Complementary Chromatographic Strategies for Enhanced Impurity Analysis and Purification of Peptides: A comparative study of HILIC, SFC, and RP-LC.

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Cleaner peptides, faster: how HILIC and SFC complement RP-LC to find and purify impurities

Peptides are promising drugs, but they often come with structurally similar impurities that are hard to spot and remove. This project compares three analytical methods used to analyse and purify peptides: the standard reversed-phase liquid chromatography (RP-LC), and two complementary methods - hydrophilic interaction liquid chromatography (HILIC) and supercritical fluid chromatography (SFC). The aim was to show when and how HILIC and SFC can give different separation of peptide mixtures (orthogonal selectivity).

Why this matters:

- Peptide mixtures can contain almost-identical impurities. If we can separate them better, medicines can be made more safely and efficiently.
- RP-LC is the standard method, but it sometimes leads to co-elution of impurities and the target peptide. HILIC and SFC can offer different separation mechanisms providing complementary merits in removing impurities.
- HILIC and SFC allow replacement of hazardous solvent and easier post-purification processing, which helps the environment, safety, and costs.

What was done:

- Nine peptides were analysed by RP-LC, HILIC, and SFC to compare different elution profiles.
- In HILIC, acetonitrile (ACN) and methanol (MeOH) were tested as solvents.
- HILIC and RP-LC selectivity was compared by analysing degradation- and synthesis-related impurities elution relative to the target peptide of the samples.
- In SFC, a CO_2-rich mobile phases were used along with three acids (trifluoroacetic acid, ethane- and methanesulfonic acids), that change how peptides travel through the column.

Key findings:

- HILIC often separated peptides in a different order than RP-LC. In several instances it was able to separate impurities from the target peptide when RP-LC failed to do so.
- In HILIC, ACN usually gave cleaner separations than MeOH, which typically led to early elution of the tested peptides.
- In SFC, the choice of acid mattered: ethane- and methanesulfonic acids usually improved the analysis process when compared to trifluoroacetic acid.

Take-home message:

- HILIC and SFC are complementary methods to standard RP-LC for peptide analysis and purification. The results offer a starting point for future exploration of this topic.

Abstract

Introduction: Peptide therapeutics are becoming increasingly important across multiple disease areas, bringing increased structural diversity and a broader range of physicochemical properties. This diversity, together with more complex synthetic- and product-related impurities, raises the bar for liquid chromatography methods that should be efficient and sustainable. Reversed-phase liquid chromatography (RP-LC) is widely used; however, it may have limited resolving power for closely related impurities. Hydrophilic interaction liquid chromatography (HILIC) and supercritical fluid chromatography (SFC) offer complementary selectivity that may improve impurity resolution and workflow efficiency.

Background: Despite recent studies on the topic, systematic guidance for applying HILIC and SFC to peptide samples is limited; the influence of organic modifier, buffer conditions, and stationary-phase chemistry on selectivity and robustness remains greatly unexplored.

Aim(s): To compare HILIC and SFC with RP-LC for peptide analysis and purification, establish orthogonality, and identify practical method-development guidelines to apply to diverse peptide profiles.

Methods: A set of nine commercially available peptide drugs were analysed by RP-LC, HILIC and SFC for comparison. The effects of chromatographic variables on retention, elution order, peak shape and resolution were investigated.

Results: HILIC exhibited distinct and often inverted elution orders relative to RP-LC, consistent with their different retention mechanisms. Some of the HILIC methods resolved several synthesis-related deletion impurities that co-eluted in RP-LC.
In SFC, additive identity governed retention via ion pairing: average retention followed TFA < ESA < MSA for most peptides. The columns CN, GSBC and GSFS provided the most balanced SFC performance across additives; highly basic and polar peptides (e.g., ACTH, liraglutide) retained strongly across phases.

Conclusion and future perspective: HILIC provided robust, orthogonal selectivity to RP-LC for peptide mixtures, potentially improving impurity detection and purification. In SFC, peptide retention and peak quality were influenced by the additives, as well as peptide and column chemistries.

Keywords: Hydrophilic-interaction liquid chromatography (HILIC), Method Development, Orthogonal separation method, Peptide analysis and purification, Supercritical fluid chromatography (SFC).

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Content Table

1. List of abbreviations

SPPS: Solid-phase peptide synthesis
Fmoc: N^{α}-fluorenylmethoxycarbonyl
Boc: N^{α}-tertiarybutyloxycarbonyl
HPLC: high performance liquid chromatography
m/z: mass-to-charge ratio
RP-LC: reversed-phase liquid chromatography
RP: reversed phase
LC: liquid chromatography
MS: Mass spectrometer
NP-HPLC: normal-phase liquid chromatography
HILIC: hydrophilic interaction liquid chromatography
SFC: Super-Critical Fluid Chromatography
MP: mobile phase
SP: stationary phase
TFA: Trifluoroacetic acid
MSA: Methanesulfonic acid
ESA: Ethanesulfonic acid
ACN: Acetonitrile
MeOH: Methanol
RPLC/AEX: mixed mode Reverse-Phase/Anionic Exchange Chromatography
HCl: Hydrochloric acid
NaOH: Sodium Hydroxide
H_2O_2: Hydrogen Peroxide
NH_4OAc: Ammonium acetate
TPSA: Topological Polar Surface Area
SSL: Separation Science Lab

2. Introduction

Nowadays, peptide-based drugs represent a novel and ever-growing therapeutic option to treating multiple diseases, such as cancer, HIV and type 2 diabetes mellitus[1]. Compared to small molecules drugs, these short aminoacidic sequences (typically lower than 50 residues) possess greater selectivity and specificity to effectively modulate protein-protein interactions (PPIs). Moreover, peptides also have advantages over biologics alternatives such as proteins, e.g. less immunogenicity, lower cost of manufacture and enhanced tissue penetration.[2]
Apart from the tailored sequence design targeting specific PPIs, peptide structures can also be chemically modified in the backbone or side chains to increase the potency and their physicochemical stability, further increasing their potential as therapeutic agents.[2] However, the diversified designs bring increased structural heterogeneity and broaden the range of physicochemical properties, which together with the process-related impurity, make the development of efficient liquid chromatography methods more challenging.
As of today, multiple chromatographic techniques have been investigated for the analysis and purification of peptides, such as Reversed Phase Liquid Chromatography (RP-LC), Ion-Exchange Chromatography (IEX), and Size-exclusion Chromatography (SEC)[3]; nonetheless, techniques like Hydrophilic-Interaction Liquid Chromatography (HILIC) and Super-Critical Fluid Chromatography (SFC) still remain less explored in peptide application, their potential is yet to be fully grasped.

2.1 Peptide synthesis

Currently, peptide synthesis is performed mainly through two techniques: Solid Phase Peptide Synthesis (SPPS) and Liquid-phase peptide synthesis (LPPS).
Merrifield´s SPPS technique involves building the target peptides by sequential addition of aminoacidic residues. The peptide's C-terminal residue is covalently anchored to a cross-linked polymeric resin, and standard protecting group strategies are applied to mask the α-amino functionality, enabling iterative chain elongation on the solid support.[4] Widely used protecting groups are *N$^{\alpha}$-fluorenylmethoxycarbonyl* (Fmoc) and N$^{\alpha}$-tertiarybutyloxycarbonyl (Boc). Fmoc is removable under basic conditions, whereas Boc is cleaved under acidic conditions and therefore necessitates acid-resistant side chain protecting groups to preserve the peptide integrity.[4] Deprotection of the α-amino group by elimination reaction precedes coupling with the new protected aminoacidic residue; these steps are repeated until completing the target peptide sequence. Finally, cleaving of the crude peptide from the support and removal of side chain protective groups takes place. [4] SPPS is a high-performing and automated process, characterised by high yield and purity which allows for large-scale production [5]. Moreover, SPPS is easily automated and supports high-throughput, parallel work, allowing robust processes.[4]
LPPS is an alternative approach to peptide synthesis. It consists of a progressive aminoacidic addition to a peptide molecule in stepwise fashion. However, the difference with SPPS stands in a liquid-phase system synthesis[5]; this makes LPPS ideal for the synthesis of long peptide sequences where resin swelling would hinder the SPPS synthesis, and/or demand of environmentally friendly procedures [5].
In the present study, the peptides used for the investigation were synthesized by SPPS.

