), and coelution test results of sample and IsoCysA (bottom).

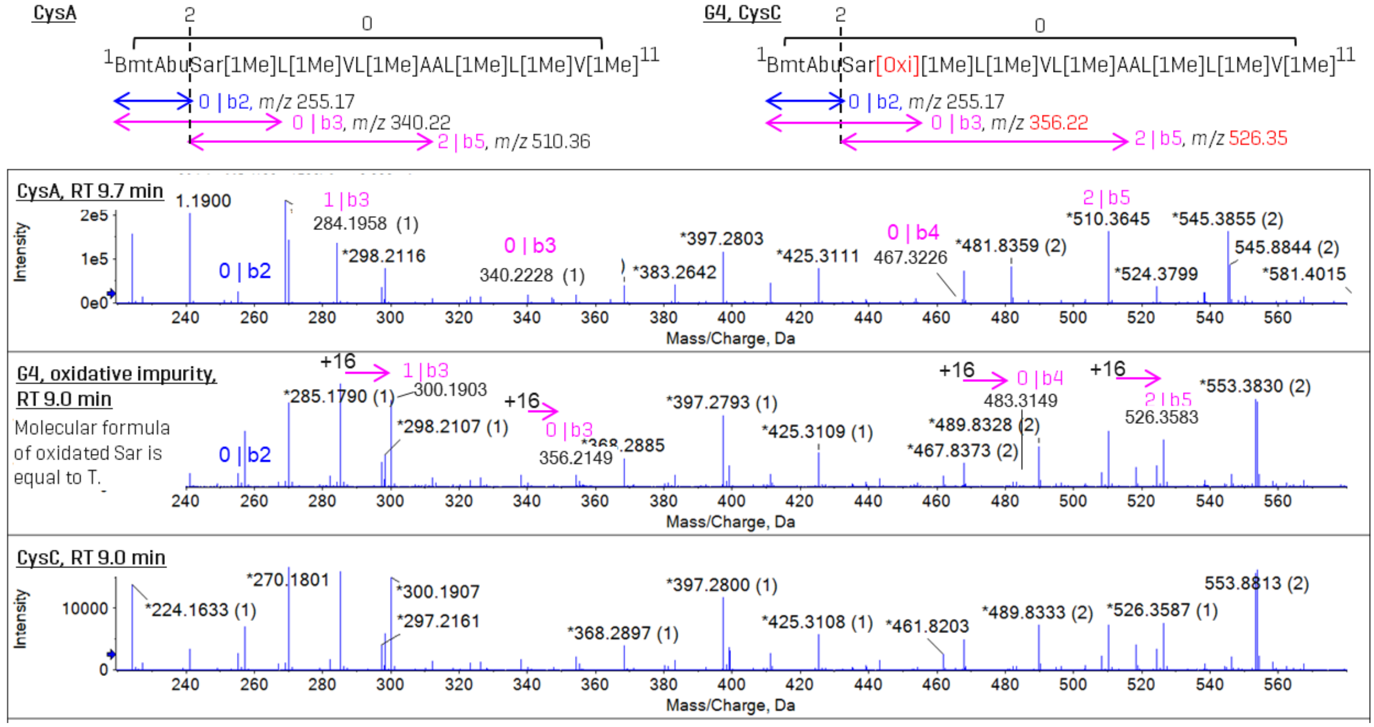
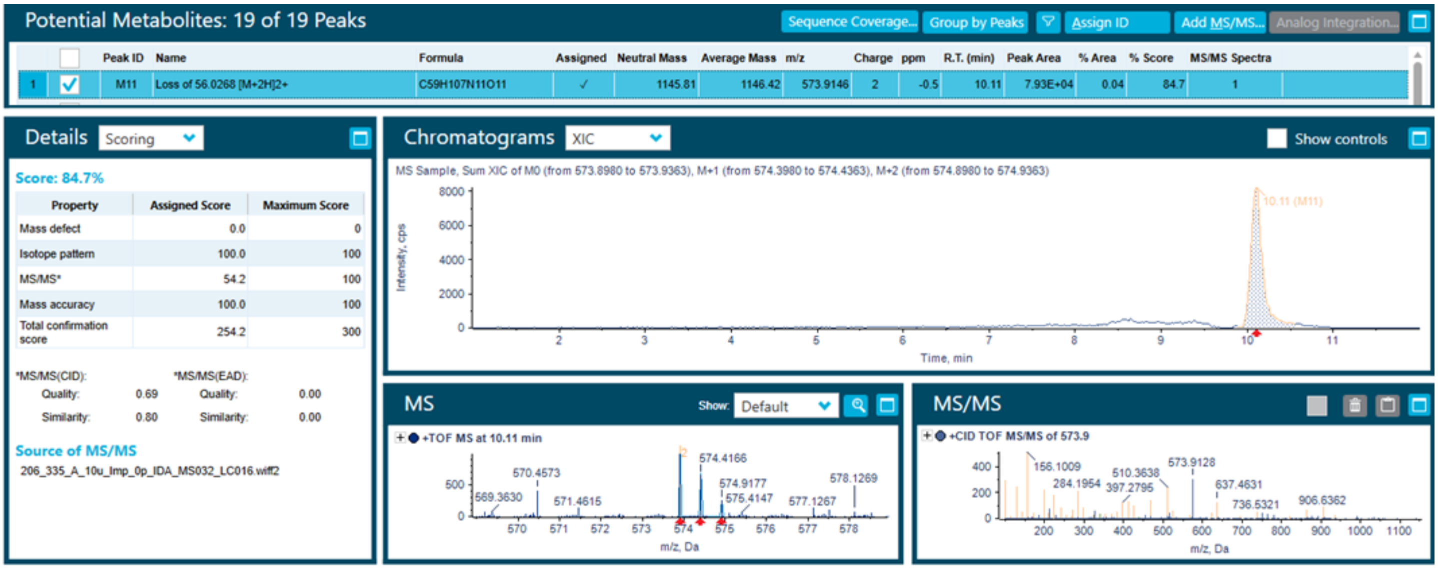


