

## Introduction

Protein quantification plays a critical role in biopharmaceuticals and in vitro diagnostics. Traditional primary or authoritative methods for protein quantitation, such as the mass balance method or quantitative nuclear magnetic resonance method, are only applicable to solid samples or small peptides. For protein quantitation in solution, isotope dilution mass spectrometry (IDMS) remains the sole primary method, which lacks orthogonal method validation and may introduce bias.

To address these limitations, our research group has developed three novel potential primary methods for protein quantification based on spectral techniques: high-performance liquid chromatography - circular dichroism or optical rotation dispersion (HPLC-CD or HPLC-ORD), and gas chromatography - isotope dilution infrared spectroscopy (GC-IDIR). These new methods use chiral isomers or isotopic labelled amino acids as standards or internal standards for quantification, respectively. In a non-chiral environment, these compounds share the same physicochemical properties as the analytes, and remain together throughout sample preparation and separation analysis, which helps to minimize both systematic and random errors, ensuring accurate measurement results.

## Instruments and Methods

An Agilent 1260 LC was connected to a Jasco J815 CD spectrometer with an ORD accessory for HPLC-CD or HPLC-ORD analysis. Human insulin was hydrolyzed with 500  $\mu$ L of 8 mol/L HCl at 110°C for 72 h. After hydrolysis, the sample was mixed with different amounts of solutions containing known amounts of D-Val, D-Leu, D-Ile, and D-Phe for sample analysis, while using 0.01 mol/L HCl as blank control. The sample was separated by a Acclaim™ RSLC PA2 column and the mobile phase was water: acetonitrile: TFA: heptafluorobutyric acid = 91:9:0.1:0.2 with isocratic elution. If the CD/ORD values of the mixed sample fell within 3 times of the blank control value (near zero), the concentrations of L-amino acids in the hydrolysate were deemed to be equal to those of D-amino acids in standard solution and used for calculating protein concentration, otherwise the sample was mixed with difference amount of D-amino acids again and repeated for analysis until the criteria achieved.

A GC system (Trace 2000 series) was connected to a Fourier transform infrared spectrometer (Thermo Nicolet IS50 St) for GC-IDMS analysis. [Glu<sup>1</sup>]-fibrinopeptide B with isotopic labelled Gly, Val and Phe was hydrolyzed with 500  $\mu$ L of 6 mol/L HCl at 110°C for 48 h. After hydrolysis, the sample was dried and derivatized with [BSTFA + 1% TMCS (99:1 v/v)] and methoxylamine hydrochloride. A DB-1701 column (60 m, 0.250 mm, 0.25  $\mu$ m) was used for separation. The initial oven temperature was 100 °C for 3 min and then raised to 230 °C at 5 °C/min. The chromatogram with IR detector was recorded; Then the IR spectra of each amino acid peak were extracted and deconvoluted to obtain the peak area of both natural and isotopic amino acids for further calculating protein purity.

## Conclusion

HPLC-CD, HPLC-ORD and GC-IDIR have been developed for protein purity assessment, which showed similar average and uncertainty compared with IDMS, making them potentially use for value assessment or orthogonal method validation.

