

Mass Spectrometry for the Analysis of Payloads and Related Impurities in Antibody–Drug Conjugates

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Abstract

In antibody–drug conjugate research, the small molecule impurities requiring evaluation include not only free payloads, but also potential payload-related small molecule impurities. In this study, high-resolution mass spectrometry and triple quadrupole mass spectrometry were employed for systematically and comprehensively investigate small molecule related impurities in antibody–drug conjugate product. First, the quantitation of free payload was analyzed. High-resolution mass spectrometry enabled quantification at both precursor ion and product ion levels, the free payload exhibited good linearity over the concentration range of 0.05–100 ng/mL ($R^2 \geq 0.991$), with a lower limit of quantification of 0.05 ng/mL. The accuracy ranged from 80% to 120%, and the precision was below 5%. The high-resolution mass spectrometry results were confirmed using triple quadrupole mass spectrometry. Second, other potential payload related small molecule impurities in the antibody–drug conjugate was investigated in depth. Multiple possible hydrolysis pathways of the payload were predicted based on molecular structure analysis, and the corresponding payload related impurities were subsequently identified using triple quadrupole mass spectrometry in accordance with the predicted precursor–product ion information. Third, stability studies of the antibody–drug conjugate were conducted under forced degradation conditions. Qualitative analyses were performed to characterize unknown payload related degradation products generated under stress conditions, including light exposure, heat, acidic, and alkaline environments. The major degradation products and their relative abundances were identified, with alkaline conditions exerting the greatest impact on sample stability. Clinical trial number: not applicable.

1 Introduction

Rapid progress in the biopharmaceutical market has led to the emergence of antibody–drug conjugate (ADC) drugs as a new approach to cancer treatment. ADCs link cytotoxic small-molecule drugs to monoclonal antibodies via chemical bonds. The high affinity of antibodies for specific tumor cell surface antigens enables the precision delivery of cytotoxic drugs to cancer cells, enhancing efficacy and reducing damage to normal tissues. Several generations of development have occurred since the concept of ADCs was first proposed. The first-generation ADC is represented by Pfizer's Mylotarg (gemtuzumab ozogamicin). Mylotarg, the first ADC worldwide, was approved in 2000, representing the first clinical use of this drug type. Mylotarg is formed by conjugating an anti-CD33 monoclonal antibody to the antitumor antibiotic calicheamicin via a cleavable hydrazone linker and is used to treat CD33-positive acute myeloid leukemia (AML) [1]. However, in clinical practice, linker stability was found to be poor in the blood circulation; furthermore, calicheamicin was prone to early release, leading to severe off-target toxicity, a narrow therapeutic window, and limited patient benefit. Thus, Mylotarg was withdrawn from the market in 2010.

The issues encountered with this first-generation ADC provided direction for subsequent research and development. Second-generation ADCs are optimized with respect to linkers, antibodies, and the cytotoxic payload. The linker is designed to be more stable, for example, using non-cleavable linkers or modifications to the structure of cleavable linkers that improve their stability in the blood circulation, reducing the risk of early release. Regarding antibodies, humanized or fully human antibodies are often

used to reduce immunogenicity [2–3]. For example, Adcetris (brentuximab vedotin) and Kadcyla (ado-trastuzumab emtansine), which are representative second-generation ADCs for treating solid tumors, have cleavable linkers that couple different toxin molecules [4–5].

Continuous technical progress has enabled the development of more innovative, third-generation ADCs that also retain the advantages of the previous two generations. For example, regarding the coupling technique, the site-specific conjugation method results in a more even drug-to-antibody ratio (DAR), enhancing product quality and stability and improving the ADC's pharmacokinetic properties. For example, Daiichi Sankyo's Enhertu (trastuzumab deruxtecan) is formed by coupling trastuzumab and a topoisomerase I inhibitor (exatecan derivative) via a cleavable linker. This ADC has demonstrated excellent efficacy in treating several cancers, including HER2-positive breast cancer and gastric cancer, expanding the applicable target population of this drug type [6–7]. To date, a total of 19 antibody–drug conjugates (ADCs) have been approved globally, covering a broad range of indications for hematological malignancies and solid tumors. In addition, approximately 40 ADC candidates worldwide are currently undergoing phase III clinical trials. Given their therapeutic potential in oncology, ADCs are expected to experience rapid and transformative growth in the near future. [8–10].

On April 23, 2021, loncastuximab tesirine was approved by the United States Food and Drug Administration (FDA) for the treatment of relapsed or refractory diffuse large B-cell lymphoma (DLBCL) after two or more systemic therapies. On December 10, 2024, loncastuximab tesirine was approved by the National Medical Products Administration (NMPA) of China, representing the first CD19 ADC drug approved and marketed in China. Loncastuximab tesirine comprises a humanized IgG1 kappa monoclonal antibody attached to SG3249 (tesirine) by cysteine, and its DAR value is approximately 2.3 [11–12]. SG3249 comprises the pyrrolo–benzodiazepine (PBD) dimer SG3199, para-aminobenzoic acid (PABA), a dipeptide (Val-Ala), a PEG8 spacer to increase solubility, and a maleimide linker. Its structure is shown in Fig. 1. This study focuses on this product.

ADC products require multidimensional quality analysis during research and manufacturing. In particular, comprehensive quality characterization of the naked antibody is essential, including extensive structural and physicochemical analyses such as amino acid sequence determination, glycosylation profiling, and higher-order structure characterization, to ensure the correctness and stability of the antibody structure. Meanwhile, free payload and payload-related impurities are also critical quality attributes (CQAs), as they directly determine the safety and efficacy of the drug product. Quality control of small-molecule toxins and linkers involves structural confirmation and limit testing, among other requirements [13–15], all of which rely on appropriate analytical methodologies.

In studies of drug metabolism and pharmacokinetics (DMPK), the quantification of free payload requires substantially higher analytical sensitivity due to its low in vivo concentrations and pronounced matrix effects. To date, the most widely reported approach for quantifying free payloads in blood samples is triple quadrupole mass spectrometry, operated in the multiple reaction monitoring (MRM) mode. From the perspective of in vivo drug metabolism studies, this method has been extensively applied to characterize the time-dependent release profiles of ADC payloads in circulation [16–18]. In certain investigations of

ADC in vivo metabolism, the lower limit of quantification for unconjugated cytotoxic drugs in blood has been reported to reach 0.015 ng/mL using triple quadrupole mass spectrometry [19]. Owing to the excellent selectivity of the MRM quantification mode, triple quadrupole mass spectrometry is also applicable to quality control of ADC products, where it can be employed to monitor free payload levels and thereby assess drug safety and efficacy [20–21]. As the sensitivity requirements for quantifying free payloads in in vitro ADC products are generally lower than those for in vivo bioanalysis, high-resolution mass spectrometry (HRMS) is also suitable for meeting analytical needs in ADC product testing. With its superior mass resolution, HRMS enables quantification based on both full-scan (TOFMS) data and fragment ions in MS/MS-based quantitative workflows, such as high-resolution multiple reaction monitoring (MRM^{hr}). Reported studies have demonstrated that the sensitivity of HRMS-based methods for quantifying free payloads in ADCs can reach the ppb level or even lower [22].

