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Optimized HPLC method development of Tirzepatide using Kinetex PS C18: Assay, Impurity Profiling, and Stability Indicating for Degradation products.

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Introduction

Tirzepatide functions as a dual agonist for the glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) receptors. The increasing interest in these dual GIP/GLP-1 receptor agonists stems from their potential as novel treatments for regulating blood glucose levels and managing body weight, demonstrating enhanced glycemic control and weight reduction when compared to selective GLP-1 receptor agonists.

Tirzepatide is a synthetic linear peptide consisting of 39 amino acids, chemically modified through the incorporation of a C20 fatty diacid moiety. The separation of impurities associated with Tirzepatide presents significant challenges due to the intricate nature of peptide-related impurities. These impurities typically encompass truncated sequences, oxidized or reduced forms, as well as variants resulting from deamidation, phosphorylation, and glycosylation. To achieve effective separation, a systematic methodology and careful selection of the stationary phase are essential. Enhanced retention and selectivity are vital for distinguishing structurally similar peptide impurities.

In this technical application note, precise high-performance liquid chromatography (HPLC) techniques were established for the quantitative analysis of Tirzepatide and its nine related substances, utilizing the Kinetex PS C18 HPLC column. The Kinetex PS C18 column is characterized by its high efficiency and 100 % aqueous stability, providing multi-interaction selectivity due to its positive surface modification. This column exhibits improved peak shape for basic compounds and enhanced retention for acidic analytes. The core-shell particle structure reduces analyte diffusion, resulting in narrower peaks and delivering exceptional resolution and peak shape, particularly for peptide molecules.

Forced degradation studies were conducted to further assess the stability-indicating nature of the method. Tirzepatide was subjected to various stress conditions, including:

- Acidic Degradation (0.1 mL of 1 N Hydrochloric Acid at RT for 4 Days)
- Basic Degradation (0.1 mL of 0.1 N Sodium Hydroxide at RT for 1 hour)
- Oxidative Degradation (0.1 mL of 10 % Hydrogen Peroxide at RT for 4 Days)
- Thermal Degradation (80 °C water bath for 18 hours)

This approach successfully isolated Tirzepatide from its 9 identified and at least 18 unidentified degradation impurities, showcasing both specificity and its ability to indicate stability. The assessment of peak purity utilizing a PDA detector validated the homogeneity of the Tirzepatide peak across all degradation samples.

LC Conditions

Parameter	Assay	Related Substances	Stability Indicating
Column:	Kinetex 2.6 µm PS C18 **Part No. 00F-4780-E0**	Kinetex 2.6 µm PS C18 **Part No. 00F-4780-E0**	Kinetex 2.6 µm PS C18 **Part No. 00G-4780-E0**
Dimensions:	150 x 4.6mm	150 x 4.6 mm	250 x 4.6 mm
Mobile Phase:	**A**: 0.1 % Trifluoroacetic Acid in milli Q water **B**: 0.1 % Trifluoroacetic Acid in Acetonitrile		
Diluent:	Acetonitrile and water (50:50 v/v).		
Gradient:	**Time (min) / MP A (%) / MP B (%)** 0.0 / 65 / 35 3.0 / 65 / 35 9.0 / 56 / 44 30.0 / 56 / 44 35.0 / 30 / 70 40.0 / 30 / 70 41.0 / 65 / 35 45.0 / 65 / 35	**Time (min) / MP A (%) / MP B (%)** 0.0 / 67 / 33 3.0 / 67 / 33 9.0 / 57 / 43 48.0 / 56 / 44 55.0 / 54 / 46 60.0 / 40 / 60 68.0 / 30 / 70 69.0 / 67 / 33 72.0 / 67 / 33	**Time (min) / MP A (%) / MP B (%)** 0.0 / 67 / 33 5.0 / 67 / 33 15.0 / 57 / 43 80.0 / 56 / 44 90.0 / 54 / 46 100.0 / 40 / 60 104.0 / 30 / 70 105.0 / 67 / 33 112.0 / 67 / 33
Flow Rate:	1.2 mL/min	1.0 mL/min	1.0 mL/min
Temperature:	60 °C	60 °C	60 °C
Injection Volume:	10 µL	10 µL	10 µL
LC System:	Waters Arc HPLC		
Detector:	215 nm	215 nm	215 nm

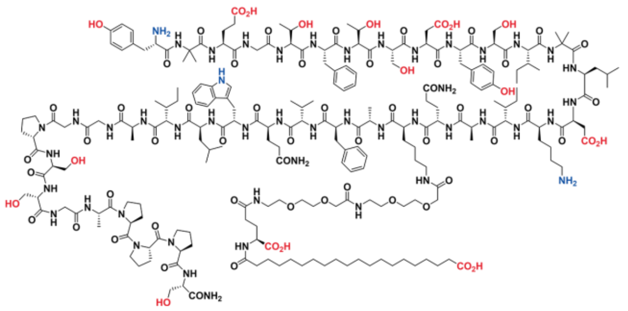
Standard and Impurity Standard Preparation

Standard solution:	1.0 mg/mL of Tirzepatide in Diluent for RS and 0.4mg/ml for Assay.
Impurity standard stock:	1.0 mg/mL in Diluent
Spiked sample:	0.3 %, 0.6 %, 1 % and 1.5 % of each impurity spiked in Tirzepatide standard with respect to 1.0 mg/mL test concentration.
Impurities:	The standard and impurities were purchased locally from ManoTri Pharma and Clearsynth Labs Limited.