2.2 Analytical and purification challenges

In early drug discovery, the development of rapid and efficient analytical and purification methods for synthetic peptides is essential to support screening assays and other intended studies. Namely, it is critical to identify and remove impurities from the crude peptides to guarantee the desired level of product quality[6].

The common impurities in peptide crudes can be categorised into the following types:

- Synthesis-Related impurities: arise from the peptide synthesis process and are typically structurally similar to the target peptide. These impurities may result from deletion, truncation or insertion sequences, incomplete deprotection, peptide racemization, and other synthetic by-products.

- Non-Peptide Impurities: are compounds that do not originate from the peptide itself and may include residual solvents, reagents, and other process-related contaminants.

The synthesis-related and degradation-related impurities usually share structural homology or/and physicochemical property similarity with the target peptide, which makes the separation process rather difficult[6].

2.3 Liquid Chromatography: principles and different modes

Liquid Chromatography (LC) is a widely used and effective technique for peptide analysis and purification. In principle, different LC modes share a common underlying mechanism: analytes partition between the mobile phase (MP) and the stationary phase (SP) according to their intermolecular interactions[7]. The SP is typically an adsorbent solid that retains analytes through various molecular interactions, whereas the MP is the carrier liquid that solubilises and transports them. As MP passes through the column, each compound in the sample partitions differently between the two phases based on their physicochemical properties and molecular structure, resulting in distinct elution profiles that enable separation and resolution[8] .

Chemical characteristics of the SP and the MP can be selected to separate specific classes of compounds, consequently giving rise to different LC modes. Operating conditions can further modulate separation performance; parameters such as column temperature, MP flow rate, injection volume and analyte concentration can be adjusted to enhance resolution or shorten analysis time[7]. Furthermore, the MP can be modified using buffers to adjust ion strength and pH, potentially improving the solubilisation or elution profile of the analyte. [7]

The main chromatographic approaches for peptide analysis and purification are discussed below.

2.3.1 Reverse Phase Liquid-Chromatography (RP-LC)

In RP-LC, the MP composition is constituted by water and a water-miscible organic solvent, typically MeOH or ACN. The SP is usually low-polarity, chemically bonded inorganic oxide. Common examples include organosilanol-based networks where alkyl chains are covalently attached to silanol groups on the silica surface; among them, C18 (Octadecylsilane) is the most common one.[7] The separation of the analytes lies largely on their differential affinity for the SP, hence, on their hydrophobicity. Under gradient elution, RP-LC general operating principle is starting with a low organic content and progressively increase it to reduce the polarity difference between the SP and the MP. By doing so, the peptide-SP interaction weakens, allowing the peptides to be eluted from the column in order of increasing affinity for the SP.[7]

RP-LC allows for the analysis of compounds in a wide range of polarity and size. Its versatility derives from diverse stationary-phase manufacturing and surface treatments; measures such

as end-capping and ligand shielding improve silica stability and can extend usable pH toward and occasionally beyond pH 8.[9] Even so, RP-LC can struggle with the analysis of large biomolecules, such as proteins and peptides, due to their size and structure complexity. Moreover, highly polar species may exhibit weak retention, and structurally similar components in peptide crudes can be difficult to resolve under standard RP-LC conditions[10]

2.3.2 Hydrophilic Interaction Liquid Chromatography (HILIC)

HILIC chromatographic mode consists of a polar stationary phase covered by a water-rich layer and a MP rich in organic solvent. The relative distribution of water and organic solvent within the column is crucial for separation in HILIC. Under gradient elution, a high percentage of organic solvent is operated from the start and is progressively decreased over time. Commonly used SP are made of bare silica or modified with for example amino, cyano, diol or zwitterionic functionalities.[7]

HILIC has been widely used for the analysis of pharmaceuticals and compounds of biomedical relevance.[11] Its strong retention of highly polar compounds makes it a well-established orthogonal analytical technique to RP-LC, where retention of these analytes is insufficient.

However, general guidelines for HILIC application remain limited, including column selection, buffer composition, organic solvent and gradient, as well as choice of diluent and injection volume.

The complex nature of HILIC retention makes the prediction of analytes elution inherently difficult. McCalley proposed a “mixed mode” retention mechanism[11], that theorise the co-existence of multiple retention phenomena. These includes (1) partitioning of the analyte between the water-rich layer on the SP and the organic-rich MP, and (2) adsorption processes such as hydrogen bonding, dipole/dipole interactions, and electrostatic forces.

In addition, the high organic content typically used in HILIC eluents can simplify downstream handling, facilitate rapid solvent removal, and improve lyophilisation efficiency, which can shorten post-purification timelines and reduce processing costs.

In summary, despite its challenges HILIC constitutes a valuable alternative for peptide analysis and purification. It can also be employed as an orthogonal method to RP-LC or IEX in the context of 2D-LC, further improving peptide separation and purification.

2.4 Super Critical Fluid Chromatography (SFC)

Besides traditional LC techniques, alternative approaches like Supercritical fluid chromatography (SFC) are being developed for peptide analysis and purification.

In SFC, the mobile phase is based primarily on carbon dioxide (CO_2), which is used under compressed conditions, typically at or above its critical temperature and pressure. Under these conditions, the mobile phase combines liquid-like density with gas-like viscosity and diffusivity, which can enable rapid mass transfer and efficient separations.[12] Although the apolar nature of CO_2 might seem counterintuitive for peptide separations, adding polar organic modifiers (e.g., MeOH or ACN) enables effective peptide solubilisation and tuning of elution strength. This way the characteristic low-viscosity, high-efficiency performance of the CO_2-rich mobile phase is preserved, altogether supporting retention control of peptides.[6] Building on this, additives represent an effective way to further improve the solubilisation of polar analytes, potentially by aggregating in the cybotactic region around the analyte. Their use in SFC has been proven to influence chromatographic features such as retention or peak shape.[13] Indeed, additives alter the apparent pH of the MP, which in turns influence the protonation state of the SP and the

analytes based on their pKa values. Moreover, additives can “mask” charged sites on the peptides and/or SP, inhibiting electrostatic attraction/repulsion between species.
The apparent pH of the mobile phase in SFC varies from slightly to moderately acidic when adding modifiers and/or acidic additives. Indeed, when introducing MeOH and/or water in the mobile phase, methoxylcarbonic acid and carbonic acid form from the reaction of CO_2 with respectively MeOH and water, lowering the apparent pH of the MP.[13]
SFC is compatible with a broad range of packed columns bearing polar to non-polar ligands, which support robust operation across several modifiers, additives and temperatures. Commonly, polar stationary phases are employed as in NP-LC; however, apolar RP-LC columns also find an application in SFC.[14]
Overall, in virtue of its remarkable versatility and high efficiency, SFC represents a promising chromatographic mode for peptide analysis and purification. Moreover, CO_2 constitutes a greener alternative to organic solvents, as it is typically sourced from industrial by-product streams and can be safely captured and recycled at the end of the chromatography. In addition, the use of organic solvent is limited in comparison to HPLC, reducing exposure to hazardous chemicals and solvent consumption.[12] This makes SFC an attractive chromatographic method from sustainability perspective.