Researchers have also used specialized pretreatment methods to quantify the free ADC payload using an optimized liquid chromatography (LC) method; for example, samples labeled with fluorescent dye during pretreatment, purified using solid-phase extraction (SPE), and then quantified by LC [22]. However, the pretreatment required for this method is complicated, recovery is unstable, and sensitivity is limited, usually in the range of nanograms per milliliter to micrograms per milliliter. TQMS is the most reported method for quantifying the free ADC payload in drug metabolism and pharmacokinetics studies. *In vivo* drug metabolism research has been employed to demonstrate the difference in the released ADC payload content over time [23–27]. This technique, using the targeted quantification mode, is also used to quantitatively analyze the free payload in ADC quality control. Similarly, HRMS has been used to quantify the free payload in ADC drugs; the free payload is measured while the ADC product is characterized [28–30]. For example, Leanne combined HRMS and TQMS to quantitatively analyze the free payload in ADC products. However, deeper-level structural characterizations and drug stability analyses remain lacking. Forced degradation experiments are critical core components in the study of ADC drug stability. In the context of drug stability, artificial applications of extreme conditions, such as high temperatures, acidic or basic environments, and oxidative conditions, can systematically reveal potential degradation pathways and key degradation products, providing the critical evidence needed for drug formulation optimization, packaging material selection, and storage condition determination.

In this study, we analyzed the newly approved ADC loncastuximab tesirine using multiple HRMS quantification methods. In addition to the secondary quantification of free payload using MRM^{hr}, the super-high-resolution capability of HRMS was exploited to achieve primary free payload quantification. Experimental validation revealed a good quantification range. TQMS was applied in the same context. In the quantitative free-payload analysis of the ADC product, the HRMS primary and secondary quantification results were consistent with those obtained via TQMS, with coefficients of variation below 10%. In the study of payload stability, specific ions were assessed via TQMS in product-ion scan mode to identify hydrolysis products predicted from hydrolysis-prone sites in the chemical structure, providing reliable data to support the quantification of the payload and its potential hydrolysis products. In the complete stability study, the ADC was subjected to various forced degradation conditions, including strong acidic, strong basic, heated, and light-illuminated environments. For the first time, we employed HRMS coupled with

nanoflow liquid chromatography to analyze the degradation products of the ADC drug payload. The degradation pathways of the ADC products under various extreme conditions were elucidated according to the number of degradation products and their common products, providing data to support drug stability studies.

2 Materials and samples

2.1 Materials

Liquid chromatography–mass spectrometry (LC–MS) grade acetonitrile and water were purchased from Thermo Fisher; 10 kDa ultrafiltration membrane filters were purchased from Sartorius.

2.2 Samples

The ADC product loncastuximab tesirine and N-acetylcysteine-modified tesirine (NAC-SG3249) were sourced as retained from the National Institutes for Food and Drug Control (China). In the ADC manufacturing process, N-acetylcysteine (NAC) was used as the antioxidant, which binds to free tesirine; thus, the free payload exists as NAC-SG3249.

2.2.1 Preparation of the NAC-SG3249 standard

The NAC-SG3249 standard was sequentially diluted with LC–MS-grade water, and standard curve gradient concentrations were prepared as follows: 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 ng/mL.

2.2.2 ADC sample preparation

The sample was ultrafiltered using a 10 kDa ultrafiltration membrane. Subsequently, the filtrate was evaporated to dryness and reconstituted in a 1/10 volume of LC–MS water to prepare the test sample. The free NAC-SG3249 content in the test sample was measured.

2.2.3 Stress test sample preparation

Four forced degradation modes were designed to process the ADC sample: acid treatment (pH adjusted to 2–3 using formic acid), base treatment (pH adjusted to 11–12 using aqueous ammonia), strong light illumination, and heating at 60°C. The four conditions were maintained in an enclosed environment for one day. Subsequently, the samples were ultrafiltered using a 10 kDa membrane. The filtrate was then evaporated to dryness and reconstituted in a 1/10 volume of LC–MS-grade water to prepare the test samples. The potential degradation products in the test samples were measured.

3 Methods

Liquid chromatography–high-resolution mass spectrometry (LC–HRMS) was used to quantitatively analyze NAC-SG3249, and the results were compared with those from the LC–TQMS method. The potential hydrolysis products were identified and structurally characterized by TQMS. The degradation impurities in

the payload under forced degradation conditions were analyzed by HRMS coupled with nanoflow liquid chromatography.

3.1 LC method

A SCIEX ExionLC™ AE system was employed using a Waters ACQUITY UPLC Peptide BEH C18 chromatography column (130 Å, 1.7 µm, 2.1 × 100 mm) under the following conditions: mobile phase A, water, 0.1% formic acid; mobile phase B, acetonitrile, 0.1% formic acid; column temperature, 40°C; and injection volume, 20 µL. The gradient elution program is shown in Table 1.

Table 1
Liquid chromatography gradient elution program

Time (min)	Flow (mL/min)	B. Conc (%)
0.0	0.3	10.0
1.0	0.3	10.0
7.0	0.3	95.0
10.0	0.3	95.0
10.1	0.3	10.0
12.0	0.35	10.0

A Waters ACQUITY MCLASS LC system was employed using a Waters ACQUITY UPLC Peptide BEH C18 chromatography column (1.7 µm, 75 µm × 25 cm) under the following conditions: mobile phase A, water, 0.1% formic acid; mobile phase B, acetonitrile, 0.1% formic acid; column temperature, 60°C; and injection volume, 20 µL. The gradient elution program is shown in Table 2.

Table 2
Nanoflow liquid chromatography gradient elution program

Time (min)	Flow (µL/min)	B. Conc (%)
0.0	0.3000	3.0
1.0	0.3000	8.0
41.0	0.3000	45.0
43.0	0.3000	95.0
50.0	0.3000	95.0
51.0	0.3000	3.0
60.0	0.3000	3.0

3.2 QTOF method

A SCIEX ZenoTOF 7600 system was employed for MRM^{hr} data collection in positive-ion mode. The conditions were as follows: nebulizer gas (GS1), 70 psi; auxiliary gas (GS2), 20 psi; curtain gas (CUR), 35 psi; ion source temperature (TEM), 500°C; ionization source voltage (IS), 5500 V; and collision gas (CAD), 7 psi. MRM^{hr} ion pair data are shown in Table 3.

Table 3
MRM^{hr} ion pairs and MS parameters

Compound	Precursor ion	product ion	Accumulation time (ms)	Declustering potential	Collision energy
NAC-SG3249-1	830.3729	358.1073	25	80	46
NAC-SG3249-2	830.3729	585.2713	25	80	30
NAC-SG3249-3	830.3729	908.4179	25	80	26

The SCIEX ZenoTOF 7600 system was also employed for information-dependent acquisition (IDA) of data in positive-ion mode. The conditions were as follows: GS1, 8 psi; CUR, 25 psi; TEM, 175°C; IS, 4000 V; and CAD, 7 psi. For the TOF MS scan, the range was 350–1500, the accumulation time was 0.25 s, and the maximum number of candidate ions was 20. Exclude Former candidate ions were excluded for 3 s. For the TOF MSMS scan, the range was 100–1500, and the accumulation time was 0.05 s. Zeno pulsing was enabled, and the Zeno threshold was set to 100,000. The dynamic collision energy (CE) spread was 10, and the time bins were set to sum 8.