Analytes

No.	Analyte	IUPAC (Sequence)
1	Des Side chain-Tirzepatide	H-Tyr-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Ile-Aib-Leu-Asp-Lys-Ile-Ala-Gln-Lys-Ala-Phe-Val-Gln-Trp-Leu-Ile-Ala-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH_2
2	Des Tyr (10)-Tirzepatide	H-Tyr-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Ser-Ile-Aib-Leu-Asp-Lys-Ile-Ala-Gln-Lys (AEEAc-AEEAc-γ-Glu-18-carboxy nonadecanoyl)-Ala-Phe-Val-Gln-Trp-Leu-Ile-Ala-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH2
3	Des-Tyr(1)-Aib(2)-Tirzepatide	H-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Ile-Aib-Leu-Asp-Lys-Ile-Ala-Gln-Lys(AEEAc-AEEAc-γ-Glu-18-carboxy nonadecanoyl)-Ala-Phe-Val-Gln-Trp-Leu-Ile-Ala-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH2
4	Endo-AEEAc-Tirzepatide	H-Tyr-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Ile-Aib-Leu-Asp-Lys-Ile-Ala-Gln-Lys(AEEAc-AEEAc-AEEAc-γ-Glu-18-carboxy nonadecanoyl)-Ala-Phe-Val-Gln-Trp-Leu-Ile-Ala-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH2
5	Endo-Gly(4)-Tirzepatide	H-Tyr-Aib-Glu-Gly-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Ile-Aib-Leu-Asp-Lys-Ile-Ala-Gln-Lys(AEEAc-AEEAc-γ-Glu-18-carboxy nonadecanoyl)-Ala-Phe-Val-Gln-Trp-Leu-Ile-Ala-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH2
6	Endo-Thr(5)-Tirzepatide	H-Tyr-Aib-Glu-Gly-Thr-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Ile-Aib-Leu-Asp-Lys-Ile-Ala-Gln-Lys (AEEAc-AEEAc-γ-Glu-18-carboxy nonadecanoyl)-Ala-Phe-Val-Gln-Trp-Leu-Ile-Ala-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH2
7	Endo-Tyr(1)-Tirzepatide	H-Tyr-Tyr-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Ile-Aib-Leu-Asp-Lys-Ile-Ala-Gln-Lys (AEEAc-AEEAc-γ-Glu-18-carboxy nonadecanoyl)-Ala-Phe-Val-Gln-Trp-Leu-Ile-Ala-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH2
8	Des-AEEAc-Tirzepatide	H-Tyr-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-D-Ser-Ile-Aib-Leu-Asp-Lys-Ile-Ala-Gln-Lys(AEEAc-γ-Glu-18-carboxy nonadecanoyl)-Ala-Phe-Val-Gln-Trp-Leu-Ile-Ala-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH2
9	Des-γ-Glu-Tirzepatide	H-Tyr-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Ile-Aib-Leu-Asp-Lys-Ile-Ala-Gln-Lys(AEEAc-AEEAc-18-carboxy nonadecanoyl)-Ala-Phe-Val-Gln-Trp-Leu-Ile-Ala-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH2

Structure of Tirzepatide

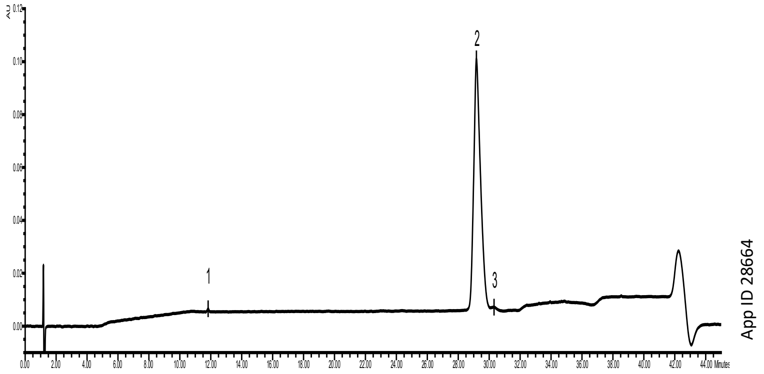


Results and Discussion

Assay

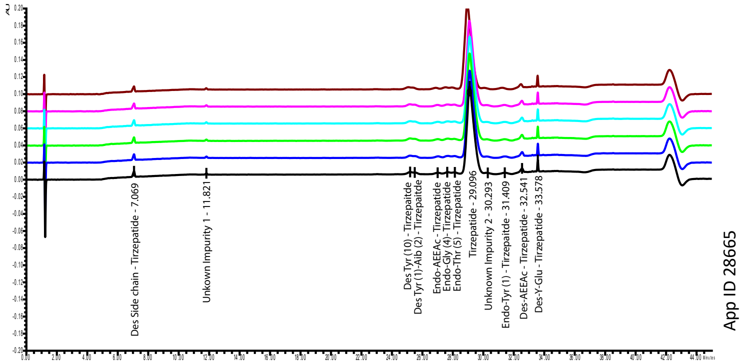
The Kinetex 2.6 µm PS C18 column (150 × 4.6 mm) was employed to develop a method for the assay of Tirzepatide. The objective of this assay is to accurately quantify Tirzepatide, necessitating the effective separation of Tirzepatide from its impurities. The chromatogram representing the Tirzepatide standard is presented in **Figure 1**. It was noted that the standard displayed two unidentified impurities at low concentrations, which were also successfully separated from Tirzepatide. In the assay method development, the Tirzepatide standard was spiked with nine known impurities at a concentration of 1 %. **Figure 3** illustrates the chromatographic separation of Tirzepatide from the nine known and two unknown impurities. All identified impurities were distinctly resolved from the Tirzepatide peak and confirmed using individual standards. All peaks showed peak tailing values within the USP requirements between 0.8-1.8. The system suitability was validated through six replicate injections of the sample solution, showcasing high reproducibility in retention times and peak areas, as depicted in **Figure 2**.

