Application Note

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Enhanced Peptide Characterization and Stability Assessment

UV-visible second-derivative spectroscopy on an Agilent Cary 3500 Multicell UV-Vis Spectrophotometer

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Abstract

Monitoring the stability of peptide therapeutics is vital in biopharmaceutical development. Traditional ultraviolet-visible (UV-Vis) absorption spectroscopy techniques commonly used in stability studies often do not provide sufficient wavelength resolution, especially for biological molecules such as proteins and DNA which typically have broad peaks. This makes it challenging to detect subtle structural changes, particularly when spectral features overlap. This application note demonstrates the benefits of using UV-Vis second-derivative spectroscopy with the Agilent Cary Multicell 3500 UV-Vis Spectrophotometer to improve peptide characterization and stability assessment. The advanced features of the Cary 3500 Multicell UV-Vis Spectrophotometer, including multicell measurement and built-in derivative calculation, streamline data collection and analysis, making it a valuable tool for peptide stability studies in biopharmaceutical applications.

Introduction

Synthetic peptides are a growing class of biotherapeutics. Glucagon-like peptide-1 (GLP-1) agonists are synthetic peptides that mimic natural GLP-1 hormone and play an important role in metabolic regulation and help in both insulin secretion and the promotion of weight loss.[1] Because the GLP-1 molecule is prone to various degradation pathways, assessing the quality and stability of GLP-1-based therapeutics is crucial to ensuring their effectiveness.

Peptide stability is commonly measured by observing changes in the absorbance spectra produced by ultraviolet spectroscopy.[2] Because it is non-destructive, the technique offers significant workflow advantages. For example, valuable samples remain intact after analysis and can be subsequently analyzed by complementary techniques such as liquid chromatography-mass spectrometry. Additionally, UV-Vis spectroscopy is fast and easy, making it accessible to a wide range of users.

However, conventional UV-Vis absorption spectra can be difficult to interpret when monitoring for subtle changes such as small shifts in peak positions or minor intensity variations, especially for biomolecules exposed to environmental factors like oxidation stress. Poor resolution of spectral features is also a challenge when biomolecule spectra consist of multiple overlapping components. Derivative spectroscopy is a powerful technique that addresses these limitations by enhancing subtle features, allowing resolution of overlapping peaks that would be indistinguishable in a traditional absorption spectrum.

The Agilent 3500 Multicell UV-Vis spectrophotometer with its multicell module is a powerful tool for biomolecule analysis using second-derivative measurements. The instrument allows simultaneous measurement of multiple samples — including standards, samples, and controls — under the same conditions. The Agilent Cary UV Workstation software that controls the Cary 3500 Multicell UV-Vis spectrophotometer includes a built-in equation function that automatically produces second-derivative spectra after absorbance measurement to minimize calculation error and streamline data processing.

This application note demonstrates use of the advanced capabilities of the Cary 3500 Multicell UV-Vis Spectrophotometer and UV-Vis second-derivative spectroscopy to improve the resolution of overlapping absorption peaks collected from peptides and aromatic amino acids.

Experimental

Materials

Tryptophan (W), tyrosine (Y), phenylalanine (F), α-melanocyte-stimulating hormone (MSH), melittin, liraglutide, angiotensin II, 30% (v/v) hydrogen peroxide (H_2O_2), and sodium phosphate were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

All peptide samples including tryptophan (0.15 mM), tyrosine (0.5 mM), and phenylalanine (4.0 mM) were prepared in sodium phosphate buffer, pH 7.4, at a concentration of 1 mg/mL. For the stress conditions, the peptides were incubated with 0.07% H_2O_2 overnight at room temperature. No other sample preparation before UV-Vis analysis was required.

Instrumentation

Data acquisition was performed by the Cary 3500 Multicell UV-Vis Spectrophotometer equipped with the Cary Workstation software using the parameters listed in Table 1. The multicell design allows for simultaneous measurement of up to seven samples along with a reference sample. Measurements were carried out using an ultra-microvolume rectangular cuvette with a path length of 10 mm (Agilent part number 5062-2496). Spectra were averaged from multiple runs (n = 5) to ensure reproducibility and reduce noise.