2.3.3 Triple quad method

The Triple Quad™ 6500 + system, a SCIEX localized product in China, was used; the data collection mode was MRM, and the positive ion mode was employed. The conditions were as follows: GS1, 70 psi; GS2, 20 psi; CUR, 35 psi; TEM, 500°C; IS, 5500 V; and CAD, 7 psi. The MRM ion pair data are shown in Table 4.

Table 4
MRM ion pairs and MS parameters

Compound	Precursor ion	Product ion	Dwell time (ms)	Declustering potential	Entrance potential	Collision energy	Collision cell exit potential
NAC-SG3249-1	830.7	358.1	20	100	10	50	10
NAC-SG3249-2	830.7	585.2	20	100	10	30	10
NAC-SG3249-2	830.7	908.4	20	110	10	30	10

3 Results and discussion

3.1 Quantitative analysis of payload (NAC-SG3249) in the ADC product

The molecular formula of payload (NAC-SG3249) is $C_{80}H_{110}O_{26}N_{10}S$. The information-dependent acquisition (IDA) mode in HRMS was used to identify the quantitative ion pairs (Fig. 2). The mass-to-charge ratio (m/z) of the doubly charged ion detected by the TOFMS was 830.3741, and this precursor ion displayed the highest response, showing only 1.44 ppm in the mass error of the theoretical m/z . Thus, it could be used for primary HRMS quantification. In the analysis of the fragments, the characteristic fragment ions of 358.1072 (m/z), 585.2708 (m/z), and 908.4172 (m/z), whose corresponding structural information could be found in the structural elucidation, were detected. The corresponding ion pairs could act as the theoretical ion pairs set in the MRM^{hr} method of HRMS and the MRM method of TQMS.

TOFMS and TOFMS/MS analyses were performed to quantitatively detect the NAC-SG3249 in the ADC sample according to the identified ion pairs. NAC-SG3249 gradient standard samples were used to investigate linearity to verify the method's reliability (Fig. 3). In the TOFMS and TOFMS/MS analyses, the concentration gradient for NAC-SG3249 was set to 0.05–100 ng/mL, and the linear correlation coefficients (R^2) were 0.991 and 0.996, respectively. We performed the TQMS quantitative analysis in parallel to verify the accuracy of the HRMS results. The linear correlation coefficient (R^2) reached 0.999. The limit of quantitation values for HRMS and TQMS in the NAC-SG3249 analysis were 0.05 ng/mL and 0.005 ng/mL, respectively. Furthermore, the accuracy values at all linear concentration points stabilized within 80%–120%, indicating that both methods are suitable for quantitative analyses.

In addition to assessing the linear relationship, detection limit, and accuracy, we conducted a systematic analysis of precision and recovery across different instruments and analytical methods. Precision was investigated using low, medium, and high concentration gradients; three injections were conducted per concentration point (Table 5). The precision values of all concentration points were below 5%, indicating

good repeatability. Furthermore, recovery was stable across all concentrations, at 80%–120%, further validating the method's reliability and accuracy.

Table 5
Precision analysis results obtained with different techniques and different instruments

Concentration point	Precision investigation		
	TOFMS	TOFMSMS	Triple Quad MS
0.5 ng/mL	1.33%	3.03%	2.82%
5 ng/mL	1.38%	2.73%	1.07%
50 ng/mL	0.84%	0.41%	0.48%

We employed three methods to analyze payload (NAC-SG3249) in ADC samples: TOFMS quantification, TOFMS/MS MRM^{hr}, and TQMS MRM; measured concentrations of NAC-SG3249 residue in the ADC samples were 812.5 ng/mL, 846.6 ng/mL, and 781.4 ng/mL, respectively. The three quantification methods showed good consistency, with an RSD below 10%. These findings indicate that HRMS can reach the same level of detection as TQMS for the free payload in ADC samples.

3.2 Assessment of NAC-SG3249 stability

In the *in vitro* synthesis of NAC-SG3249, NAC attacks the double-bonded carbon atom on the maleimide ring of SG3249 via a nucleophilic reaction mechanism, thus combining with SG3249 to form NAC-SG3249. We assessed NAC-SG3249 stability via molecular structure analyses, revealing relatively low amide and ester bond energies, indicating bonds prone to hydrolysis or cleavage (Fig. 4). Notably, the specific cleavage sites of the three characteristic ions used in the quantification analysis could be determined by analyzing the NAC-SG3249 hydrolysis products.

Furthermore, we investigated the potential hydrolysis products of NAC-SG3249 during the stability analysis. Six possible hydrolysis products were predicted according to the sites prone to hydrolysis (Fig. 5). MRM mode was employed in the TQMS measurements. The predicted hydrolysis product was selected as the precursor ion, and the fragment ion 358.1 m/z was selected as the product ion. The molecular weight of predicted hydrolysis product A was too small for analysis; thus, this product was not investigated. The precursor ion signal of 756.3 m/z was detected at 3.8 min (as shown in Fig. 6), indicating the hydrolysis product of NAC-SG3249 (Fig. 4B). The retention behavior of this hydrolysis product on the C18 chromatography column revealed that its hydrophilicity was even stronger than that of NAC-SG3249, consistent with the theoretical structure characteristics. Following MRM mode application, an information-dependent acquisition (IDA)-enhanced product ion (EPI) was set to conduct fragmentation treatment on this hydrolysis product, and the characteristic ions of 696.55 m/z, 710.76 m/z, and 738.48 m/z were detected in the product ion signal (Fig. 7). Additionally, the cleavage positions corresponding to these fragments could be found in the structure of this hydrolysis product, and they were related to three

different cleavage positions (Fig. 7D). Thus, it can be determined that this substance is an effectively detectable hydrolysis product of NAC-SG3249.

3.3 Stability Study of Small Molecules with Forced Degradation in ADC Samples

To assess the stability of the ADC product, we designed four forced degradation experiments with different conditions: acid treatment, base treatment, light illumination, and heating treatment. The metabolites of the payload in the ADC drug were analyzed under each condition. HRMS coupled with nanoflow liquid chromatography in IDA scan mode was employed to measure metabolites in the different forced degradation samples. Under acid, base, light illumination, and heating conditions, 23, 39, 26, and 28 types of small molecular related impurities were detected. The greatest number of small molecular related impurities was observed with base treatment. Twelve common small molecular related impurities were identified across the four forced degradation conditions (Fig. 8). The common small molecular related impurities are shown in Table 6.