## Human insulin purity assessment by LC-CD or LC-ORD

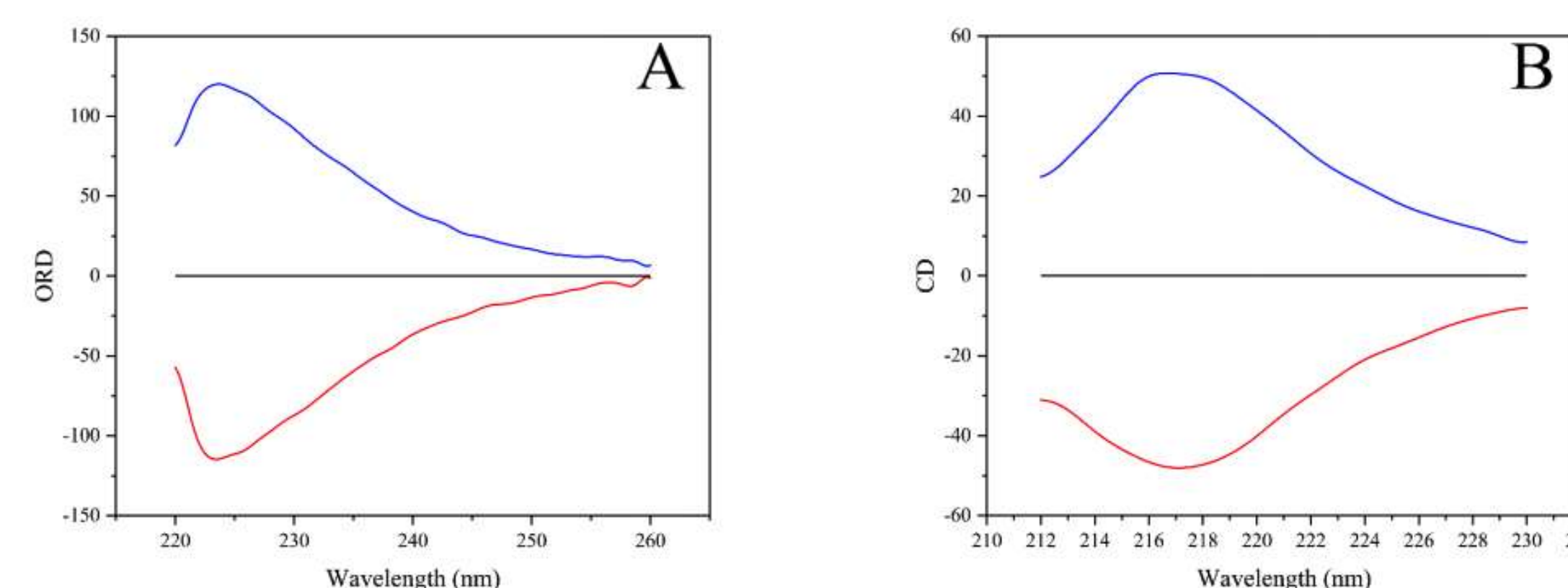


Figure 1 ORD(A) and CD(B) spectra of Phe

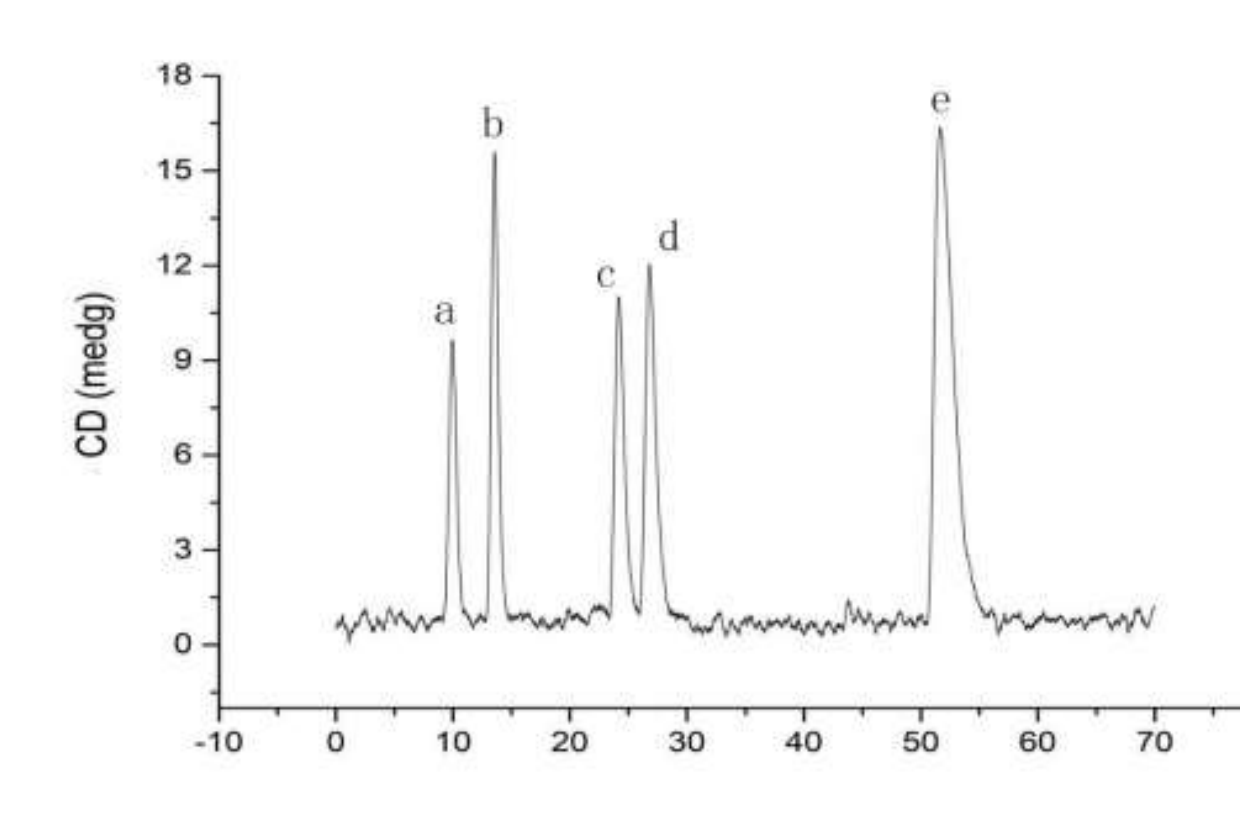


Figure 2 Separation of Pro(a), Val(b), Ile(c), Leu(d) and Phe(e)