Table 1. Agilent Cary 3500 Multicell UV-Vis Spectrophotometer method parameters.

Parameter	Value
Collection Mode	Scan
Scan Range	200 to 500 nm
Averaging Time	0.02 s
Data Interval	1.00 nm
Scan Rate	3,000 nm/min
Spectral Bandwidth	2.00 nm
Derivative	Second derivative
Smoothing Filter	9

Results and discussion

UV-visible spectra of aromatic amino acids

Tryptophan, phenylalanine, and tyrosine are aromatic amino acids that are commonly found in peptides and proteins and have characteristic UV-Visible absorption profiles. Figure 1A shows the UV-Vis zero-order spectra of tryptophan, phenylalanine, and tyrosine. The overlapping peaks in the zero-order spectra make it difficult to distinguish subtle spectral features in the amino acids.

To enhance the resolution and accuracy of the absorption spectra, second-order derivative spectra were analyzed based on the Savitzky-Golay method. Figures 1B, 1C, and 1D show the second-order derivative UV-Vis spectra of tyrosine, phenylalanine, and tryptophan. After the sample measurements were acquired, the second-order spectra were automatically generated using the built-in calculation function in the Cary UV Workstation software. The second-order derivative spectra display peaks with maxima and minima (points of inflection) which can be used to distinguish the different spectral absorbance bands of the various aromatic amino acids, enabling study of the minor spectral changes that occur when biomolecules experience stress conditions. The absorption maxima of the amino acids were:

- **Tryptophan:** Absorption maximum at approximately 273, 280, and 288 nm
- **Tyrosine:** Absorption maximum at approximately 275 and 282 nm
- **Phenylalanine:** Absorption maximum at approximately 246, 252, 257, 264, and 268 nm