Figure 1. Standard Solution (0.4 mg/mL) for Assay on Kinetex 2.6 µm PS C18, 150 x 4.6 mm



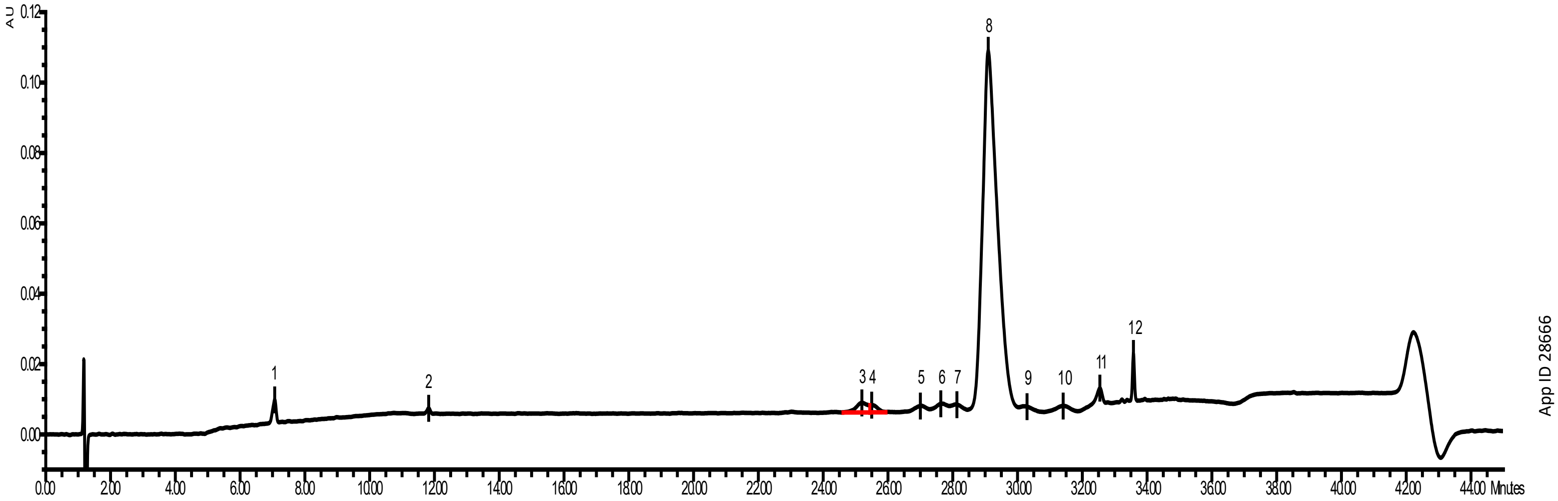
	Analyte	Retention Time	Area	% Area	USP Resolution	USP Tailing
1	Unknown impurity 1	11.830	9244	0.32	-	1.0
2	Tirzepatide	29.177	2872778	99.26	32.7	1.3
3	Unknown impurity 2	30.315	12074	0.42	1.7	1.3

Figure 2. Overlayed chromatogram of six replicate injections of impurities spiked in the standard solution (0.4 mg/mL) on Kinetex 2.6 µm PS C18, 150 x 4.6 mm



	Tirzepatide	
	Retention Time	Area
Mean	29.177	3160054
SD	0.025	43199
% RSD	0.1	1.4
N=6 Injections		

Figure 3. Chromatogram of Tirzepatide standard spiked with known impurities at 1 % level on Kinetex 2.6 µm PS C18, 150 x 4.6 mm