Table 6
Identification of the common degradation products using four forced degradation method

No.	Name	Formula	Neutral Mass	Average Mass	m/z	ppm
1	Loss of C ₄₄ H ₄₇ N ₅ O ₁₀ and C ₁₂ H ₁₄ N ₂ O ₆ S+Demethylation to Carboxylic Acid [M + H] ⁺	C ₂₄ H ₄₇ N ₃ O ₁₂	569.3148	569.4921	570.3221	-2.1
2	Loss of C ₅ H ₇ N ₃ O ₃ and C ₄₉ H ₅₆ N ₆ O ₁₁ + Thioalcohol to Alcohol [M + H] ⁺	C ₂₆ H ₄₇ N ₃ O ₁₃	609.3091	609.5364	610.3164	-3
3	Loss of C ₁₂ H ₁₅ N ₃ O ₆ S and C ₄₉ H ₅₆ N ₆ O ₁₁ + Amine to Carboxylic Acid [M + H] ⁺	C ₂₀ H ₃₈ O ₁₁	454.2398	454.4053	455.2471	-3.5
4	Loss of C ₅₂ H ₆₁ N ₇ O ₁₂ and C ₁₂ H ₁₄ N ₂ O ₆ S+Oxidation [M + H] ⁺	C ₁₆ H ₃₅ N ₉ O	385.2298	385.3778	386.2371	-3.5
5	Loss of C ₁₂ H ₁₅ N ₃ O ₆ S and C ₄₉ H ₅₆ N ₆ O ₁₁ + Oxidation [M + H] ⁺	C ₁₉ H ₃₉ N ₁₀ O	441.256	441.4348	442.2633	-3.1
6	Loss of C ₁₂ H ₁₅ N ₃ O ₆ S and C ₅₂ H ₆₁ N ₇ O ₁₂ + Oxidation [M + H] ⁺	C ₁₆ H ₃₄ O ₉	370.2195	370.3921	371.2267	-2.2
7	Loss of C ₅₂ H ₆₁ N ₇ O ₁₂ and C ₁₂ H ₁₄ N ₂ O ₆ S [M + H] ⁺	C ₁₆ H ₃₅ N ₈ O	369.2348	369.6364	370.2421	-4
8	Loss of C ₄₉ H ₅₆ N ₆ O ₁₁ and C ₁₄ H ₁₉ N ₃ O ₇ S+Amine to Carboxylic Acid [M + H] ⁺	C ₁₈ H ₃₄ O ₁₀	410.2135	410.3629	411.2208	-4
9	Loss of C ₁₂ H ₁₅ N ₃ O ₆ S and C ₅₆ H ₆₉ N ₇ O ₁₄ + Glucose Conjugation [M + H] ⁺	C ₁₈ H ₃₆ O ₁₁	428.2247	428.383	429.232	-2.4
10	Loss of C ₄₉ H ₅₆ N ₆ O ₁₁ and C ₁₆ H ₂₃ N ₃ O ₈ S+Amine to Carboxylic Acid [M + H] ⁺	C ₁₆ H ₃₀ O ₉	366.1877	366.3189	367.195	-3.4
11	Loss of C ₄₉ H ₅₇ N ₇ O ₁₁ and C ₁₆ H ₂₃ N ₃ O ₇ S+Oxidation [M + H] ⁺	C ₁₅ H ₃₀ O ₉	354.1885	354.3265	355.1958	-1.2
12	Loss of C ₃₄ H ₃₆ N ₄ O ₈ and C ₂₆ H ₄₃ N ₃ O ₁₂ S+Glucose Conjugation [M + H] ⁺	C ₂₆ H ₄₁ N ₃ O ₁₁	571.2721	571.473	572.2793	-3.6

4 Conclusions

This study employed high-resolution mass spectrometry (HRMS) and triple quadrupole mass spectrometry (TQMS) as the core analytical technologies to comprehensively investigate the payload and payload-

related small-molecule impurities in the commercially available antibody–drug conjugate (ADC) product loncastuximab tesirine. The study focused on the quantitative determination of the free payload (NAC-SG3249), the characterization of potential small-molecule impurities associated with the payload, and the investigation of payload-related impurities generated under forced degradation conditions. The objective of this work was to establish a clear and reliable analytical strategy for payload analysis in ADC products.

For the quantitative analysis of the free payload, characteristic precursor–product ion transitions of NAC-SG3249 were selected. Both TOFMS and TOFMS/MS quantification approaches were evaluated by taking advantage of the superior mass resolution of high-resolution mass spectrometry, and the analytical methodology was systematically assessed. The results demonstrated excellent linearity across different concentration levels, with coefficients of determination (R^2) greater than 0.99. The lower limit of quantification (LLOQ) was determined to be 0.05 ng/mL. Method accuracy and precision were evaluated at multiple concentration levels; accuracy ranged from 80% to 120%, and precision showed relative standard deviations (RSDs) of less than 5%. Recovery values also fell within the acceptable range of 80%–120%. The quantitative results obtained by HRMS were consistent with those generated using triple quadrupole mass spectrometry. For the determination of free payload (NAC-SG3249) in ADC samples, the deviation among the three analytical approaches was less than 10%.

In the study of potential impurities associated with the payload (NAC-SG3249), labile bonds within the molecular structure that are susceptible to hydrolysis were systematically analyzed. Using the MRM-IDA-EPI acquisition mode of triple quadrupole mass spectrometry, hydrolysis products of NAC-SG3249 were accurately detected and structurally characterized. These findings provide a theoretical basis for the subsequent quantitative analysis of payload-related impurities.

For the stability assessment of the ADC samples, forced degradation studies were conducted under acidic, alkaline, photolytic, and thermal conditions. Leveraging the high sensitivity and mass accuracy of high-resolution mass spectrometry, trace-level degradation products were detected and confidently identified through matching experimental data with theoretical mass calculations using dedicated data analysis software. Among the four forced degradation conditions, alkaline treatment had the most pronounced impact on sample stability, generating a total of 39 degradation products. Twelve degradation products were found to be common across all four stress conditions. These results enable the elucidation of degradation pathways of the payload and provide a solid foundation for subsequent stability evaluation and impurity control strategies.

Abbreviations

ADC

Antibody–drug conjugate

NAC

N-acetylcysteine

CQAs

Constitute critical quality attributes

MS
Mass spectrometry
LC
Liquid chromatography
HRMS
High-resolution mass spectrometry
TQMS
Triple quadrupole mass spectrometry
TOFMS
Time of flight mass spectrometry
TOFMS/MS
Tandem time-of-flight mass spectrometry
MRM^{hr}
High-resolution multiple reaction monitoring
MRM
Multiple reaction monitoring
RSD
Relative standard deviation
CD33
Cluster of differentiation 33
DAR
Drug-to-antibody ratio
IDA
Information-dependent acquisition
EPI
Enhanced product ion
FDA
United states food and drug administration
NMPA
National medical products administration

Declarations

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not applicable.

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Competing Interests

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Author's Contribution

GW and GX conceived and designed the research; YZ, TG and XL collected and analyze the data; YN, HC and ML wrote the manuscript; JD and CY revised the manuscript and gave meaningful discussion and suggestions. All authors have read and agreed to the published version of the manuscript.

Data Availability

Data are available from the corresponding authors on reasonable request.