2.5 Research gap and research questions

Although HILIC and SFC have previously been applied to peptide analysis and purification, systematic guidance on how chromatographic parameters such as stationary-phase chemistry, mobile-phase composition, and modifier selection influence peptide separation remains limited. Consequently, the practical use of these techniques for impurity analysis and purification of synthetic peptides is still not well established.
The present study aims to provide a comprehensive evaluation of HILIC and SFC as alternative and complementary chromatographic methods to RP-LC for the analysis and purification of synthetic peptides. Specifically, the study investigates the extent to which HILIC and SFC provide orthogonal selectivity relative to RP-LC and identifies the conditions under which such complementary selectivity can be achieved.
For HILIC, the study evaluates the influence of organic solvent composition, with particular emphasis on ACN and MeOH, on chromatographic performance and selectivity. Furthermore, the separation behavior of synthesis-related and degradation-related peptide impurities is compared between HILIC and RP-LC to assess the potential advantages of orthogonal selectivity in the context of impurity identification and purification.
For SFC, the study investigates the applicability of the technique to peptide analysis and examines the impact of acidic modifiers, stationary-phase chemistry, and peptide physicochemical properties on chromatographic performance.
Ultimately, the work seeks to establish a starting point for the screening and purification of synthetic peptides using HILIC and SFC and to contribute to the development of general guidelines for their implementation in peptide analysis.
It should be noted that part of the evaluation of HILIC operating conditions builds upon a previous master's thesis project. Unpublished data from that work are used in the present study as reference points and, where appropriate, for comparative analysis to support the conclusions drawn. Such data are clearly identified wherever cited.

3. Methods and materials

3.1 Instrumentation

For RP and HILIC the analytical runs were performed on a Waters ACQUITY UPLC system equipped with a Waters 2998 photodiode array detector and a Waters SQD-2 electrospray ionization mass spectrometer. A Xevo G3 QTof MS was used for high-resolution accurate-mass characterization of the force-degraded peptides. The data was acquired with MassLynx 4.2 software. The obtained LC-MS raw data was processed with MestreNova Gears, an automated framework within the Mnova software that allows processing for analytical data. Baseline correction, deconvolution, quantitation and reporting is possible.[15]

For SFC the analytical runs were performed on a Waters ACQUITY UPC^2 system with a Waters 2998 photodiode array detector and a Waters SQD-2 electrospray ionization mass spectrometer. The data was acquired and processed with Empower® 3 software.

ACD Labs Percepta was used to calculate the charge state of the SFC stationary phase ligands.

3.2 Peptides

The test set of peptides is reported on Table 1. The peptides were injected either as a mixture or as individual peptide solutions depending on the experiment. For HILIC experiments, all peptides were dissolved in ACN/water (1/1, v/v) at a final concentration of 0.5 mg/mL each, either in the mixture or in a single peptide solution. For SFC experiments, 1 mg/mL single peptide solutions were prepared.

Table 1. Physicochemical properties of the test set of peptides

Peptide		MW (avg.) (g/mol)	pI	HELM sequence
1	Desmopressin	1069.2	8.7	Y-F-Q-N-C(1,3)-P-dR-G-am-[AcidC3SH] (1,1)
2	Angiotensin II	1046.2	7.7	D-R-V-Y-I-H-P-F
3	Leucine Enkephaline	555.6	6.0	Y-G-G-F-L
4	ACTH Porcine	4541.1	9.4	S-Y-S-M-E-H-F-R-W-G-K-P-V-G-K-K-R-R-P-V-K-V-Y-P-N-G-A-E-D-E-S-A-E-A-F-P-L-E-F
5	Triptorelin	1311.4	7.7	5 (O) Pro-H-W-S-Y-dW-L-R-P-G-am
6	Leuprolide	1209.4	9.7	5 (O) Pro-H-W-S-Y-dL-L-R-N1 (CCC [C@H]1C(NCC) =O) [*:1]
7	Somatostatin (cyclic)	1637.9	8.9	A-G-C(1,3)-K-N-F-F-W-K-T-F-T-S-C(1,3)
8	Liraglutide analogue	3512.8	4.6	H-A-E-G-T-F-T-S-D-V-S-S-Y-L-E-G-E-A-A-K-E-E-F-I-A-W-L-V-R-G-R-G
9	Carbetocin	988.2	N/A	[* : 3] C [* : 2] (1, 3) (2, 2). Tyr(Me)-I-Q-N-C (1, 3)-P-L-G-am-[AcidC3] (2,1)

Note that Liraglutide analogue refers to the parental peptide minus the lipid moiety.

3.3 The columns

The size of the HILIC/RP-LC columns used was of 2.1 mm in inner diameter per 100 mm in length. C18 and Amide columns had particle size of 2.5 µm, while the others had 3 µm particles.

Table 2. Overview of columns used in HILIC evaluation study

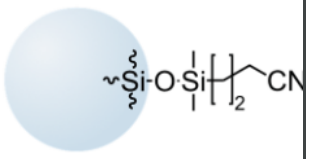
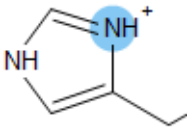
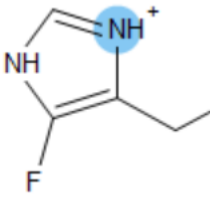
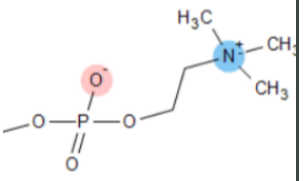
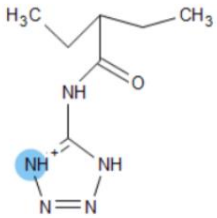
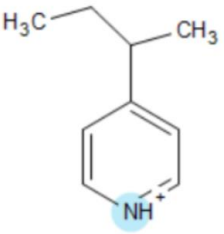
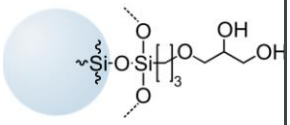
Column	**Mode**	**Stationary Phase**
Waters Xbridge BEH C18	RP	Waters proprietary BEH (Ethylene-Bridged Hybrid) C18
Waters Xbridge BEH Amide	HILIC	Xbridge BEH Amide
Phenomenex Luna Polar Pesticides	HILIC	Proprietary Polar
DAICEL Dcpak PMPC	HILIC	Poly (2-Methacryloyloxyethyl phosphorylcholine) on silica
DAICEL Dcpak PTZ	HILIC	Poly N-(1h-tetrazole-5-yl)-methacrylamide on silica
DAICEL Dcpak P4VP	HILIC	Poly (4-vinylpyridine) on silica

The size of the SFC columns used was of 4.6 mm in inner diameter per 150 mm in length. The particle size was 2.5 µm for the Kromasil-CN and Kromasil-Diol columns, while 3 µm for the remaining ones.

Table 3 – Overview of columns used in SFC evaluation study

Column	**Stationary Phase**
Kromasil-CN	Cyano propyl silane
Kromasil-Diol	2-(3-propoxy) ethane-1,2-diol silane
PerkinElmer GreenSep Basic (GSBC)	Imidazole chemistry
PerkinElmer GreenSep FluoroBasic (GSFS)	Fluorinated imidazole chemistry
DAICEL Dcpak PMPC	Poly (2-Methacryloyloxyethyl phosphorylcholine) on silica
DAICEL Dcpak PTZ	Poly N-(1h-tetrazole-5-yl)-methacrylamide on silica
DAICEL Dcpak P4VP	Poly (4-vinylpyridine) on silica

Table 4. Estimated protonation state of the stationary-phase ligands under an apparent pH of ~1, as predicted using ACD/Labs Percepta. Note that the GSBC and GSFS ligand structures are not publicly disclosed; accordingly, the image was generated with an AI model from vendor-provided descriptions.

CN	GSBC	GSFS	PMPC
			
PTZ	**P4VP**	**Diol**	
			

3.4 The mobile phases

3.4.1. HILIC mobile phases composition

Four MeOH-containing mobile phase conditions were evaluated in this study, using different pH values and buffer strengths.