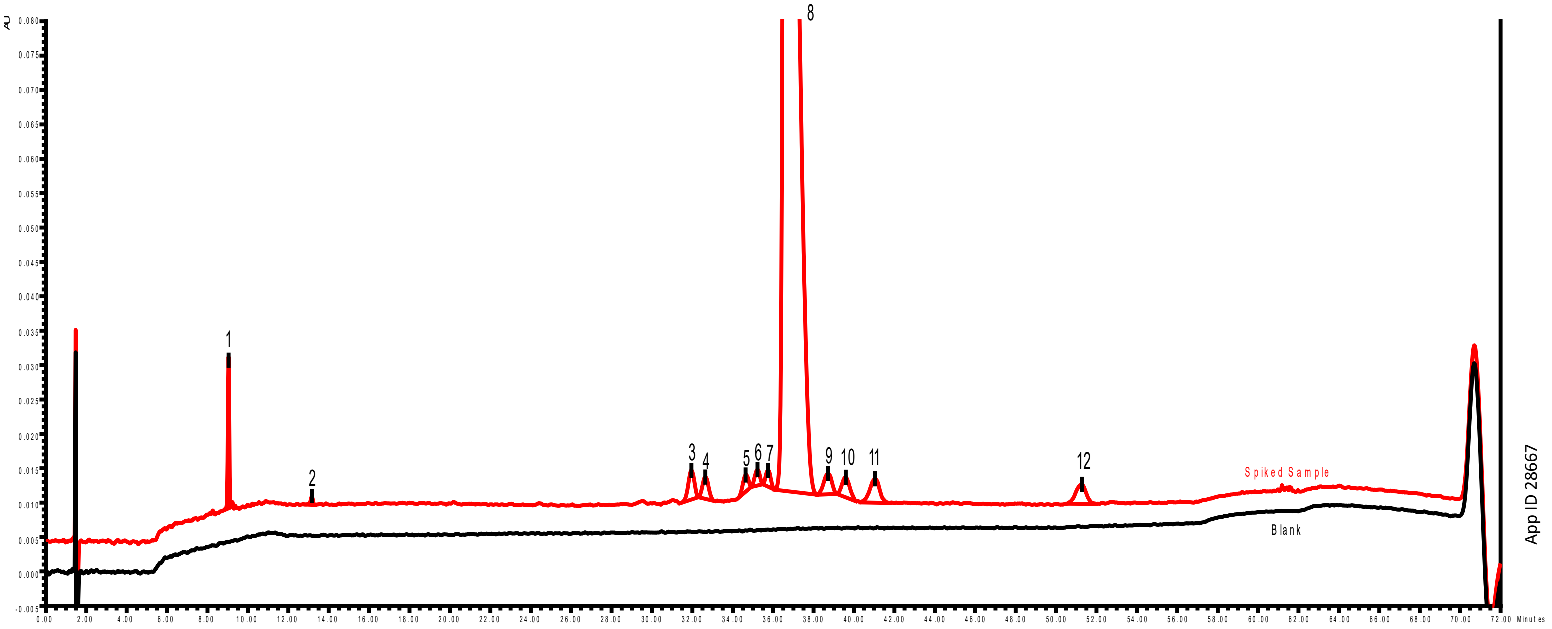


	Analyte	Retention Time	Area	% Area	USP Resolution	USP Tailing
1	Des Side chain-Tirzepatide	7.069	49157	1.36	-	0.8
2	Unknown impurity 1	11.821	11195	0.31	23.3	0.9
3	Des Tyr (10)-Tirzepatide	25.195	70166	1.94	22.7	-
4	Des-Tyr(1)-Aib(2)-Tirzepatide	25.497	33309	0.92	0.1	-
5	Endo-AEEAc-Tirzepatide	27.000	25024	0.69	0.8	0.9
6	Endo-Gly(4)-Tirzepatide	27.632	17983	0.50	1.2	0.9
7	Endo-Thr(5)-Tirzepatide	28.130	14136	0.39	1.1	1.2
8	Tirzepatide	29.096	3226373	89.18	1.4	1.4
9	Unknown impurity 2	30.293	13991	0.39	1.6	1.2
10	Endo-Tyr(1)-Tirzepatide	31.409	46693	1.29	1.7	0.9
11	Des-AEEAc-Tirzepatide	32.541	53235	1.47	2.1	0.8
12	Des-γ-Glu-Tirzepatide	33.578	56422	1.56	4.8	1.1

Related Substances

For related substances analysis, it was important to clearly separate each impurity from that of the API. The objective was also to guarantee that the resolution remained robust, even at elevated impurity concentrations. In this investigation, the gradient was meticulously adjusted to ensure dependable separation within a 72-minute total method duration. As illustrated in **Figure 4**, this approach effectively separated all impurities under examination from the Tirzepatide peak, achieving a resolution of no less than 1.2, which demonstrates remarkable selectivity. Individual impurity standards were assessed to verify the identity of each peak by matching retention times. The overlaid chromatograms for each individual standard are presented in **Figure 5**. Furthermore, six replicate injections of the spiked sample were conducted to assess the reproducibility of the method, as shown in **Figure 6**. The system exhibited consistent results, with a relative standard deviation (RSD) of less than 5 %, indicating satisfactory precision. To investigate the impact of concentration on selectivity, four spiking levels (0.3 %, 0.6 %, 1 %, and 1.5 %) of impurities were analyzed. The resulting overlaid chromatograms are depicted in **Figure 7**. It was noted that even at higher concentrations of the impurities, the resolutions achieved remained consistent.

Figure 4. Overlayed chromatogram of blank and Tirzepatide standard spiked with known impurities at 1.0 % level on Kinetex 2.6 µm PS C18, 150 x 4.6 mm



Sl. No.	Analyte	Retention Time	Area	% Area	USP Resolution	USP Tailing
1	Des Side chain-Tirzepatide	9.044	109328	1.19		0.9
2	Unknown Impurity 1	13.164	10053	0.11	24.1	0.8
3	Des Tyr (10)-Tirzepatide	31.952	88852	0.97	48.1	0.9
4	Des-Tyr(1)-Aib(2)-Tirzepatide	32.641	67290	0.74	1.2	1.1
5	Endo-AEEAc-Tirzepatide	34.633	46171	0.50	3.6	0.9
6	Endo-Gly(4)-Tirzepatide	35.224	39846	0.44	1.2	0.9
7	Endo-Thr(5)-Tirzepatide	35.752	40037	0.44	1.1	1.1
8	Tirzepatide	36.619	8366507	91.43	1.0	2.6
9	Unknown Impurity 2	38.706	83919	0.92	2.1	0.9
10	Endo-Tyr(1)-Tirzepatide	39.588	78975	0.86	1.2	1.1
11	Des-AEEAc-Tirzepatide	41.035	118080	1.29	1.8	0.9
12	Des-γ-Glu-Tirzepatide	51.274	101246	1.11	11.3	0.9

Figure 5. Overlayed chromatograms of individual impurities and Tirzeaptide standard on Kinetex 2.6 µm PS C18, 150 x 4.6 mm