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Figures

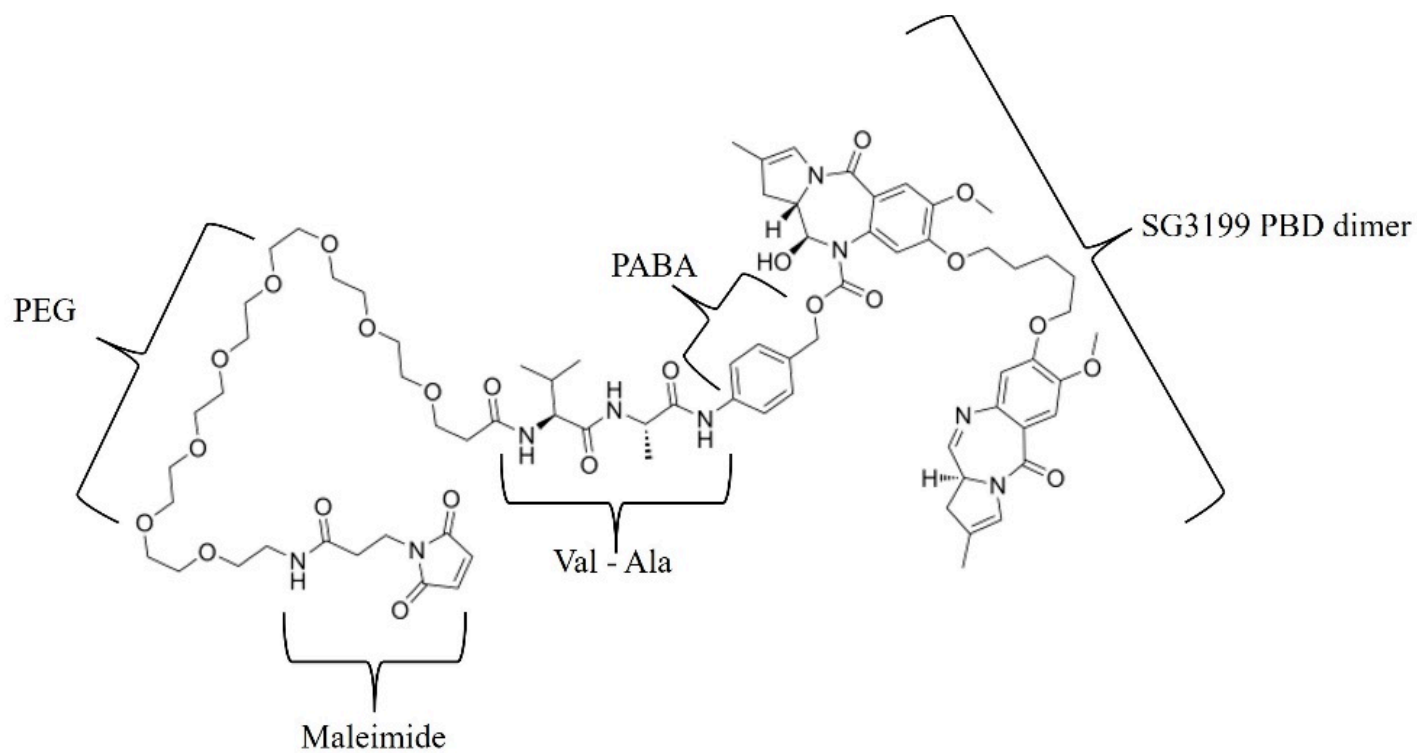


Figure 1

Schematic diagram of the SG3249 (tesirine) structure

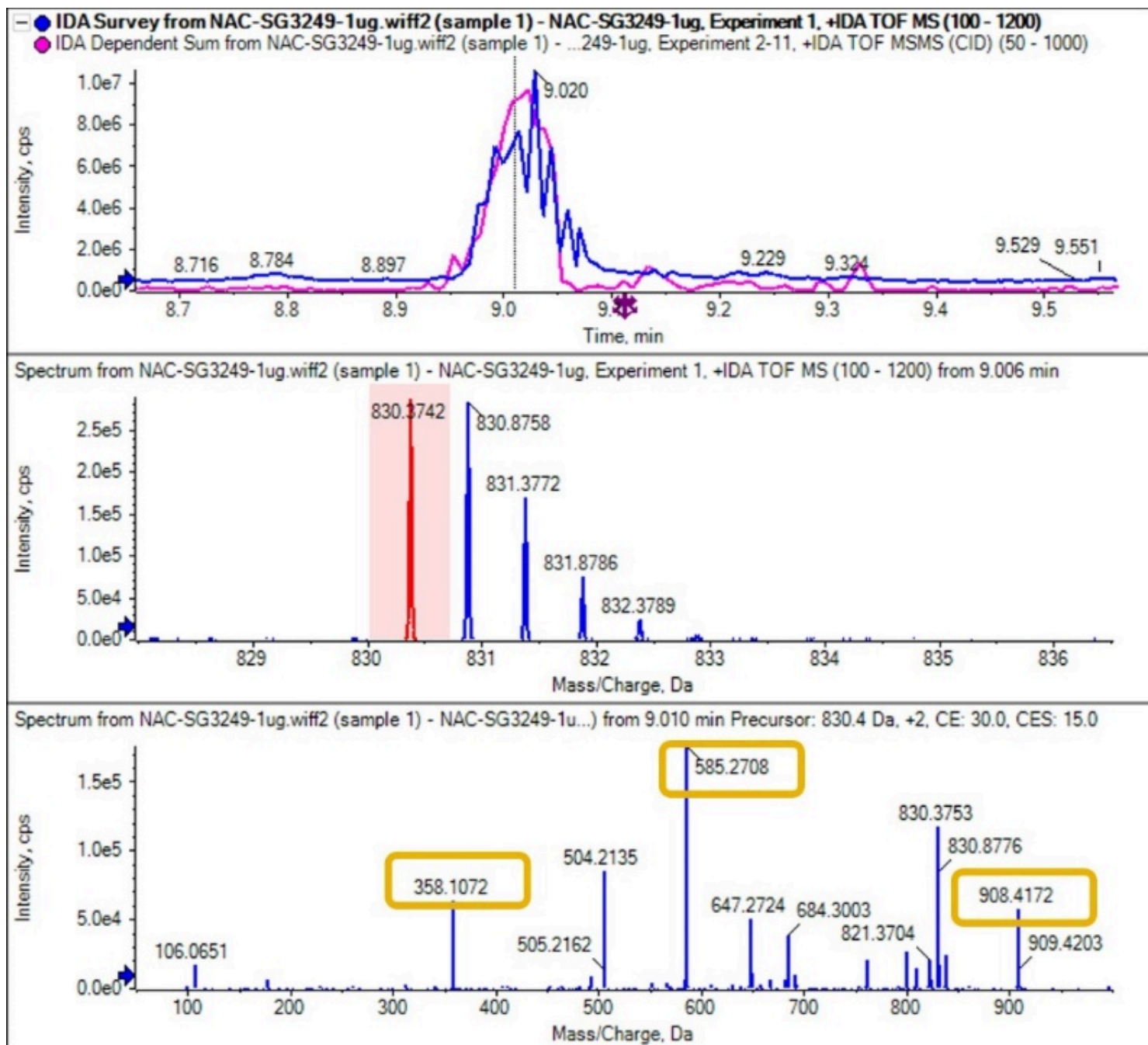


Figure 2

HRMS IDA analysis results for NAC-SG3249. A: TIC, B: TOFMS, C: TOFMSMS