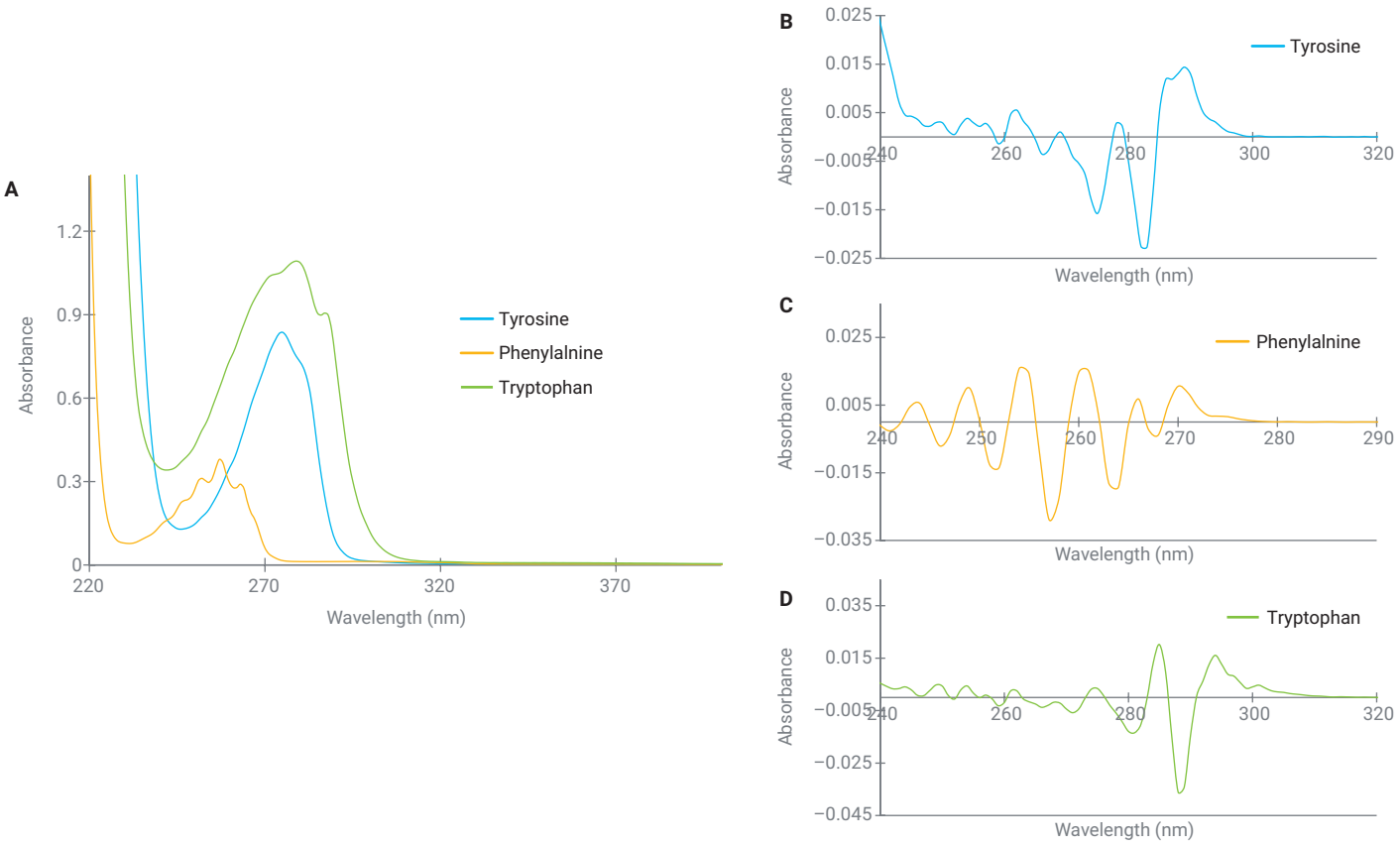

Figure 1. UV-Vis absorption spectra of aromatic amino acids. (A) zero-order and (B, C, and D) second-order derivative UV-Vis absorption spectra.

UV-visible spectra of peptides

To generate different absorption spectra for comparison, the peptides selected for study had varying proportions of aromatic amino acids. Figure 2 shows the second-order derivative absorption spectra of the four peptides melittin, MSH, angiotensin II, and liraglutide. The peak locations in the second-order derivative spectra correlated well with the known absorption features of their constituent aromatic amino acids. Overall, the second-order derivative spectra provided a more resolved view of the peptides' spectral characteristics. The number of aromatic amino acids present in the peptides studied is summarized in Table 2. Melittin showed characteristic peaks at 291, 283, and 275 nm, which were primarily attributed to tryptophan. MSH and liraglutide had peaks at 290, 283, and 266 nm, reflecting contributions from all three aromatic amino acids. Angiotensin II had peaks at 283, 276, 264, 258, and 252 nm, which was consistent with the presence of tyrosine and phenylalanine.

Table 2. Amino acid composition of peptides analyzed.

Peptides	Sequence	Number of Tryptophan (W)	Number of Tyrosine (Y)	Number of Phenylalanine (F)
Liraglutide	HAEGTFTSDVSSYLEGQAAKEEFIAWLVRGRG	1	1	1
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	1	0	0
α-Melanocyte-stimulating hormone (MSH)	SYSMEHFRWGKPV	1	1	1
Angiotensin II	DRVYIHPF	0	1	1
Substance P	RPKPQQFFGLM	0	0	2