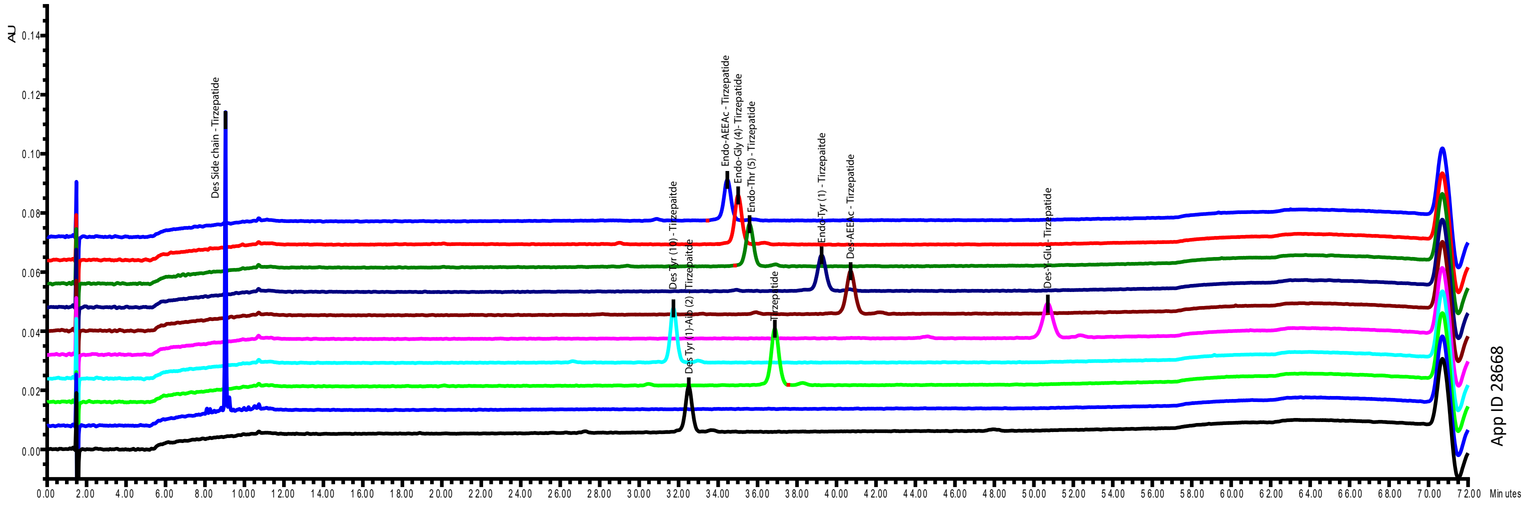


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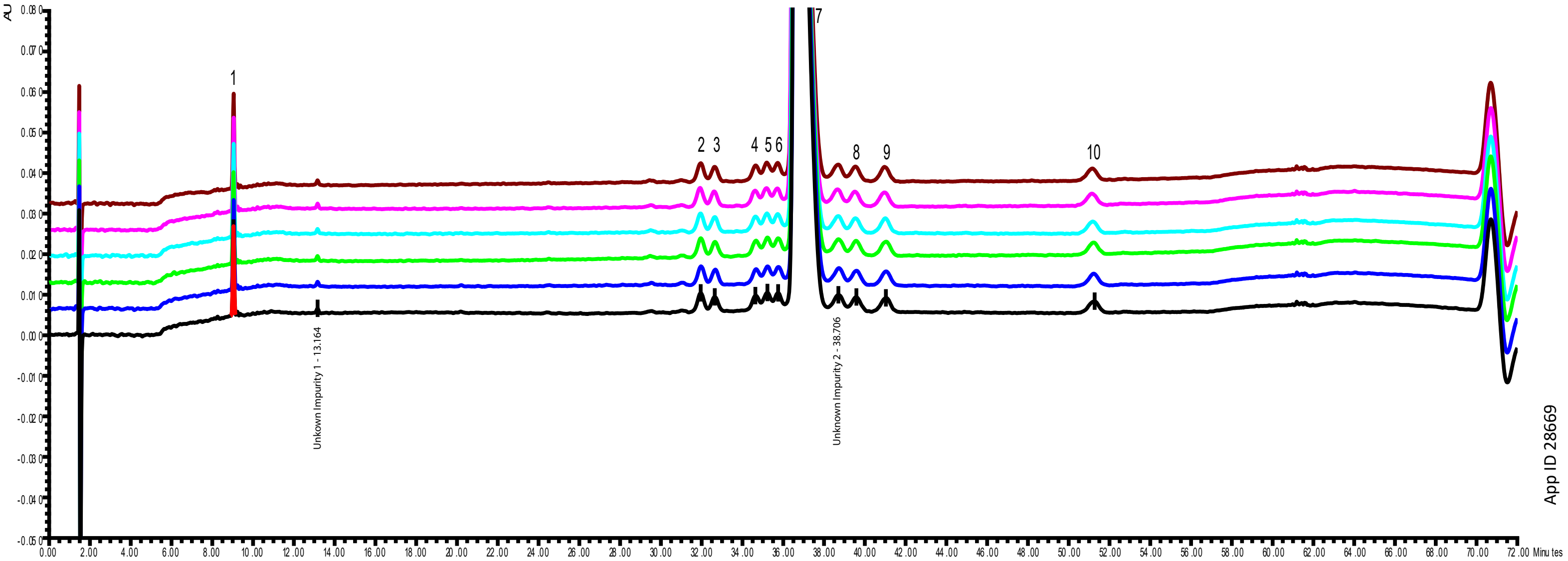
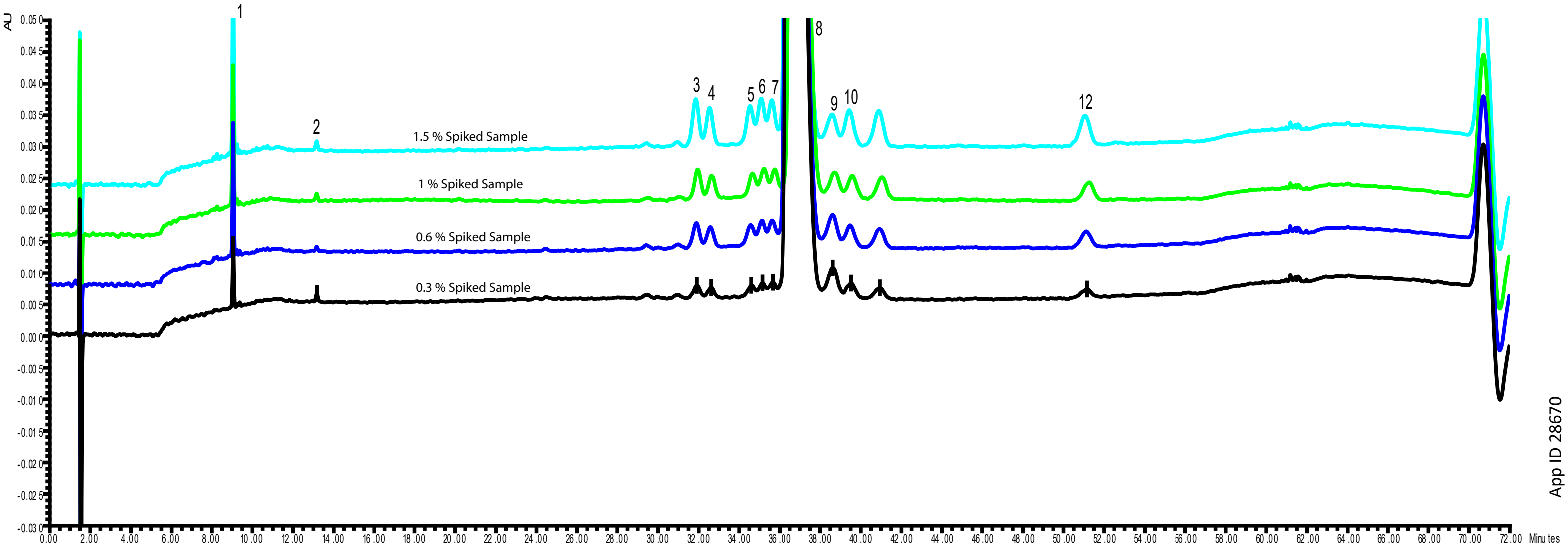
Figure 6. Overlayed chromatograms of replicate injections (N=6) of sample spiked with 1 % of known impurities on Kinetex 2.6 µm PS C18 , 150 x 4.6 mm