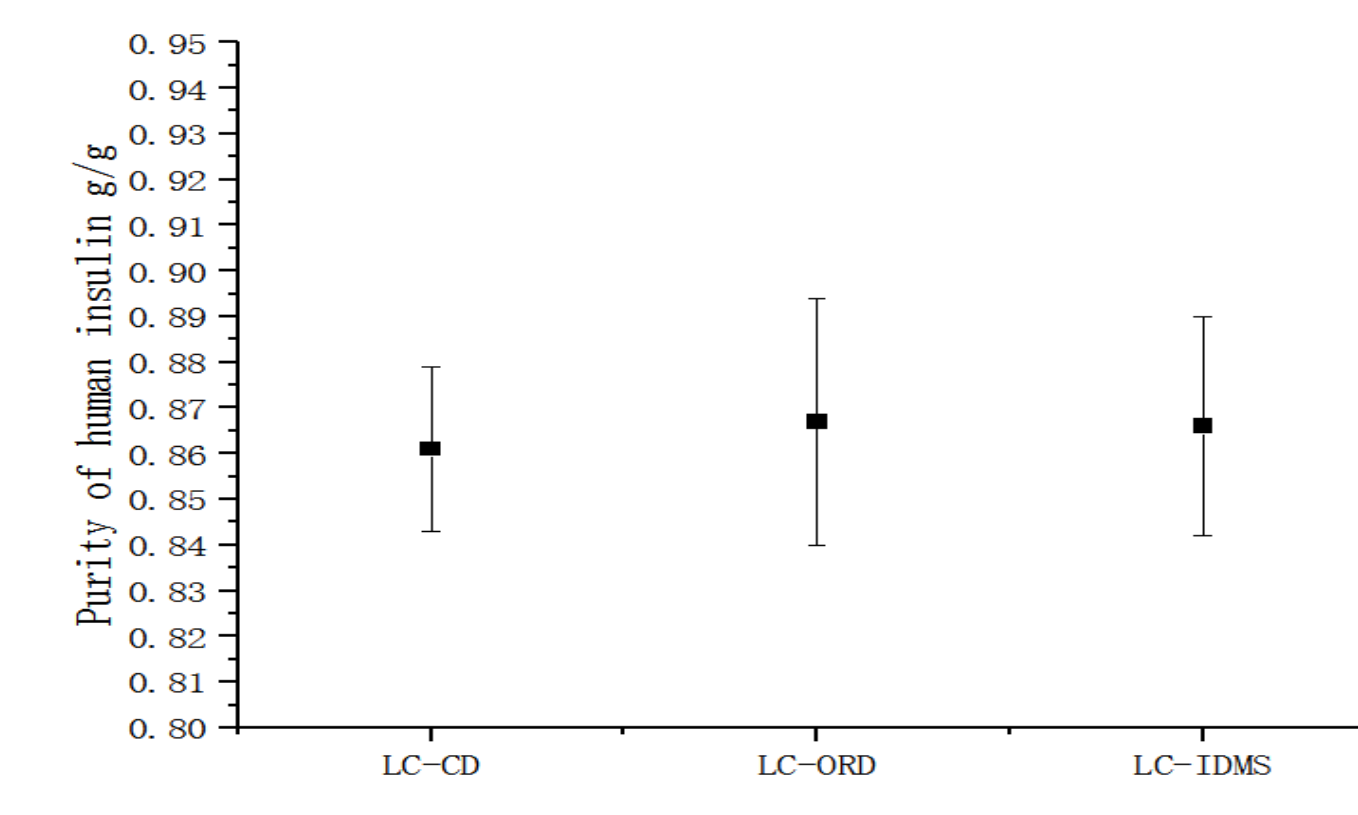


Figure 3 Human insulin purity assessment by HPLC-CD, HPLC-ORD and LC-IDMS

Table 1 Accuracy and repeatability for L-amino acid quantification by HPLC-ORD

| Conc. @0.1 mg/g           | L-Phe         | L-Val         | L-Leu         | L-Ile         |
|---------------------------|---------------|---------------|---------------|---------------|
| Certified Value of purity | (0.998±0.004) | (0.994±0.003) | (0.997±0.004) | (0.997±0.003) |
| Measured Purity           | 0.9969        | 0.9933        | 0.9972        | 0.9964        |
| Bias                      | -0.11%        | -0.07%        | 0.02%         | -0.06%        |
| RSD                       | 0.02%         | 0.06%         | 0.04%         | 0.04%         |

The CD or ORD spectra of a pair of chiral isomers typically show a pair of peaks with equal magnitude but opposite directions. When both are present in equal concentrations to form a racemic mixture, the CD or ORD signal becomes zero (Fig.1). This property is used to determine the L-amino acid concentrations in a hydrolysate using D-amino acids as standards, which allows for the measurement of protein purity or concentration, similar to a balance weighing process.

Amino acids commonly used for protein quantification, such as Pro, Val, Leu, Ile and Phe, were sufficiently separated for quantitation (Fig.2).