Table 5. Mobile phase composition and corresponding pH in the HILIC screening

A		B	pH
1	50 mM NH_4OAc in H_2O, 0.5% AcOH	MeOH, 0.5% AcOH	4.5
2	50 mM NH_4OAc in H_2O	MeOH	6.9
3	20 mM NH_4OAc in H_2O	20 mM NH_4OAc in 95/5 MeOH/H_2O	6.9
4	H_2O	MeOH	7.4

3.4.2. SFC mobile phase composition

Table 6. Mobile phase composition and corresponding pH in the SFC screening

A		B	Gradient	pH
1	CO_2	20 mM TFA in MeOH/H_2O 95/5	25-60% B in 6 min	≤1
2	CO_2	20 mM TFA in MeOH/H_2O 95/5	25-60% B in 6 min	≤1
3	CO_2	20 mM TFA in MeOH/H_2O 95/5	25-60% B in 6 min	≤1

3.5 Chromatographic conditions

For HILIC experiments, the flow rate, column temperature, injection volume, and UV detection wavelength were set to 0.4 mL/min, 45 °C, 1 µL, and 230 or 254 nm, respectively. Analyses were performed using the gradient programs indicated in the corresponding chromatograms. For SFC experiments, the column temperature, injection volume, and UV detection wavelength were set to 40 °C, 5 µL, and 230 or 254 nm, respectively. Analyses were carried out with gradient 25-60% B over 5 min. For each column, the flow rate was optimized relative to the applied backpressure.

3.6 Interpretation of the results

Method suitability will be demonstrated if: (i) resolution between Peak A and Peak B is ≥ 1.5 (only in HILIC); (ii) peak symmetry for the analyte is 0.5 ≤ As ≤ 1.5; (iii) the width at 10% height ≤ 15s.[16] The suitability of the method was established building upon previous work performed at SSL, as well as general industry guidelines.

3.7 Peptide degradation experiment

Three peptides were employed for this experiment: Carbetocin, Leuprolide and Triptorelin. The selected peptides were treated with acidic (0.1 M HCl), basic (0.1 M NaOH) and oxidative (H_2O_2, 0.3% v/v) stress conditions. The samples were incubated in a water bath at 40 °C for 4 h and 1 h, for the acidic and oxidative conditions respectively. The basic samples were exposed to room temperature for 0.5 h.

The optimal degradation conditions were determined empirically by trial and error, using Desmopressin as a model substrate. After being exposed to the degradation conditions, peptides were first analysed by HR-MS paired with a RP-LC C18 column; then, the samples were studied by RP-LC (C18) and HILIC (Amide). For each mode, MeOH and ACN were evaluated separately as the organic solvent. Gradients were selected from experimental runs that provided the best separation under the tested conditions.

3.8 Peptide crudes information

Four peptide crudes were provided by AstraZeneca, for evaluation of synthesis-related impurities. The information on the target peptides is as follows.

Table 7. Target peptides molecular weight in AstraZeneca´s crudes

Crude	**MW (avg.) (g/mol)**
Crude A	4558.8
Crude B	4445.6
Crude C	4485.5
Crude D	4485.5

Similarly to the peptide degradation experiment, each crude was examined by both RP-LC (C18) and HILIC (Amide) using both MeOH and ACN (separately) as the organic solvent.

3.9 The literature search process

Search criteria followed university guidelines and standard practices in contemporary scientific writing. The primary aim was to emphasise recent developments by limiting publications to 2010 onwards (with a small number of exceptions). Journal impact factor was not applied as an exclusion criterion; nevertheless, many sources derive from leading sites (e.g., Journal of Chromatography, Nature Reviews, Journal of the European Peptide Society).

The main data sources were Finn, Lund University Library (F), Google Scholar (GS) and PubChem (PC).

Below, an example of a search filter used for the sources is presented:

- Query “Liquid Chromatography”; source types: academic journals, e-books, reports, patents. Result: 2,606,873 records (F), 189,000 records (GS), 223,379 records.

4. Results and discussions

4.1 Evaluation of HILIC for peptide analysis

Nine peptides were screened across multiple HILIC stationary phases with MeOH- and ACN-based mobile phases to assess orthogonality to RP-LC and practical method development parameters. The complementarity of HILIC to RP-LC was evaluated in the context of pure peptides elution, separation of synthesis-related and degradation-related impurities. The results for each experiment will be systemically discussed in the sections below.

4.1.1 Assessment of MeOH as an organic modifier in HILIC

In previous studies within SSL, ACN has been comprehensively evaluated as organic modifiers for HILIC columns. MeOH can provide chromatographic performance comparable to that of ACN in some LC modes. However, the two solvents differ substantially in their physicochemical properties. MeOH is a protic solvent, whereas ACN is aprotic; MeOH also has higher polarity, viscosity, and hydrogen-bonding capacity.[7]

In the present study, MeOH was investigated as an alternative organic modifier in HILIC to assess its applicability and its impact on peptide separation performance relative to ACN. Four MeOH-based mobile phases were tested, covering pH ranges from acidic to neutral.

A peptide mixture containing 9 peptides were injected in this screening experiment. The elution of peptides was indicated in the chromograms as numbers. The numbering of peptides is listed in Table 1.

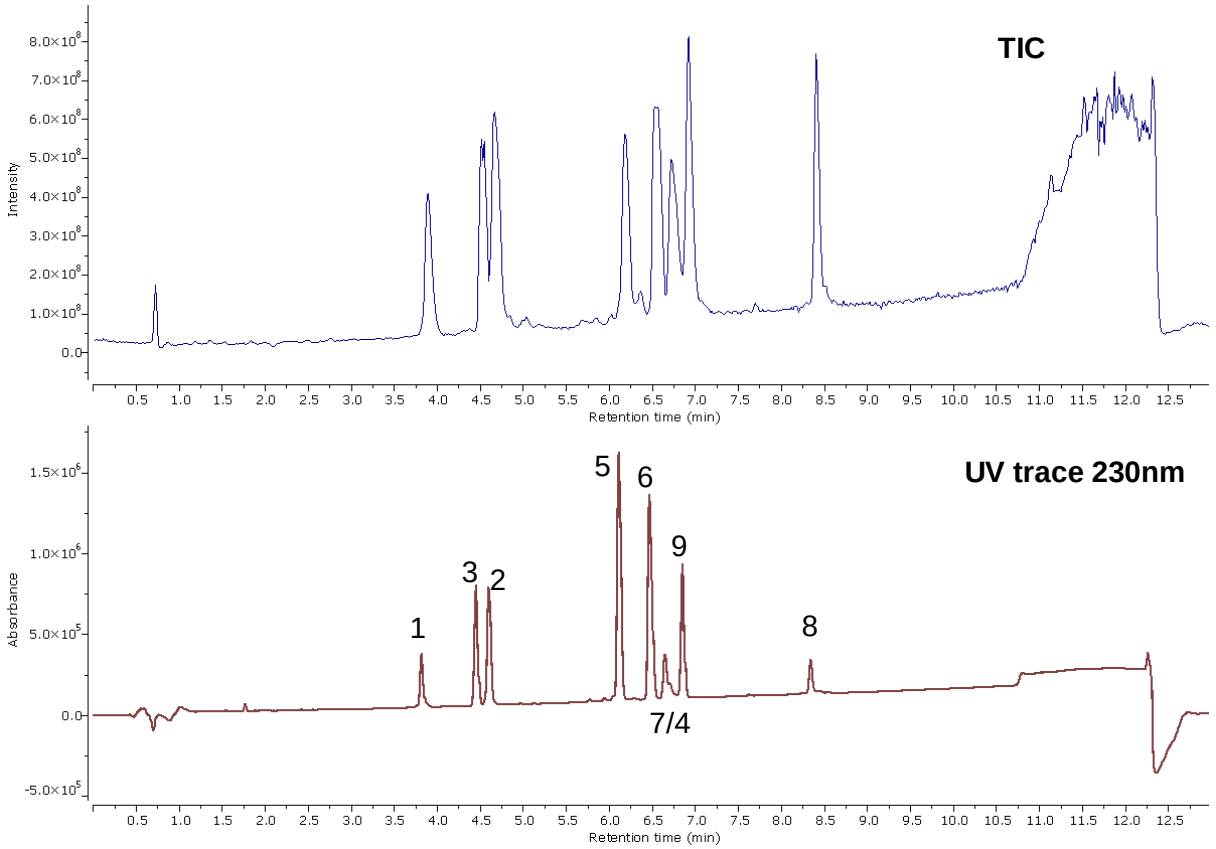


Figure 1. Peptide mixture elution profile on C18 column (RP-LC). MP1= A: 50 mM NH_4OAc in water + 0.5% AcOH (pH 4.45), B: MeOH + 0.5% AcOH. Linear gradient 30-80% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

