HPLC Purity Explainer: How to Read a Peptide COA

A guide to area%, peak shape and impurity notation on a Certificate of Analysis — what the number says, what it does not say, and how to read the chromatogram behind it.

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1. What the Purity Number Actually Measures

The number near the top of almost every research-peptide Certificate of Analysis — **Purity ≥ 99%** — is a specific, narrowly defined figure. It is the output of **reversed-phase high-performance liquid chromatography (RP-HPLC)** with ultraviolet detection, typically read at **214–220 nm**, where the peptide bond itself absorbs.[5] [#references]

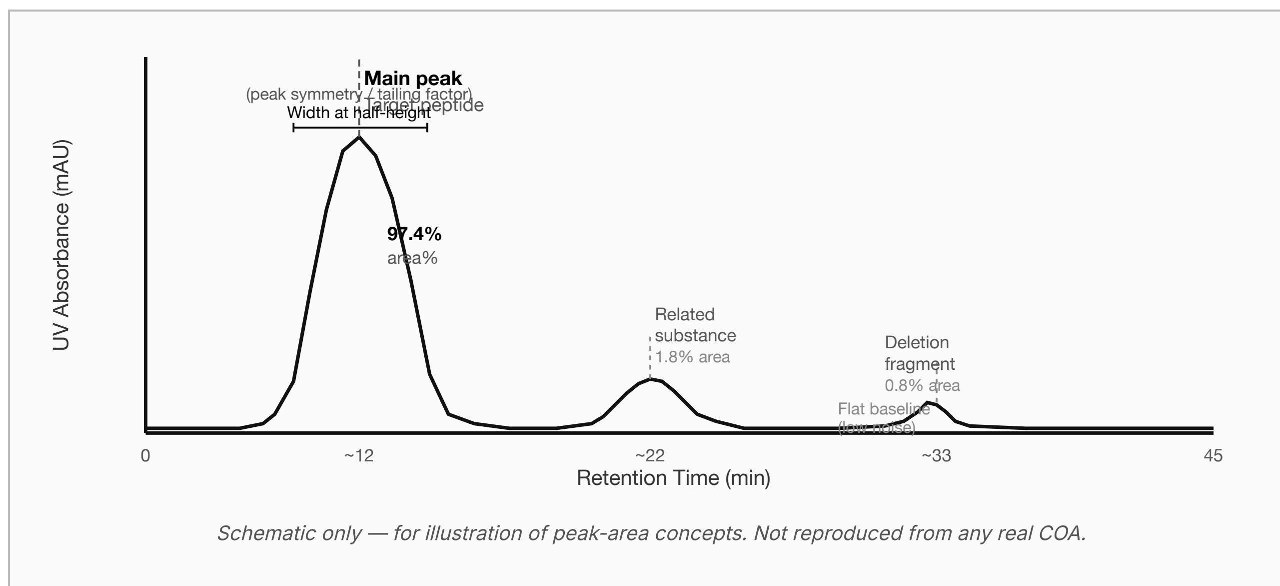
The instrument dissolves the sample and pumps it through a column packed with a non-polar (C18 or similar) stationary phase. Different molecules bind to that phase with different affinities and so emerge — *elute* — at different times, each registering as a peak on the UV detector. The software integrates the area under every detected peak. The purity figure is then simply:

> **Area%**
>
> **Purity (%) = (Area of main peak ÷ Total area of all detected peaks) × 100.**
> This is a *relative* measurement: the main peak as a fraction of everything the UV detector picked up. It is defined and governed by chromatographic conventions set out in pharmacopoeial general chapters such as USP <621>[3] [#references] and ICH Q6A.[1] [#references]

The critical implication is what the denominator excludes. Any species that **does not absorb UV at 214–220 nm**, or that never fully elutes from the column, is simply not counted. The purity percentage is honest about the chromatogram and silent about everything outside it.

2. How to Read a Chromatogram

A chromatogram is the raw output: a time-axis plot with peaks rising from a baseline. Here is a schematic of what you should expect to see on a high-quality peptide COA — and what each feature means:



Schematic only — for illustration of peak-area concepts. Not reproduced from any real COA.

What to look for on a real chromatogram:

- **One dominant main peak** with a clean, approximately symmetrical shape. The tailing factor ($T = W_{0.05} / 2f$) should be close to 1.0; values above 1.5 indicate peak asymmetry that can mask co-eluting impurities.
- **Flat, stable baseline** before and after all peaks. Baseline drift or noise can inflate or deflate the integrated areas of small peaks.
- **Well-resolved impurity peaks**, not merged shoulders on the main peak. USP <621> specifies a minimum resolution (R_s) between adjacent peaks as a system-suitability criterion; co-elution hides impurities under the main-peak area.[3] [#references]
- **A related-substances table** listing every peak above the reporting threshold (typically 0.05–0.1% area), with its retention time and area%. These are deletion sequences, oxidised forms, deprotection fragments, and aggregates — the ordinary by-products of solid-phase synthesis.[5] [#references]

> *"A purity figure without the chromatogram behind it is a number without a pedigree. A number without its method is not evidence — it is assertion."*

3. What HPLC Cannot Tell You: Identity

Here is the part that is most often omitted from supplier descriptions. A single, tall, symmetrical main peak at 99% area tells you the sample is **chromatographically homogeneous** — mostly one compound. It does *not* tell you **which compound** that is.