The accuracy and repeatability of the method were evaluated using a solution prepared with L-amino acid CRMs, as shown in Table 1. The results indicated that both bias and RSD were within 0.2%, demonstrating excellent method performance. The results of human insulin purity measurement by HPLC-CD, HPLC-ORD, and HPLC-IDMS are shown in Fig.3. Although the hydrolysis process introduced uncertainty and increased it, the final performance was comparable to traditional IDMS method, making them reliable methods for accurate protein purity assessment.

## [Glu<sup>1</sup>]-Fibrinopeptide B purity assessment by GC-IDIR

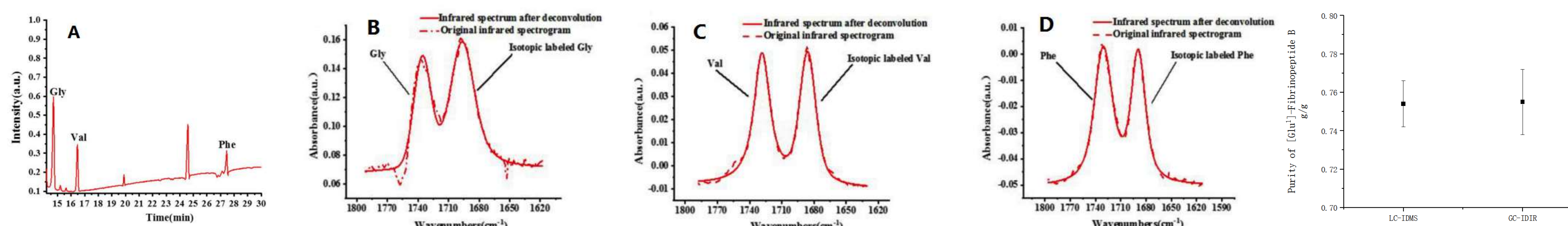


Figure 4 GC-IR chromatogram(A) and deconvoluted IR spectra of native and isotopic labelled Gly(B), Val(C) and Phe(D)

The amino acids in the hydrolysate can be easily baseline-separated on GC after derivatization due to the high column efficiency of GC (Fig.4A). With each point on the chromatogram peak, an independent infrared spectrum can be extracted for calculation, which improved the measurement accuracy.

Since isotope labeling alters the vibrational frequency of chemical bonds, natural and labelled amino acids can be distinguished on the IR spectra (Fig.4B~D). After deconvolution, the peak areas were used for purity calculation using bracket or single-point calibration. The results and uncertainties obtained by GC-IDIR and HPLC-IDMS were similar (Fig.5), making it a reliable method for accurate protein purity analysis.

Figure 5 [Glu<sup>1</sup>]-Fibrinopeptide B purity assessment by GC-IDIR and LC-IDMS

## References:

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