Figure 10. Structure confirmation of oxidative impurity G4. Sequence assignments of significant fragment ions (top), MS/MS spectra of CysA, G4 and CysC (bottom).

The methylation site in G2 was estimated to be Bmt. The fragment ions at *m/z* 394.28, 510.36 and 736.53, assigned as the 1 | b4, 2 | b5 and 4 | b7 ions of CysA, were conserved in G2, whereas the fragment ions at *m/z* 340.22 assigned as the 0 | b3 ions was detected with a +14Da shift in G2 (see Figure 13; data not shown).

Structure elucidation of unknown impurity

Figure 11 shows the results for the unexpected impurity G7. This impurity was detected as a CysA-related impurity because it generated at least five common product ions with CysA. The MS/MS of G7 was very similar to that of CysA (see the spectrum in the lower right and the score in the lower left of Figure 11). Compositional analysis using SCIEX OS software revealed that G7 was composed of CysA with less C3H4O (Figure 12). Additionally, MS/MS analysis suggested that the modification site was Bmt, as in G2, because, like G2, the fragment ions of CysA at *m/z* 394.28, 510.36, and 736.53 were conserved in G7, whereas the fragment ion at 340.22 was shifted by -56 Da (see Figure 13, data not shown).



Potential Metabolites: 19 of 19 Peaks

	☐	Peak ID	Name	Formula	Assigned	Neutral Mass	Average Mass	m/z	Charge	ppm	R.T. (min)	Peak Area	% Area	% Score	MS/MS Spectra
1	☑	M11	Loss of 56.0268 [M+2H]2+	C59H107N11O11	✓	1145.81	1146.42	573.9146	2	-0.5	10.11	7.93E+04	0.04	84.7	1

Details: Scoring

Score: 84.7%

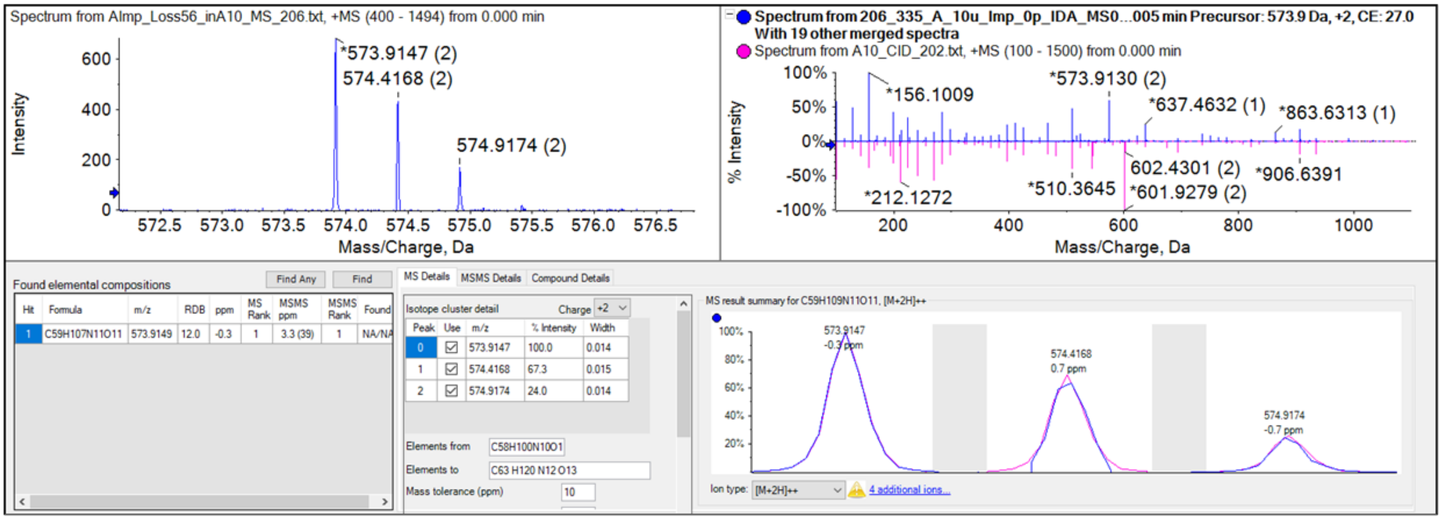
Property	Assigned Score	Maximum Score
Mass defect	0.0	0
Isotope pattern	100.0	100
MS/MS*	54.2	100
Mass accuracy	100.0	100
Total confirmation score	254.2	300