	1		2		3		4		5		6		7		8		9		10	
	Des side chain Tirzepatide		Des Tyr (10)-Tirzepatide		Des-Tyr(1)-Aib(2)-Tirzepatide		Endo-AEEAc-Tirzepatide		Endo-Gly(4)-Tirzepatide		Endo-Thr(5)-Tirzepatide		Tirzepatide		Endo-Tyr(1)-Tirzepatide		Des-AEEAc-Tirzepatide		Des-Y-Glu-Tirzepatide	
	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area
Mean	9.047	111470	31.951	89398	32.645	67014	34.640	46095	35.211	37837	35.740	39349	36.605	8385871	39.562	78329	41.012	114963	51.202	102605
SD	0.003	1689	0.015	641	0.020	1681	0.021	1190	0.021	1726	0.021	1308	0.026	48921	0.031	1597	0.033	3076	0.050	3940
%RSD	0.0	1.5	0.1	0.7	0.1	2.5	0.1	2.6	0.1	4.6	0.1	3.3	0.1	0.6	0.1	2.0	0.1	2.7	0.1	3.8
N=6 Injection																				

Figure 7. Overlayed chromatograms of Tirzepatide standard spiked with known impurities at 0.3 %, 0.6%, 1 % and 1.5 % levels on Kinetex 2.6 µm PS C18, 150 x 4.6 mm

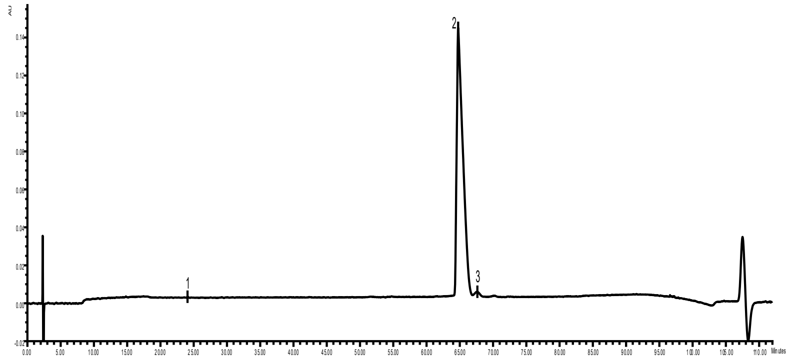


No.	Analyte	RT	USP Resolution			
			0.3% Spike	0.6% Spike	1% Spike	1.5% Spike
1	Des Side chain-Tirzepatide	9.05	25.3	27.5	24.1	25.7
2	Unknown Impurity 1	13.17	46.0	48.4	48.1	48.5
3	Des Tyr (10)-Tirzepatide	31.90	1.2	1.2	1.2	1.2
4	Des-Tyr(1)-Aib(2)-Tirzepatide	32.62	3.7	3.6	3.6	3.7
5	Endo-AEEAc-Tirzepatide	34.59	1.2	1.1	1.2	1.2
6	Endo-Gly(4)-Tirzepatide	35.14	1.3	1.1	1.1	1.1
7	Endo-Thr(5)-Tirzepatide	35.65	0.9	0.9	1.0	0.9
8	Tirzepatide	36.44	2.1	2.1	2.1	2.1
9	Unknown Impurity 2	38.61	1.2	1.2	1.2	1.1
10	Endo-Tyr(1)-Tirzepatide	39.52	1.7	1.8	1.8	1.8
11	Des-AEEAc-Tirzepatide	40.93	11.1	10.8	11.3	10.8
12	Des-γ-Glu-Tirzepatide	51.15	25.3	27.5	24.1	25.7

Forced Degradation

The HPLC analysis conducted for the forced degradation study utilized a column with dimensions of 250 x 4.6 mm. The chromatogram depicting the Tirzepatide standard is illustrated in **Figure 8**. An overlay showcasing the individual standards is provided in **Figure 9**. The system suitability was confirmed through six replicate injections of the sample solution, which exhibited high reproducibility in both retention times and peak areas, as demonstrated in **Figure 10**. This method provided exceptional chromatographic separation of Tirzepatide from nine known impurities (spiked at 1.0 % level) and two unknown impurities. The Tirzepatide standard (1 mg/mL) was subjected to a series of stress tests, which encompassed acidic degradation (0.1 mL of 1 N Hydrochloric Acid at room temperature for 4 days), basic degradation (0.1 mL of 0.1 N Sodium Hydroxide at room temperature for 1 hour), oxidative degradation (0.1 mL of 10 % Hydrogen Peroxide at room temperature for 4 days), and thermal degradation (80 °C water bath for 18 hours). The results of the chromatographic analyses for each sample post-treatment are illustrated as an overlay chromatogram in **Figure 11**. As anticipated, the control sample (Tirzepatide standard without treatment) revealed only two unknown impurities. During acid degradation, a total of 17 degradation peaks were noted; during base degradation, three degradation peaks were noted. Oxidative and thermal degradation samples showed 18 degradation peaks each and were found to be distinct from each other with respect to their retention times.