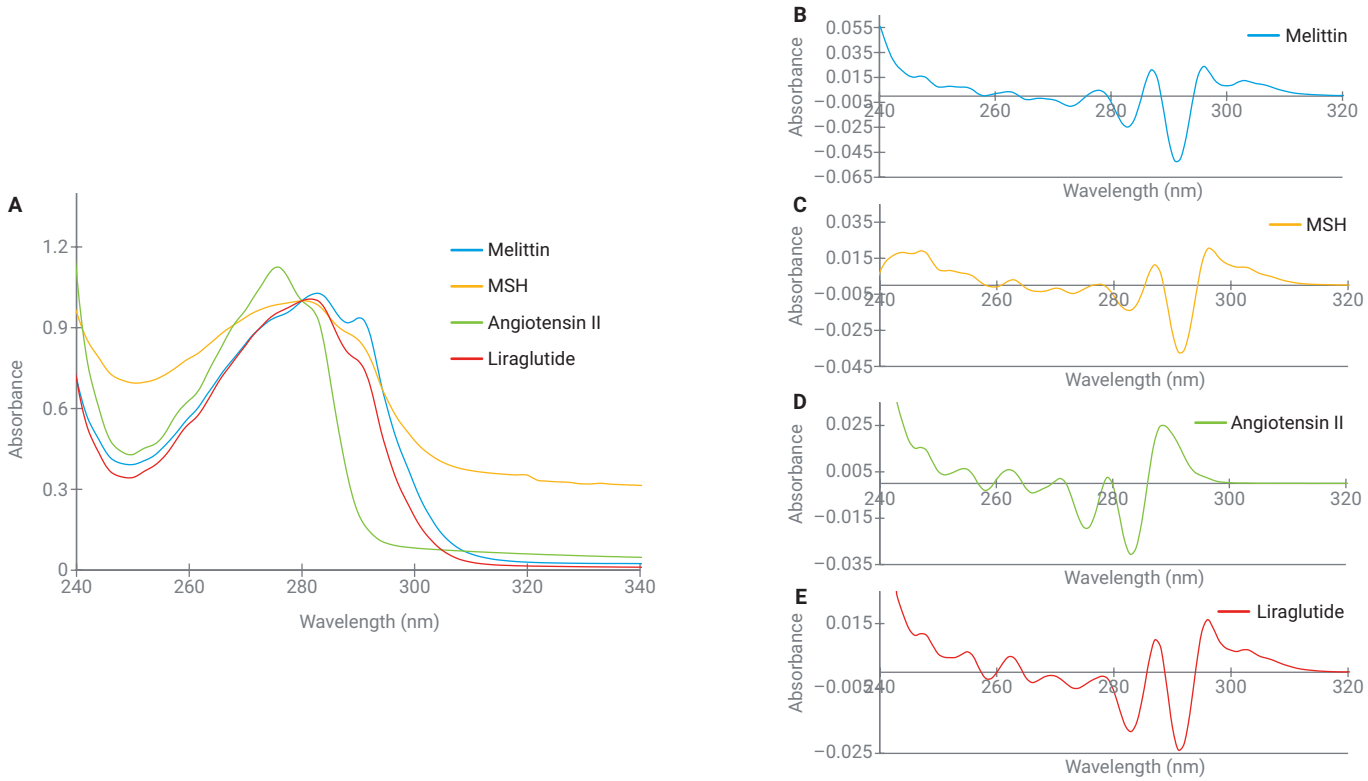

Figure 2. UV-Vis absorption spectra of peptides. (A) zero-order and (B, C, D, and E) second-order derivative UV-Vis absorption spectra.

Monitoring peptide stability under oxidation stress

To assess the utility of derivative spectroscopy for stability monitoring, the peptides were treated with H_2O_2. Figure 3 shows the comparison of second-order derivative UV-Vis spectra of untreated and H_2O_2 treated peptides. Minute changes in peptide wavelength absorbance due to H_2O_2 treatment were effectively captured in the second-order derivative spectra. Angiotensin II peptide lacks the tryptophan residue, so no significant change was observed around the 291 nm region after H_2O_2 treatment. Minimal changes were observed in melittin's second-order derivative spectrum, indicating it is less prone to oxidation. Tryptophan-containing MSH and liraglutide showed a noticeable loss in the magnitude of the 291 nm peak after H_2O_2 treatment. This indicates the compounds' susceptibility to oxidation, which primarily affects tryptophan residues, further demonstrating the specificity of the second-derivative method for detecting tryptophan oxidation. To further confirm the oxidation status of liraglutide, H_2O_2 treated samples were analyzed by liquid chromatography quadrupole time-of-flight mass spectrometry (LC/Q-TOF-MS). Figure 4 shows the total ion chromatogram (TIC) of H_2O_2 treated liraglutide. The deconvolved mass spectrum confirmed the oxidation forms showing mass shifts of +16 Da and +32 Da that correspond to mono and di oxidation products, respectively.[3]