Retention time is characteristic but not definitive. A different peptide of similar hydrophobicity can elute in the same region of the chromatogram. HPLC answers the question *"how pure?"* It cannot, on its own, answer *"pure what?"*[5] [#references]

The identity question belongs to **mass spectrometry**. Two methods are standard in peptide COAs:

Method	What it proves	What it does NOT prove
RP-HPLC (UV 214–220 nm)	Relative chromatographic purity — main peak as % of total UV-detected peak area[3] [#references]	Molecular identity; absolute concentration; anything that doesn't absorb UV or doesn't elute[5] [#references]
UPLC / UHPLC	Same purity question at higher resolution — better separation of closely related impurities[3] [#references]	Identity; non-chromophoric impurities; endotoxin or water content[5] [#references]
ESI-MS	Identity — observed mass vs calculated mass of the intended sequence[5] [#references]	Quantitative purity %; trace related-substance levels; biological contaminants[2] [#references]
MALDI-TOF	Identity / molecular weight confirmation, robust for intact peptides[5] [#references]	Fine purity quantitation; counter-ion, water and endotoxin content[5] [#references]

Purity (HPLC/UPLC) and identity (ESI-MS/MALDI-TOF) answer different questions. A credible COA reports both, in line with ICH Q6A specification practice[1] [#references] and the EMA's synthetic-peptide guideline.[4] [#references]

Mass spectrometry ionises the molecule and measures its **mass-to-charge ratio (m/z)**. The observed mass is then compared against the **calculated theoretical mass** of the intended amino-acid sequence. A match within instrument tolerance is positive evidence of identity. A mismatch renders the purity figure irrelevant — a very pure sample of the wrong peptide is not a suitable research reagent.[5] [#references]

4. How to Read the Numbers on a COA — Annotated Example

Below is an annotated mock-up of a typical peptide COA purity section. Each field is explained.

CERTIFICATE OF ANALYSIS — REPRESENTATIVE PEPTIDE (RESEARCH GRADE) · BATCH: XXXXXX	
Purity (HPLC)	**≥ 99.0% (area%)** The main peak accounts for ≥99% of the sum of all UV-detected peak areas at 214 nm. Relative, not absolute. A different vial with 97% area% is not necessarily inferior — it depends on which impurities the remaining 3% represents and whether they are qualified per ICH Q3A.[2] [#references]
Method	RP-HPLC, C18 column, UV 214 nm, gradient 5–65% ACN/0.1% TFA, 45 min run The column chemistry, gradient, wavelength and run time determine which impurities are resolved. Always ask for method details — a 10-minute isocratic run may be insufficient to separate closely related deletion sequences.
System Suitability	Tailing factor ≤ 1.5 · Resolution (R_S) ≥ 2.0 between reference peaks · Plate count ≥ 5000 USP <621> requires these criteria to be met and recorded before the analytical result is valid.[3] [#references] If this row is absent, the result cannot be assumed to meet pharmacopoeial standards.
Related Substances	See impurity table — all individual impurities < 0.5% area; total impurities < 1.0% area ICH Q3A sets identification and qualification thresholds for impurities in drug substances.[2] [#references] For research materials, what matters is whether individual impurity peaks are listed and below meaningful thresholds — not merely that the main peak is large.
Identity (ESI-MS)	Calculated MW: 1419.54 Da · Observed MW: 1419.51 Da · ✓ Confirmed The observed mass matches the theoretical mass of the intended amino-acid sequence within instrument tolerance (~0.1 Da for small peptides). This is the identity check that HPLC cannot provide. Without this row, identity is unconfirmed.[4] [#references][5] [#references]
Water Content	≤ 5% (Karl Fischer) Lyophilised peptides are hygroscopic. Water is real mass in the vial that the HPLC area% figure does not count. A high water content means the vial contains less actual peptide than the nominal fill weight implies.[5] [#references]
Counter-Ion Content	TFA salt form / Acetate salt form (ion-exchange conversion) Synthetic peptides are typically isolated as TFA (trifluoroacetate) salts from the purification step. TFA mass is real mass in the vial not reflected in the area% purity. Ion-exchange to acetate form reduces this. Some COAs report net peptide content (corrected for salt and water); most do not.[5] [#references]
Endotoxins	Reported separately — see Endotoxin / Sterility section Bacterial endotoxins are not chromophores at 214 nm and are completely invisible to the HPLC purity assay. They require dedicated LAL (Limulus Amebocyte Lysate) or recombinant factor C testing. Their presence or absence says nothing about, and is not captured by, the purity percentage.[5] [#references]