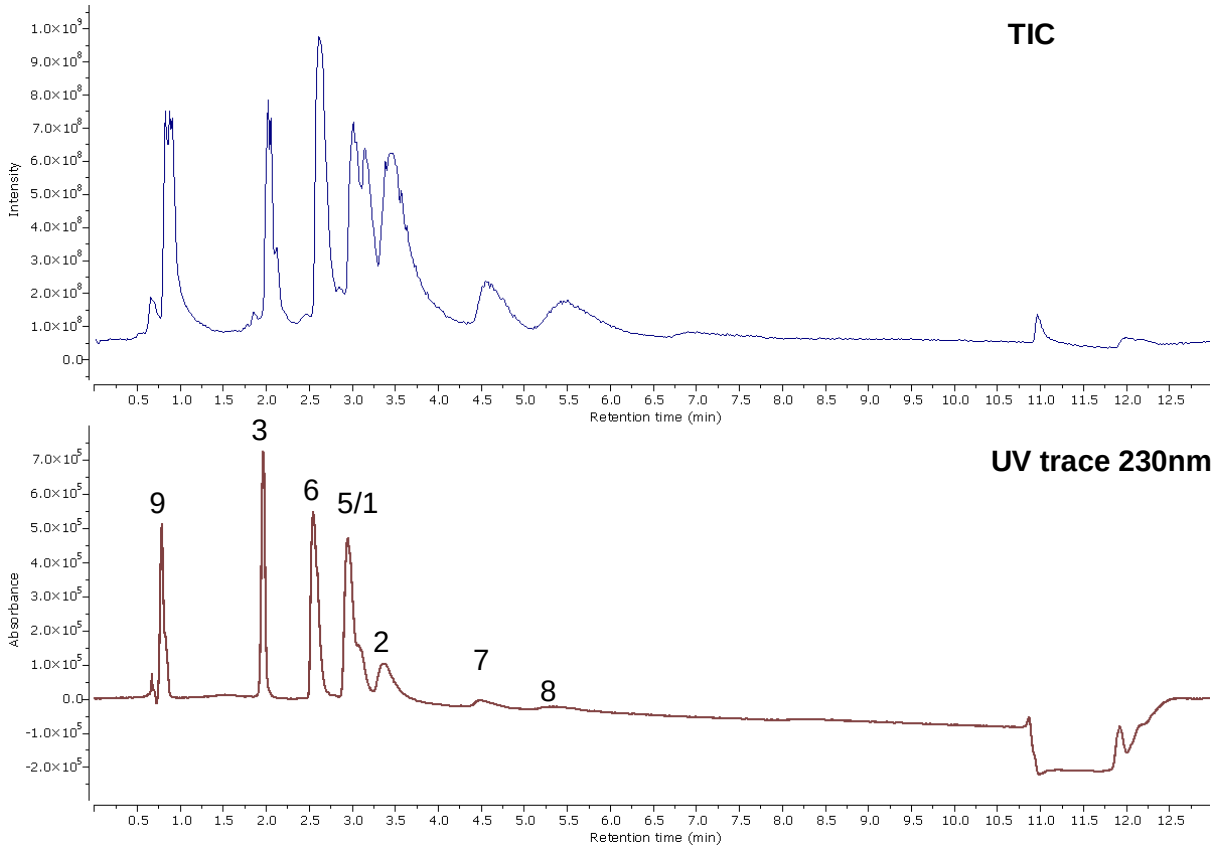


Figure 2. Peptide mixture elution profile on Amide column. MP1= A: 50 mM NH_4OAc in water + 0.5% AcOH (pH 4.45), B: MeOH + 0.5% AcOH. Linear gradient 100-80% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

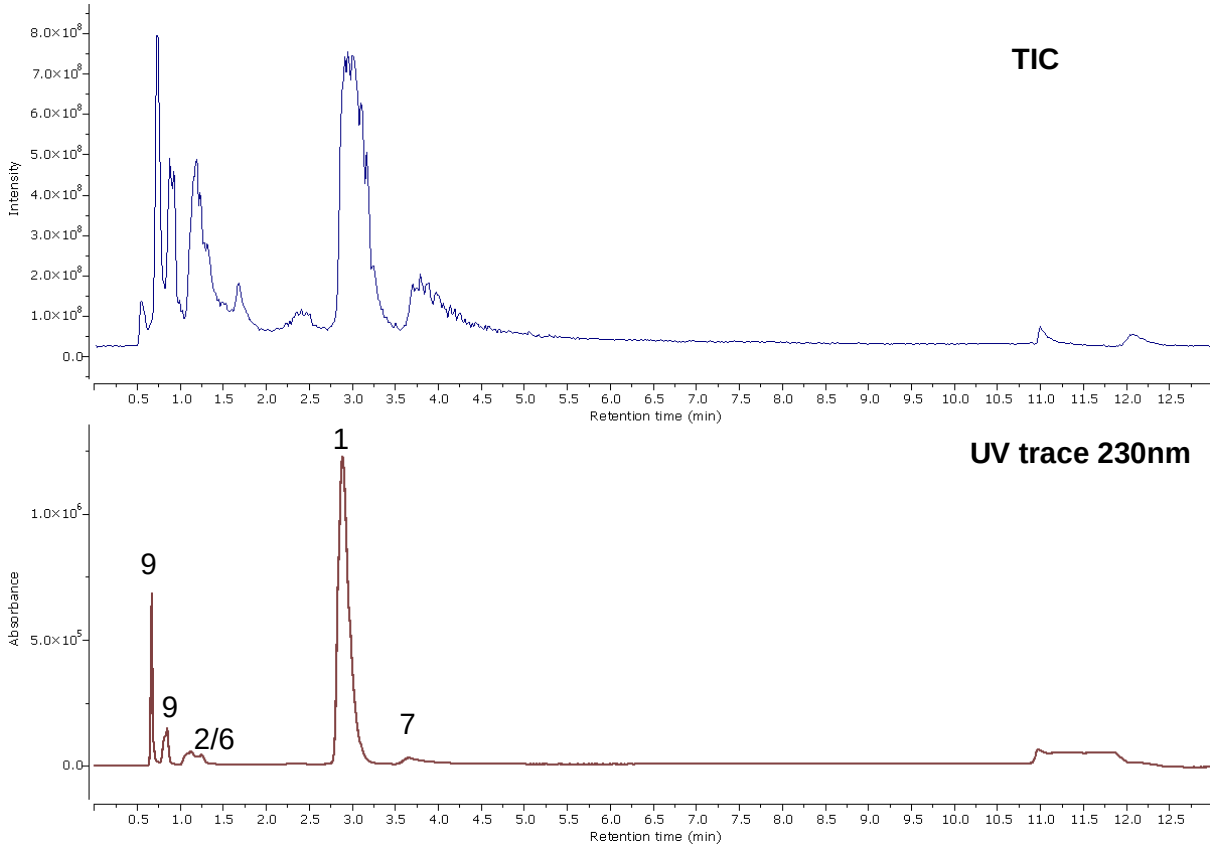


Figure 3. Peptide mixture elution profile on PMPC column. MP2= A: 50 mM NH_4OAc in water (pH 6.90), B: MeOH. Linear gradient 95-80% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