Figure 8. Tirzepatide Standard solution 1 mg/ml for related substance as a sample on Kinetex 2.6 µm PS C18, 250 x 4.6 mm



	Analyte	Retention Time	Area	% Area	USP Resolution	USP Tailing
1	Unknown Impurity 1	24.071	6393	0.07		1.2
2	Tirzepatide	64.756	8987816	98.78	35.0	2.3
3	Unknown Impurity 2	67.645	104226	1.15	1.8	1.1

Figure 9. Overlayed chromatogram of individual known impurities on Kinetex 2.6 µm PS C18, 250 x 4.6 mm

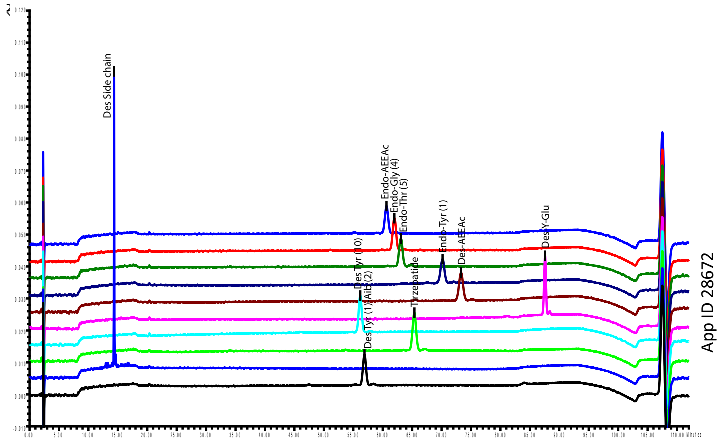
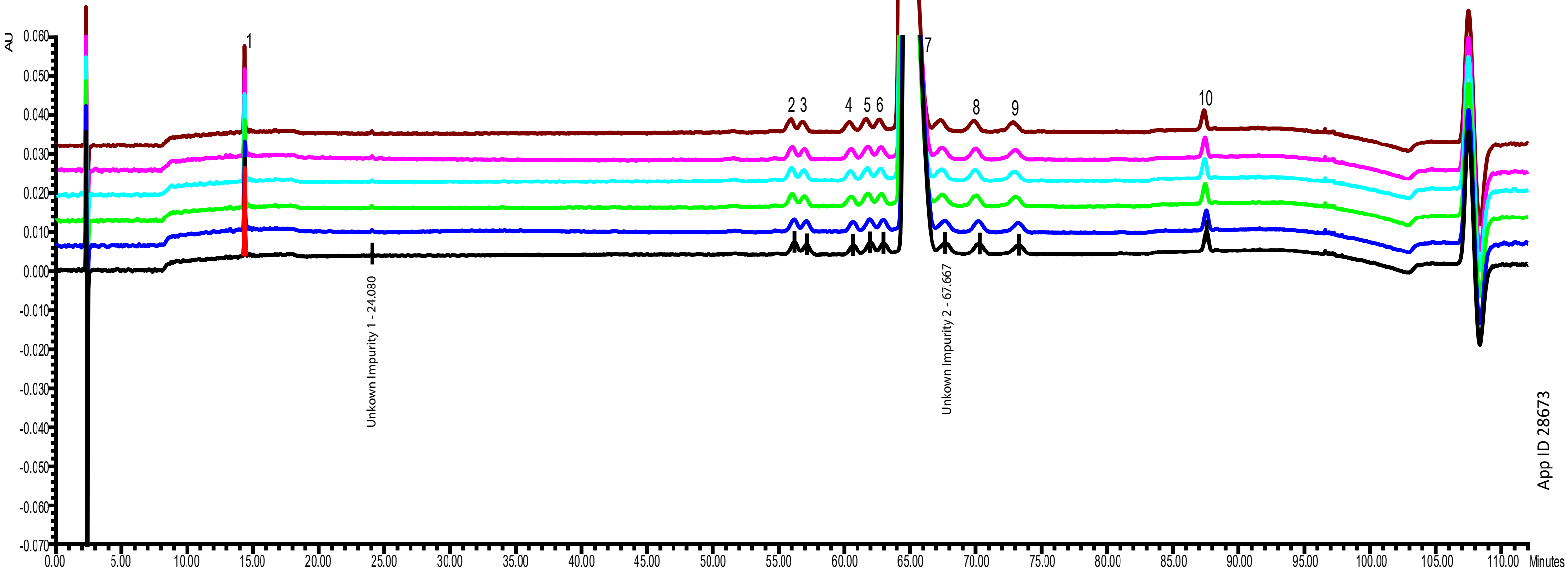


Figure 10. Overlayed chromatogram of six replicate injections of the standard spiked with known impurities at 1.0 % level on Kinetex 2.6 µm PS C18, 250 x 4.6 mm



	1		2		3		4		5		6		7		8		9		10	
	Des side chain Tirzepatide		Des Tyr (10)-Tirzepatide		Des-Tyr(1)-Aib(2)-Tirzepatide		Endo-AEEAc-Tirzepatide		Endo-Gly(4)-Tirzepatide		Endo-Thr(5)-Tirzepatide		Tirzepatide		Endo-Tyr(1)-Tirzepatide		Des-AEEAc-Tirzepatide		Des-γ-Glu-Tirzepatide	
	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area
Mean	14.365	110446	56.069	81430	57.014	63584	60.529	82284	61.816	81923	62.849	71097	64.615	9480152	70.085	136328	73.086	115170	87.481	117646
SD	0.006	1160	0.110	1986	0.106	1461	0.106	2138	0.115	3522	0.122	2563	0.134	58592	0.167	6630	0.157	4178	0.076	1040
%RSD	0.0	1.1	0.2	2.4	0.2	2.3	0.2	2.6	0.2	4.3	0.2	3.6	0.2	0.6	0.2	4.9	0.2	3.6	0.1	0.9
N=6 Injections																				