*MS/MS(CID):		*MS/MS(EAD):	
Quality:	0.69	Quality:	0.00
Similarity:	0.80	Similarity:	0.00

Source of MS/MS

206_335_A_10u_Imp_0p_IDA_MS032_LC016.wiff2

Figure 11. Results view of G7, unknown impurity. Many common product ions with CysA showed with orange in MS/MS and high MS/MS score reveals the impurity is related to CysA.



Found elemental compositions

Hit	Formula	m/z	RDB	ppm	MS Rank	MSMS ppm	MSMS Rank	Found
1	C59H107N11O11	573.9149	12.0	-0.3	1	3.3 (39)	1	NA/NA

Isotope cluster detail — Charge +2

Peak	Use	m/z	% Intensity	Width
0	☑	573.9147	100.0	0.014
1	☑	574.4168	67.3	0.015
2	☑	574.9174	24.0	0.014

Elements from: C58H100N10O1

Elements to: C63 H120 N12 O13

Mass tolerance (ppm): 10

Figure 12. Formula prediction using formula finder in SCIEX OS software. Molecular formula of G7 was predicted as C59H107N11O11 (minus C3H4O from CysA using the accurate mass), isotope pattern and MS/MS.

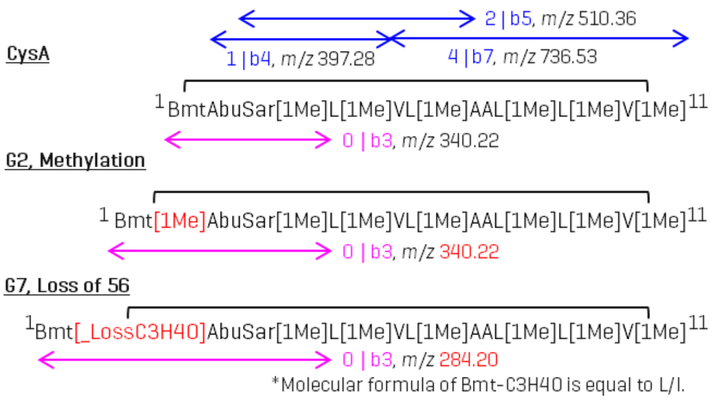


Figure 13. Estimation of modification sites of methylated impurity, G2 and unexpected impurity, G7 using product ions.

Comparison of impurities between samples

Figure 14 shows an example of impurity profile comparison between samples spiked with CysC (G4) and IsoCysA (G6) at concentrations of 0, 0.05, 0.1, 0.2, 0.5, and 1%. The areas of unspiked impurities G2 (blue) and G3 (red) do not change between samples, while the areas of G4 (purple) and G6 (green) increase with increasing spike concentration, clearly demonstrating the differences in impurity profiles. Molecule Profiler software facilitates impurity profile comparison between samples, supporting time-course analysis and lot management.

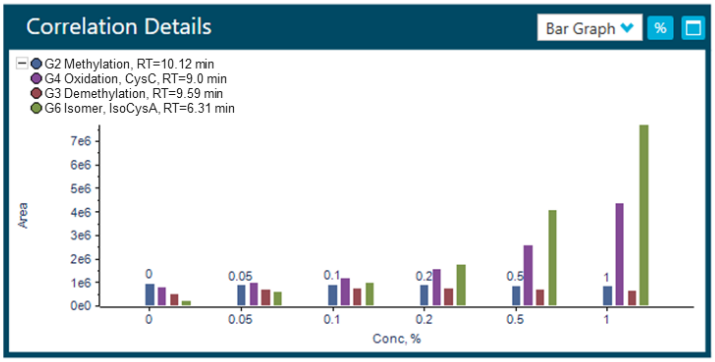


Figure 14. Correlation view of Molecule Profiler software.

Conclusions

- Confirmation of main component, and impurity analysis and profiling of cyclic peptide are shown using the ZenoTOF 7600+ system integrated with Molecule Profiler software and SCIEX OS software.
- Fast MS/MS scanning speed and high MS/MS data quality enhanced using the Zeno trap with the ZenoTOF 7600+ system, allows the identification of even very low abundant impurities.
- Analysing peptides in sequence notation simplifies the workflow, reduces manual effort, and provides clear, interpretable results. This accelerates impurity identification, modification-site localization, and correlation studies such as time-course analyses and lot-to-lot comparisons—all of which can be efficiently performed using the Molecule Profiler software

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