5. What a Purity Specification Is — and Is Not

A purity specification on a COA is a **batch-tested acceptance criterion**, not a per-vial guarantee. ICH Q6A defines it as a list of tests, procedures and acceptance criteria that a drug substance must meet.[1] [#references] The specification sets a *range* — "≥ 99%" means the tested batch met or exceeded 99%, not that every vial in that batch was measured individually.

Three structural limits follow from this:

1. **The percentage is method-dependent.** The same compound analysed by two different HPLC methods (different columns, gradients, UV wavelengths) can return different area% values. Without the method, the number is unanchored.
2. **The denominator is the chromatogram, not the vial.** Anything invisible to the method — endotoxins, counter-ions, water — is excluded from the calculation. A "99% pure" vial may be 80% peptide by true weight if water and TFA content are high.
3. **Impurity control is as important as the headline figure.** ICH Q3A requires that impurities above certain thresholds be identified, qualified and reported with limits.[2] [#references] A COA that lists only the main-peak figure without an impurity profile is not a complete characterisation document.

6. Supplier Due-Diligence Checklist

Use this checklist when evaluating any peptide COA. Every item that is missing represents a question you should put to the supplier before trusting the reagent.

- □ COA is batch-specific to the vial you are buying — not a generic product sheet reused across batches
- □ HPLC purity figure is reported as area% with method details (column, gradient, wavelength, run time)
- □ System-suitability results (tailing factor, resolution, plate count) are recorded and pass criteria per USP <621>[3] [#references]
- □ Chromatogram (the actual trace) is available on request — not just the integrated percentage
- □ Related-substances / impurity table lists all peaks above reporting threshold with area% and retention time
- □ Mass spectrometry identity data (ESI-MS or MALDI-TOF): calculated MW vs observed MW, result confirmed[4] [#references]5 [#references]
- □ Water content measured and reported (Karl Fischer titration or equivalent)
- □ Counter-ion form stated (TFA or acetate); net peptide content reported if possible
- □ Endotoxin level measured separately by LAL or rFC assay and reported[5] [#references]
- □ Testing performed by an independent, third-party laboratory (not solely in-house)

Frequently Asked Questions

Does "≥99% purity" mean the vial is 99% peptide by weight?

No. It is a relative chromatographic peak-area percentage from RP-HPLC — the main peak as a fraction of all UV-detected peaks at 214–220 nm. A vial can read 99% area% yet contain meaningfully less peptide by weight because adsorbed water and counter-ion (salt) mass are not counted in the area% calculation.[3] [#references]5 [#references]

What does "area%" mean on a chromatogram?

Area% for any given peak = (area of that peak ÷ sum of all integrated peak areas) × 100. The main target-peptide peak area% is the purity figure. All other peaks — impurities, deletion sequences, oxidised forms — appear as smaller area% values in the related-substances table. The values must sum to 100% of the detected area.[3] [#references]

Why does a clean HPLC peak not prove identity?

Retention time is characteristic but not definitive — a different peptide of similar hydrophobicity can elute at the same position. Identity requires orthogonal confirmation by mass spectrometry, which compares the calculated mass of the intended amino-acid sequence against the observed mass. A COA without that identity evidence has not demonstrated identity.[4] [#references][5] [#references]

What is a system-suitability test and why does it matter?

Before reporting a purity result, USP <621> requires the HPLC system to meet criteria such as minimum resolution between adjacent peaks, acceptable peak symmetry (tailing factor ≤ 1.5), and theoretical plate count. A purity result from a system that failed suitability is analytically invalid — it cannot be trusted to resolve closely related impurities from the main peak.[3] [#references]

What does the impurity table in a COA actually list?

The related-substances table lists every peak above the reporting threshold (commonly 0.05–0.1% area) with its retention time and area%. These are typically: deletion sequences (peptides missing one or more residues), oxidised forms of the target peptide, incompletely deprotected fragments, and synthesis by-products. ICH Q3A governs how these must be identified, qualified and assigned limits for drug substances.[2] [#references]

What do HPLC and mass spectrometry together still miss?

Both methods are blind to residual water content, counter-ion (TFA or acetate) content, and biological contaminants such as bacterial endotoxins. Endotoxins in particular carry no informative peptide-bond UV signature and pass through a purity assay entirely unseen — they require dedicated testing (LAL or recombinant factor C assay). A complete COA reports these attributes through separate, dedicated tests.[5] [#references]

References

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