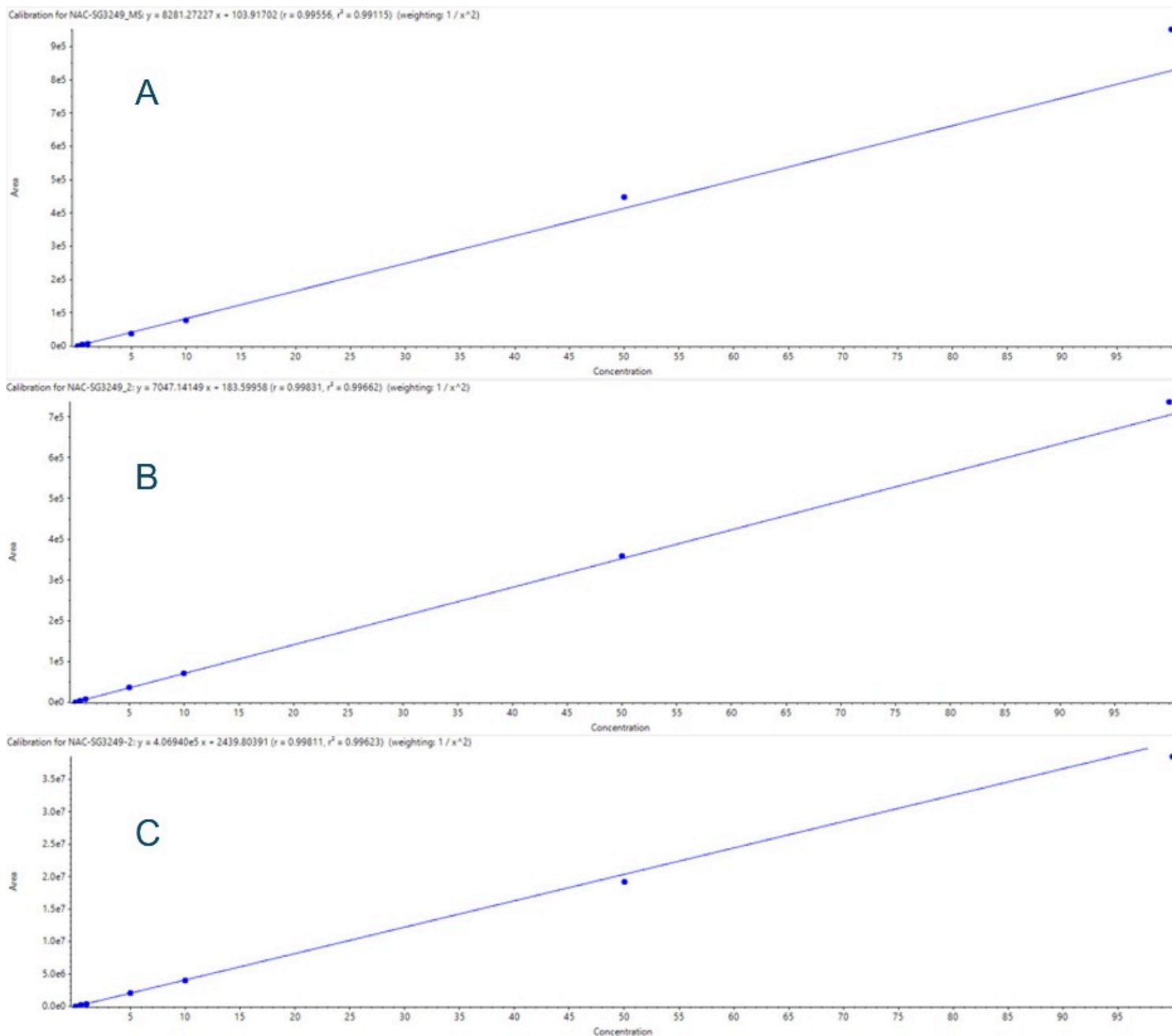


Figure 3

Linearity investigation of NAC-SG3249 using different instruments and methods. A: TOFMS, B: TOFMS/MS, C: TQMS

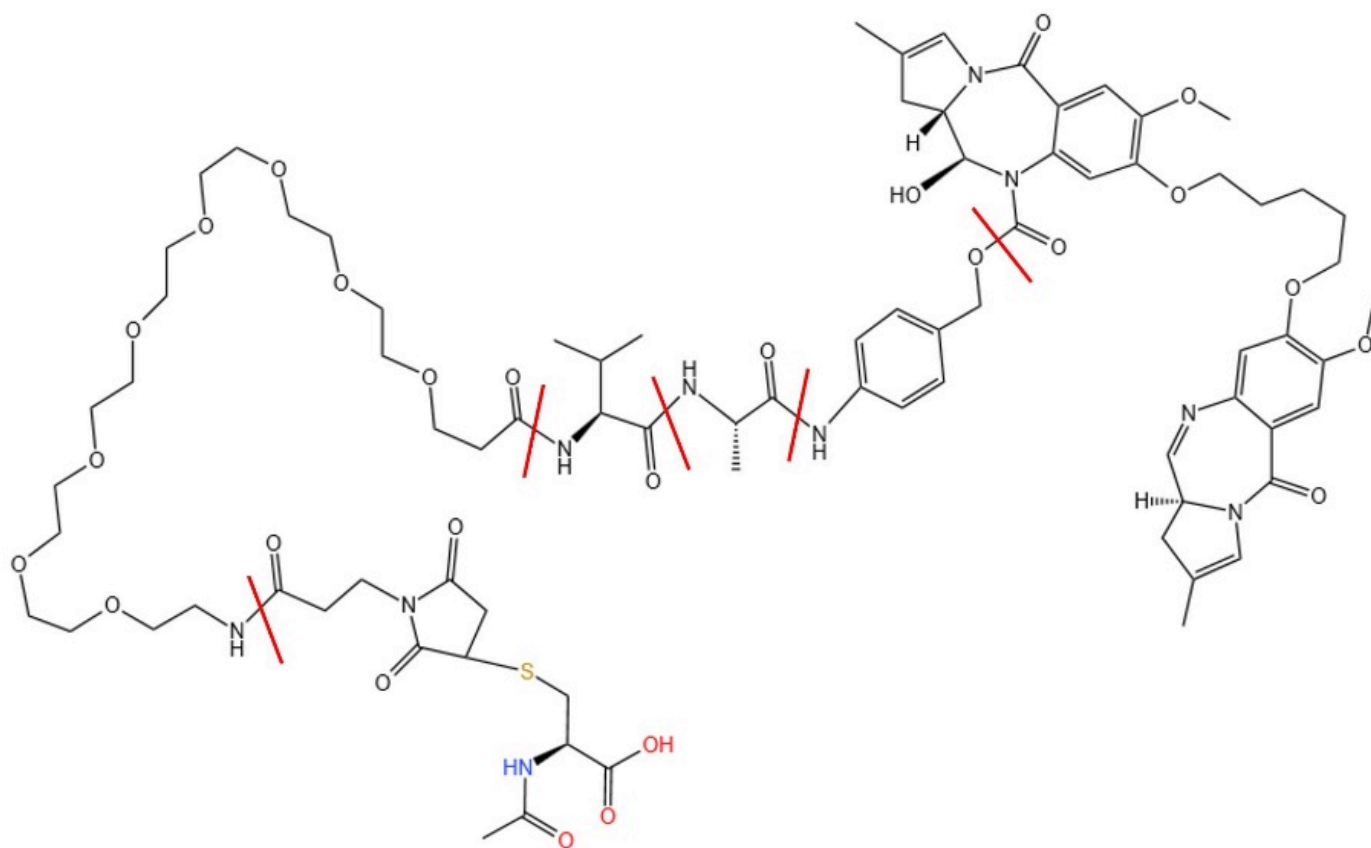
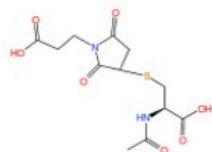