From the screening experiment, we could see different elution profiles on HILIC columns compared to RP (see Figure 2-3 compared with Figure 1). The elution orders of selected peptides were inverted, which was consistent with what was often found in the literature: RP is known to have stronger retention to more hydrophobic analytes, while the opposite is typically true for HILIC.[7, 11] Furthermore, some results highlight that HILIC exhibits complementary selectivity to RP: when looking at the for example, peptide 4 and 7 co-eluted on RP-LC (Figure 1) while well separated on HILIC columns (Figure 2-3, Amide and PMPC columns respectively). This result suggest a potential complementarity between RP-LC and HILIC, hinting at possible improvement in preparative productivity and purity of a peptide mixture when combining the techniques.[17]

Among all tested HILIC columns, the Amide column (Figure 2) gave the most comparable performance with RP in terms of number of peaks eluted and resolution. On contrary, PTZ and P4VP provided the least peptides eluted at the tested conditions (see Appendix, Figure A2-A6). This result is aligned with previous screening experiment using ACN as organic phase (unpublished data).

When comparing MeOH with ACN on the same columns, a change in elution order of the peptides is observed (see Peptides 2 and 7 in Figure 4 and Peptides 2 and 1 in Figure 5). This suggests that ACN and MeOH might not only possess distinct elution strengths, but also different selectivity; this could be connected to their molecular structure, hence, the type of intermolecular interaction established with the target peptides. In addition, when using MeOH a general early-

elution trend has been observed among the peptides; therefore, a shallower gradient was typically used than with ACN.

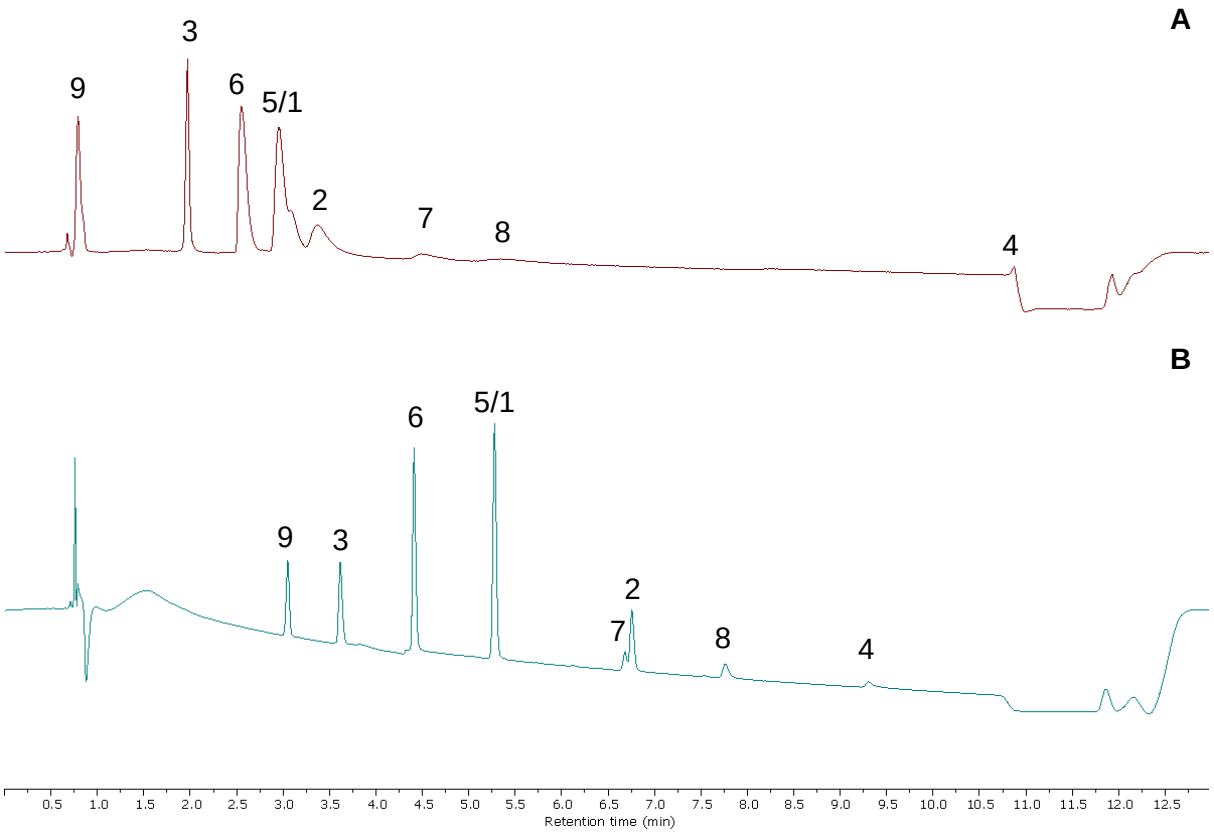


Figure 4. Peptide mixture elution profile on Amide column using MeOH (A) and ACN (B). **A**: MP1= A: 50 mM NH_4OAc in water + 0.5% AcOH (pH 4.45), B: MeOH + 0.5% AcOH. Linear gradient 100-80% B in 10 min. **B**: MP= A: 50 mM NH_4OAc in water + 0.5% AcOH (pH 4.45), B: ACN/H_2O 95/5 + 0.5% AcOH. Linear gradient 100-55% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