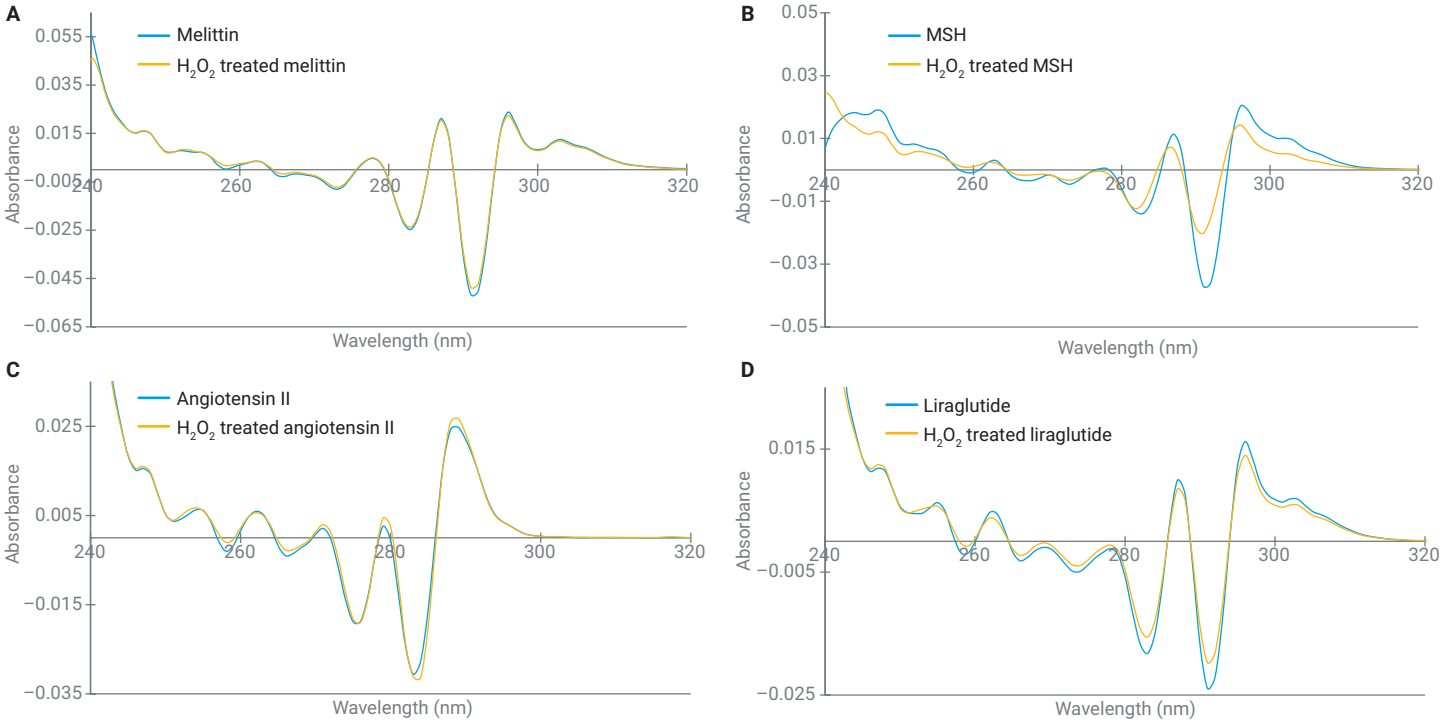

Figure 3. Second-order derivative UV-Vis absorption spectra of untreated and H_2O_2 treated peptides.