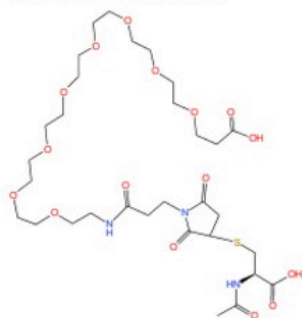
Figure 4

Structural formula of NAC-SG3249 and positions prone to cleavage or hydrolysis (marked in red).

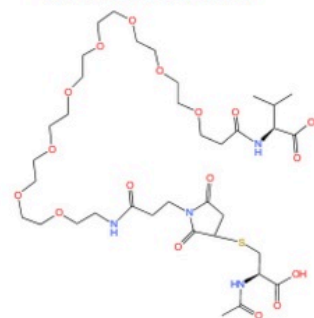
A: m/z 333.0



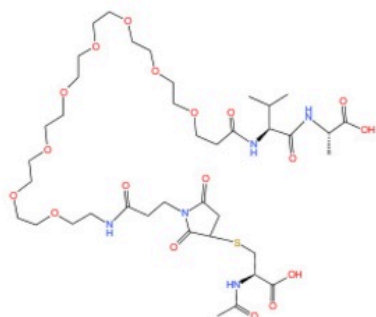
B: m/z 756.3



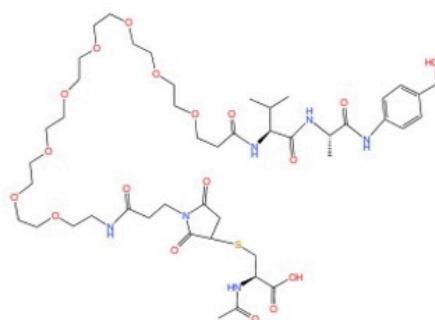
C: m/z 855.4



D: m/z 926.4



E: m/z 1031.5



F: m/z 1015.5

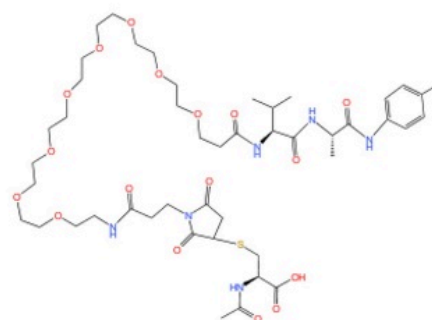


Figure 5

Predicted hydrolysis products of NAC-SG3249. The precursor ion m/z values for A–F are: 333.0, 756.3, 855.4, 926.4, 1031.5, and 1015.5, respectively.

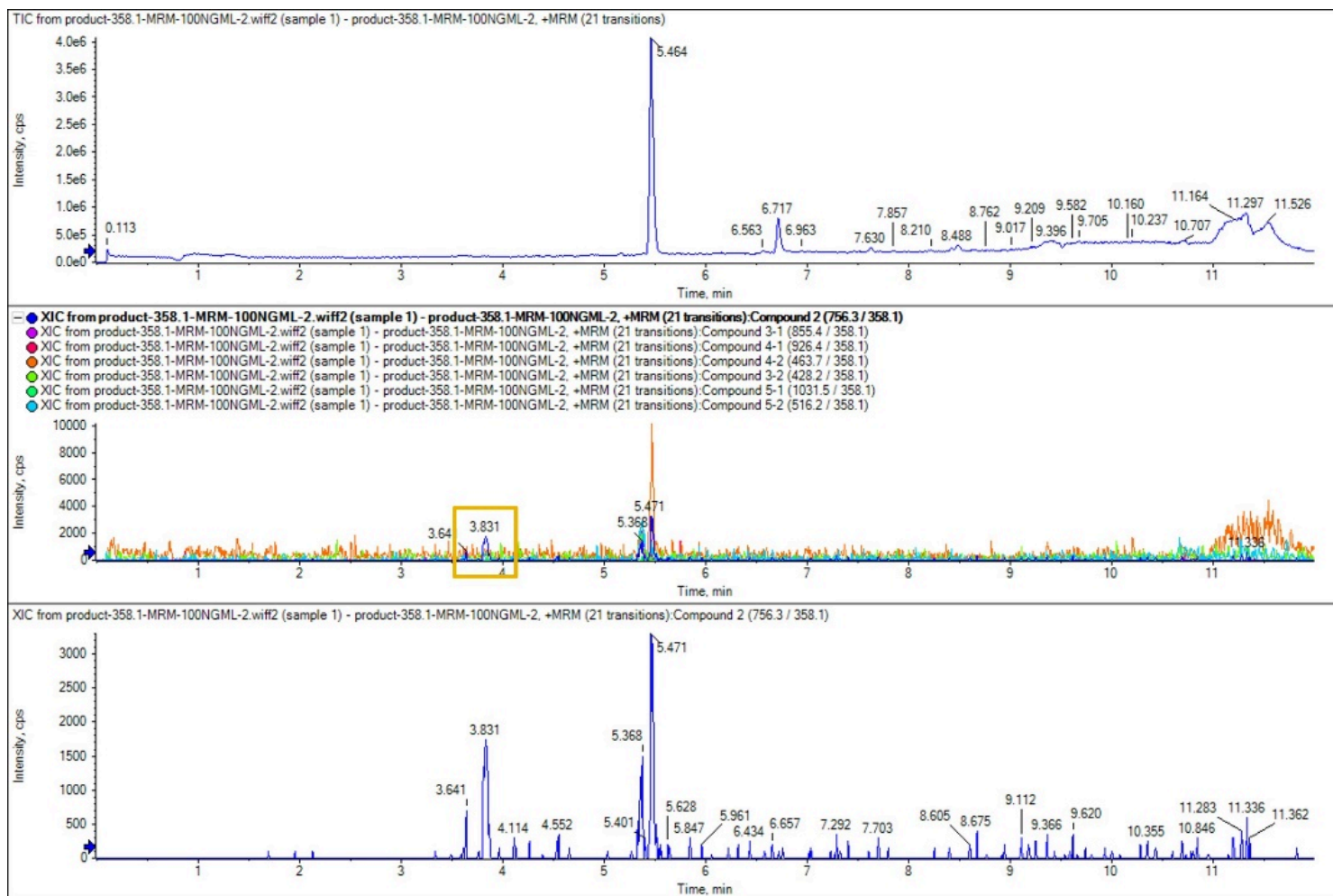


Figure 6

MS results of hydrolysis products analyzed in MRM mode using TQMS. A: TIC, B: XICs of different channels, C: XIC of m/z 756.3.