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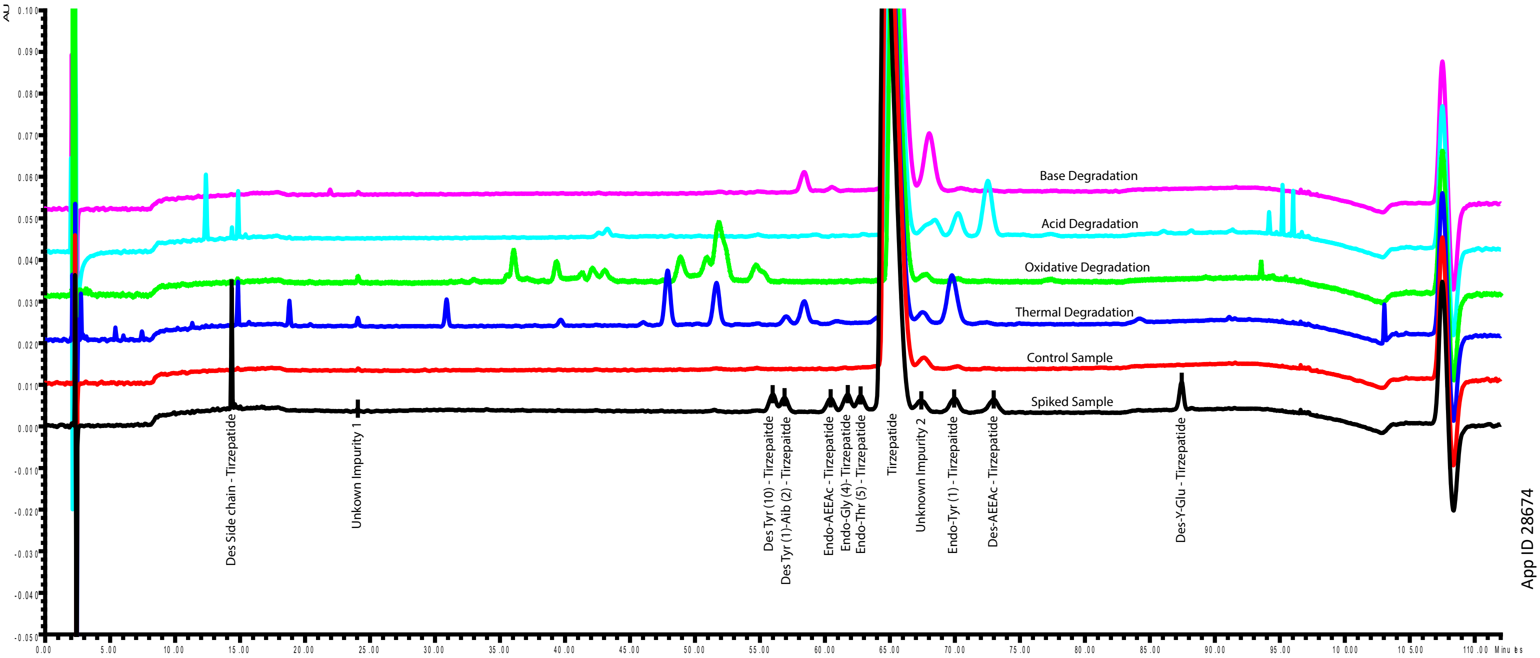
Figure 11. Overlayed chromatogram of samples exposed to varied types of forced degradation approaches on Kinetex 2.6 µm PS C18, 250 x 4.6 mm



Sample Name	Condition	% Degradation	Total number of Degradation impurity peaks
Control Sample	-	1.22	2
Acid Degradation	0.1 mL of 1 N Hydrochloric Acid for 4 Days	17.94	17
Base Degradation	0.1 mL of 0.1 N Sodium Hydroxide for 1 Hour	9.35	3
Oxidative Degradation	0.1 mL of 10 % Hydrogen Peroxide for 4 Days	23.53	18
Thermal Degradation	80°C water bath for 18 Hours	20.71	18

Conclusion

A high-performance liquid chromatographic (HPLC) reverse phase technique has been established utilizing a Kinetex PS C18 LC column for the quantification of Tirzepatide (Assay), its impurities (Related Substances), and degradation impurities. For the Assay method, a relatively rapid gradient was formulated using a Kinetex 2.6 µm PS C18 column (150 × 4.6 mm). All detected impurities were distinctly separated from the Tirzepatide peak, facilitating reliable and reproducible quantification via HPLC. Moreover, enhanced separation for each impurity was accomplished through method optimization to quantify all related substances of Tirzepatide. Both known and unknown impurities were effectively resolved and separated from Tirzepatide. These methods demonstrate reproducible results, indicating satisfactory precision through HPLC.

A stability-indicating method was further developed using a Kinetex 2.6 µm PS C18 column (250 × 4.6 mm). The objective of this method is to accurately quantify impurities and degradation products associated with Tirzepatide under various stress conditions, ensuring effective separation of Tirzepatide from its impurities. Forced degradation studies were conducted to evaluate the stability and degradation of the drug under different stress conditions, including acid, base, oxidative (peroxide), and thermal degradations. The results indicate that the developed method is highly effective in resolving both process-related impurities and those generated through forced degradation. Furthermore, the method exhibits excellent reproducibility, establishing it as a reliable tool for stability-indicating studies of Tirzepatide. This technical note represents the first report of a Tirzepatide impurity method, which we believe will serve as a foundation for the development of analytical methods for Tirzepatide.

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