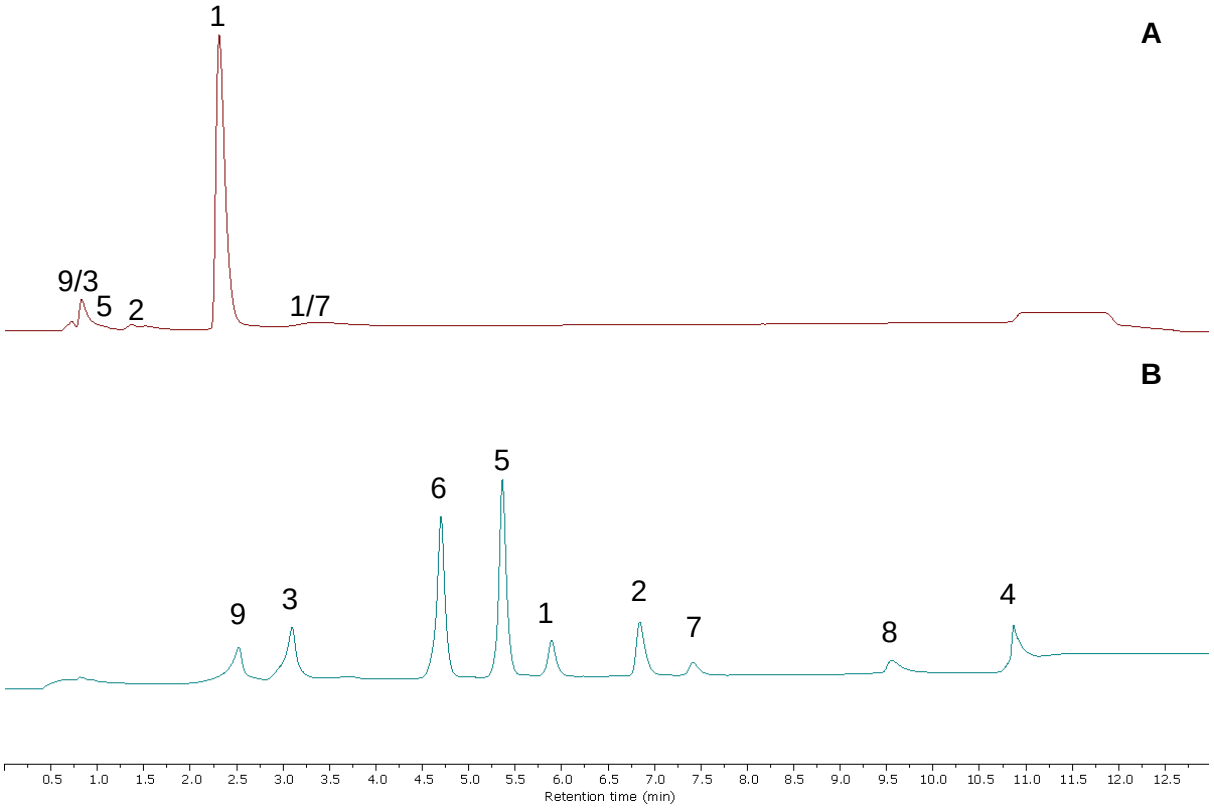


Figure 5. Peptide mixture elution profile on Luna Polar Pesticide column using MeOH (top) and ACN (down). **A**: MP1= A: 50 mM NH_4OAc in water (pH 6.9), B: MeOH. Linear gradient 100-70% B in 10 min. **B**: MP= A: 50 mM NH_4OAc in water (pH 6.6), B: ACN/H_2O 95/5. Linear gradient 95-60% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

The distinction in properties between MeOH and ACN can be summarized using Snyder´s solvent-selectivity triangle (see Figure 6), which classifies eluents based on three dominant interactions parameters: “hydrogen atom donor (HBD)”, “hydrogen atom acceptor (HBA)”, and “polarizability”. Practically, replacing an ACN-water mixture with an isoeleutropic MeOH-water mixture - as attempted in this study - increases the MP HBD and lowers its polarisability relative to ACN.[7] Consequently, aromatic/polarisable compounds tend to elute earlier in MeOH-water mixtures at the same elution strength.[7] Moreover, hydrogen-bonding between analytes and the polar stationary phases is more effectively disrupted,, leading to earlier elution of peptides with high hydrogen-bonding potential.

Nonetheless, in HILIC, MeOH performed worse than ACN. Most HILIC runs with MeOH as the organic solvent (see Appendix) failed to match RP-LC in separation and efficiency, whereas HILIC with ACN (see Figures 4–5, bottom) delivered comparable performance.
This outcome is considered to arise from differences in the solvents’ chemical properties, as mentioned earlier. In HILIC, a water-rich layer forms on the polar SP, and analytes are partitioned between this layer and the bulk organic phase[11]. With ACN, due to high dipolarity and weak hydrogen-bond donation, the interfacial water layer is thought to be typically thicker and more structured; consequently, differences in peptide polarity and hydrogen-bonding capacity could be enhanced and resolution increased. By contrast, MeOH´s HBD character tends to disrupt the water layer and to compete for polar sites on both the stationary phase and the analytes [7]. As a result, partitioning contrasts and selectivity between peptides are reduced.

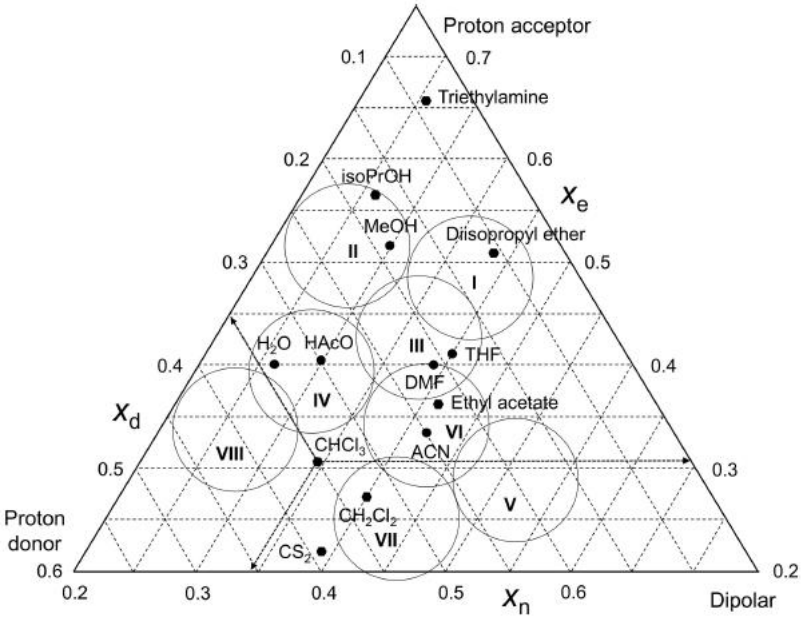


Figure 6. Snyder´s solvent selectivity triangle, based on donation of hydrogen ions, acceptance of hydrogen ions, and dipolarity.[7]

Lastly, it´s important to mention than HILIC runs typically exhibited broader peaks and increased tailing than RP (e.g. Figure A29), possibly due to the shallower gradient; however, the band broadening might be caused by mixed retention mechanisms as well[11].

4.1.2 Evaluation of HILIC methods for the separation of peptide degradation impurities

Three peptides were subjected to force-degradation experiments, and the resulted samples were analysed on both RP-LC C18 column and HILIC Amide column. The identities of the degradation impurities were confirmed with high-resolution mass spectrometry (Figure A14-A25 in the Appendix).

The basic degradation condition was confirmed to be the harshest for the tested peptides, it produced several impurities at room temperature. Typically, the acidic and basic conditions triggered the formation of impurities deriving from deamidation and/or hydrolysis, while using H_2O_2 resulted in mono and di-oxidation of the original peptide (see Figure A24 in Appendix).

Ultimately, an inversion of elution between the impurities (i.e. deamidation sites) and the target peptides can be observed between HILIC and RP (see Figure 7).