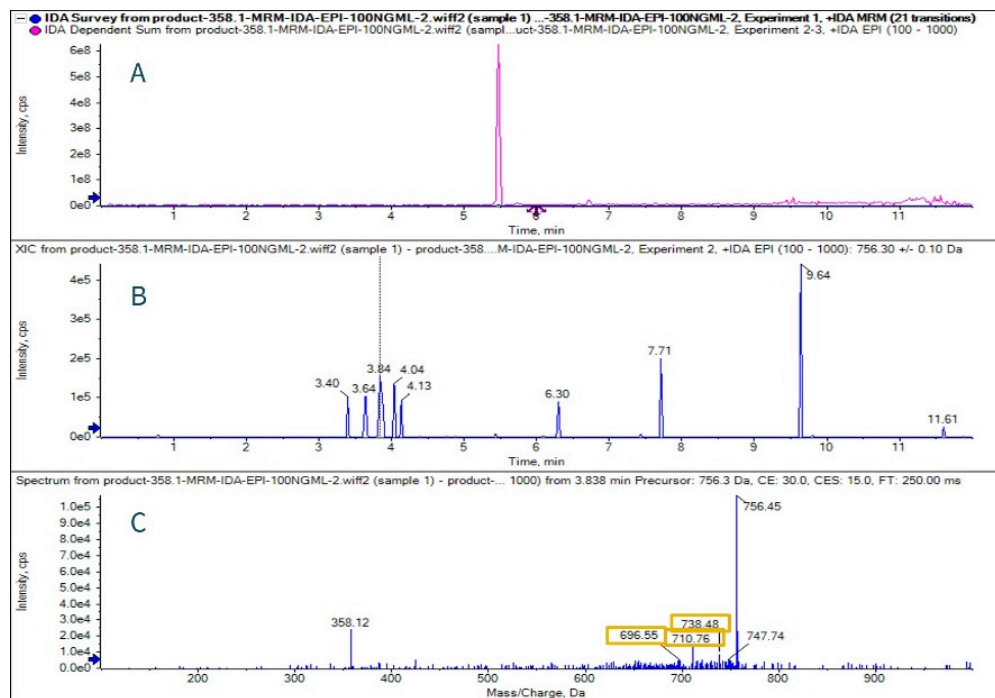


Figure 7

Secondary analysis of hydrolysis products using MRM and IDA-EPI modes. A: TIC, B: XIC of 756.3, C: Product ions of 756.3, D: Cleavage positions of the hydrolysis product (marked in red).

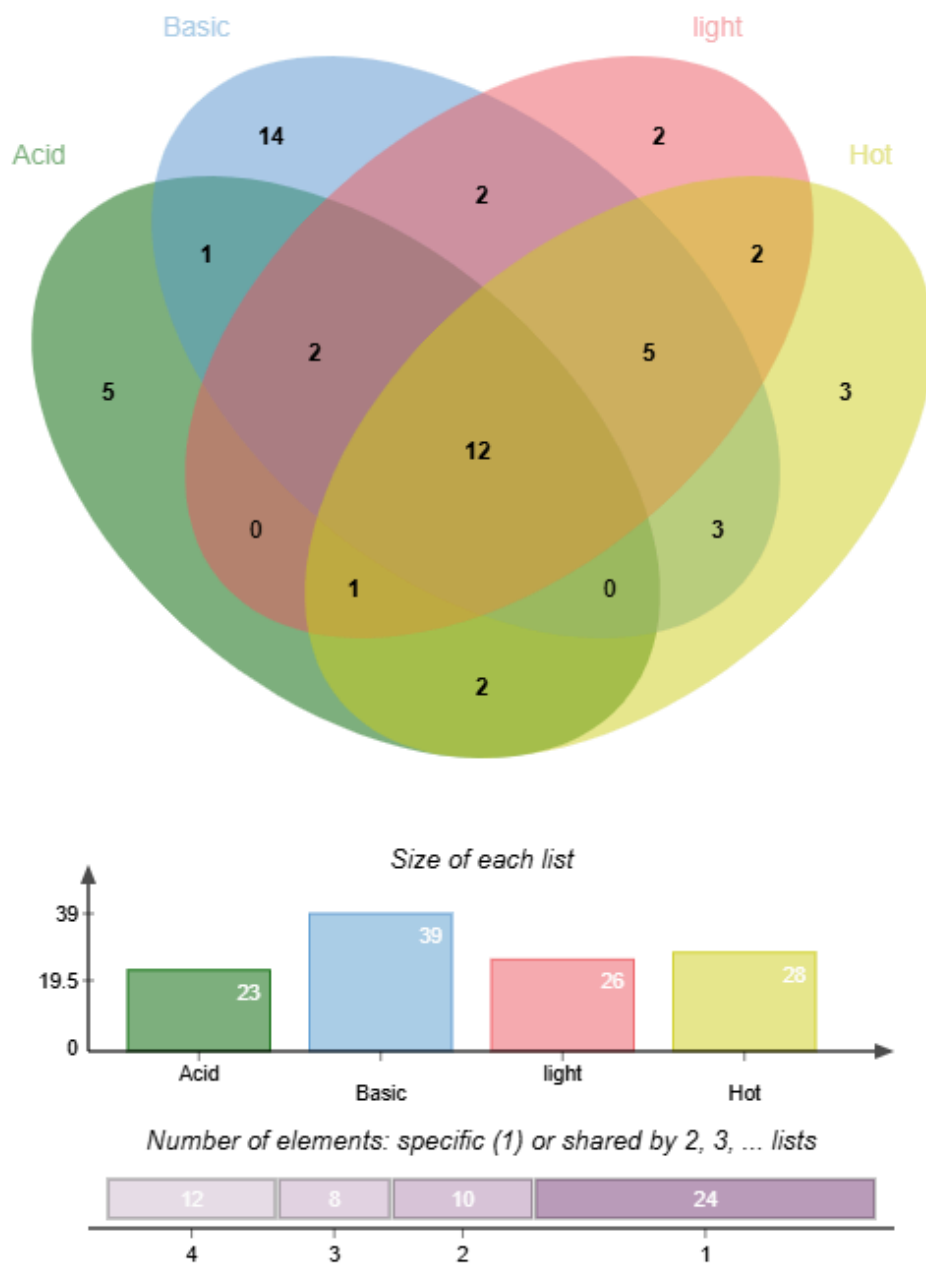


Figure 8

Distribution of the degradation product numbers under four forced degradation conditions