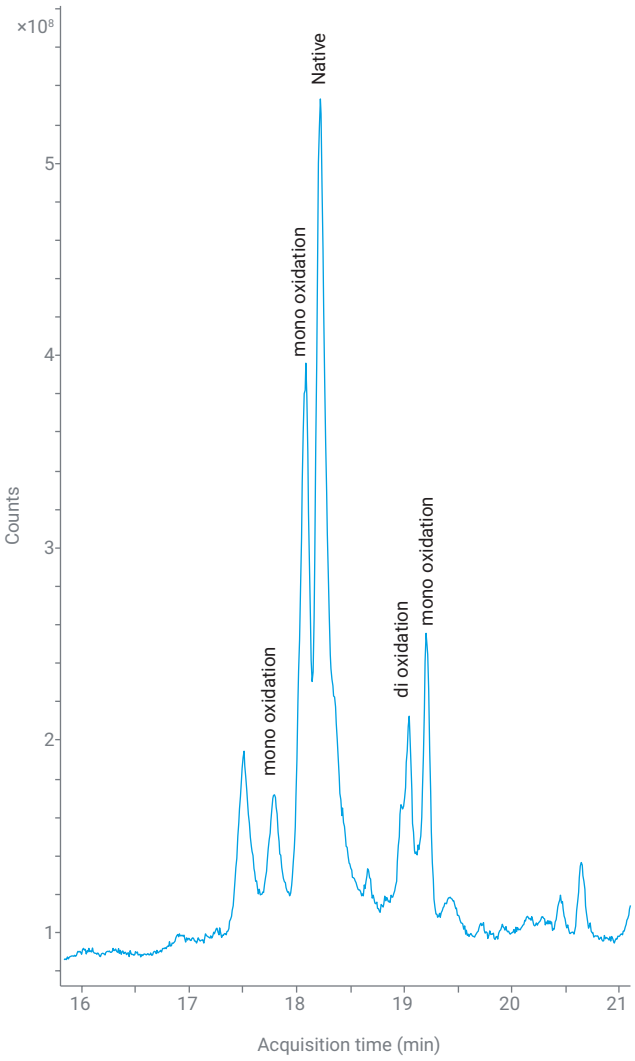

Figure 4. LC/MS total ion chromatogram of oxidated liraglutide.

Conclusion

The results of this study highlight the ability of UV-Vis second-derivative spectroscopy to precisely identify and monitor subtle spectral differences arising from changes in the aromatic residues in peptides, making it an invaluable tool for monitoring peptide stability. The Agilent Cary Multicell 3500 UV-Vis Spectrophotometer's multicell feature reduces measurement time by allowing simultaneous monitoring of eight samples. The built-in calculation feature streamlines analysis with automated calculations, making it easier for users to interpret data and make decisions. This capability is particularly valuable for biopharmaceutical analysis, where accurate and efficient monitoring of peptide stability is crucial for drug development and quality control. Non-destructive, UV-Vis spectroscopy preserves samples for subsequent analyses such as complementary LC-MS analysis. The Cary 3500 Multicell UV-Vis Spectrophotometer is an ideal choice for drug development and quality control (QC) testing laboratories, where speed, ease of use, and the ability to reuse valuable samples are paramount.

References

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2. Liyanage, M. R.; Bakshi, K.; Volkin, D. B.; Middaugh, C. R. Ultraviolet Absorption Spectroscopy of Peptides. *Methods Mol. Biol.* **2014**, *1088*, 225–236.
3. Suresh Babu, C.V. Characterization of Forced Degradation Impurities of Glucagon-Like Peptide-1 Agonists by LC/Q-TOF Mass Spectrometry. *Agilent Technologies application note* 5994-7794EN. **2025**.

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