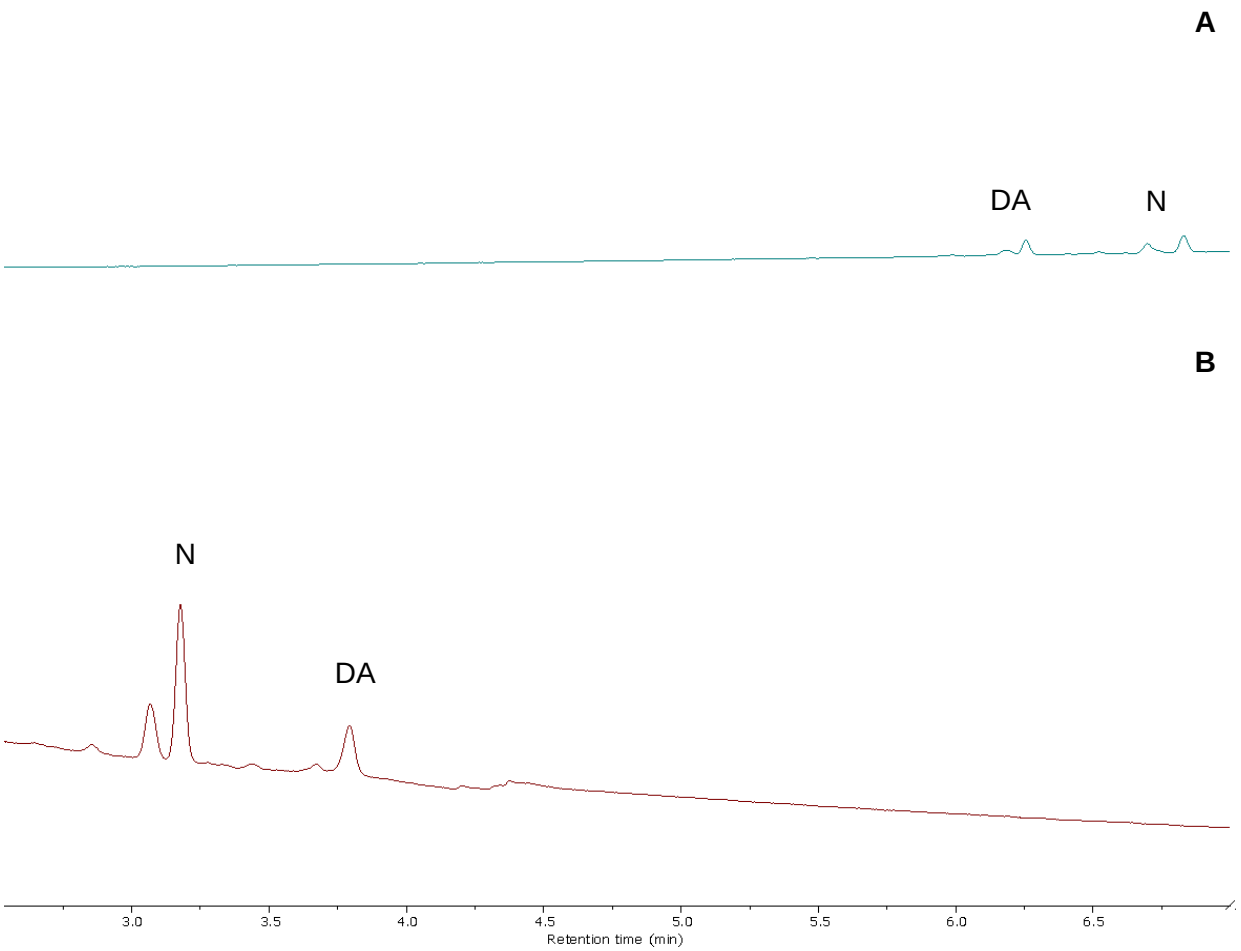


Figure 7. Elution profile of degraded Carbetocin (basic conditions) on C18-RP (**A**) and Amide-HILIC (**B**) columns using ACN. UV trace at 230 nm. "N" indicates the Native state of the peptide, while "DA" refers to the deamidated species.

Further experiments are necessary to assess HILIC's potential as a complementary, selective mode to RP-LC for separating degradation-related impurities. Regarding oxidation products of the tested peptides, the data does not provide ground for a solid conclusion on the potential of HILIC for the separation of these impurities from the native peptide (see Figure A26-A34 in the Appendix).

4.1.3 Evaluation of HILIC methods for the separation of peptide synthesis-related impurities

To assess the application of HILIC in separating synthesis-derived impurities, four crudes (see Table 7) from AstraZeneca were analysed using both RP and HILIC modes.

The crudes presented impurities mostly related to deletion involved in their synthesis, which were observed both in RP and HILIC, as well as when using MeOH or ACN (see Appendix, Figure A35-A50). These impurities were identified and correlated to specific amino acids in the crude peptides' sequences. For example, in the MS spectra of *Crude A*, an impurity corresponding to a loss of around 128 Dalton from the original mass was identified; this indicates the loss of either a Lysine (K) or Glutamine (Q) residue, which are both present in the sequence of the peptide in question.

Overall, in several instances HILIC has been proved capable to separate the deletion impurities

from the native peptide, where RP failed to do so. This is particularly evident when ACN was used as the organic solvent: in three out of the four crude peptides (see Figure 8, Figure A36, A40 and A44), HILIC succeeded in resolving deletion impurities, suggesting a complementary selectivity between HILIC and RP as chromatographic modalities. Nonetheless, further experiments involving a larger number of crudes and chromatographic conditions are required to confirm this theory.

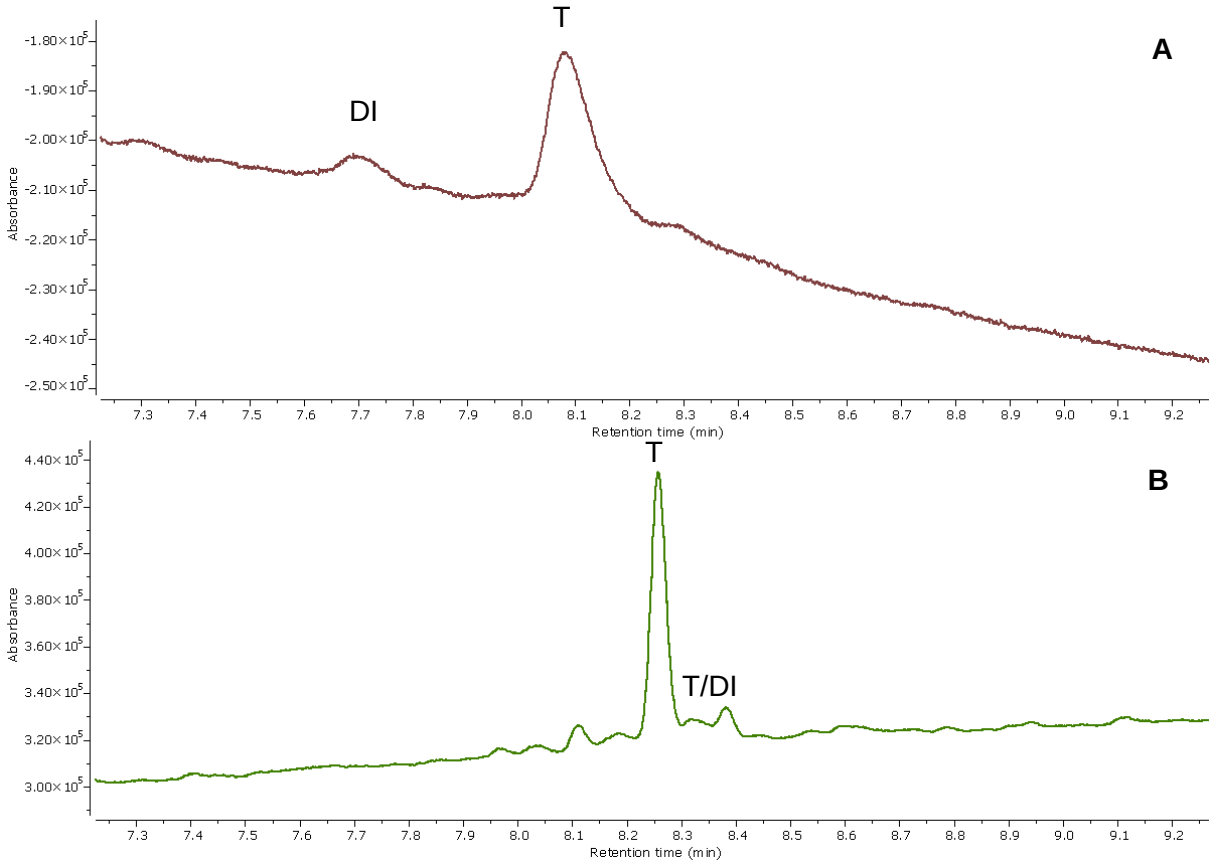


Figure 8. Comparative chromatograms of *Crude A* analysed by HILIC (**A**: upper, red trace) and RP-LC (**B**: lower, green trace) at 230 nm using ACN. For the complete chromatographic profiles, see the full-range spectra in Appendix Figures A26 and A27. "T" stands for target peptide; "DI" stands for deletion impurity; "T/D" indicates co-elution of the fractions.

4.2 Evaluation of SFC for peptide analysis

Nine peptides were tested on seven distinct SFC columns across three different acidic additives. Kromasil-CN, GreenSep Basic and GreenSep FluoroBasic columns have achieved the best results: in most cases, peptides exhibit sufficient retention to elute as well-defined peaks during the gradient, without excessive band broadening. This seems to be the case across all three mobile phases. To further investigate these differences, the analysis of the results was divided into three sections, each exploring a different aspect of the experiment: additive effects, peptide chemistry, column chemistry.

4.2.1 Additives: apparent pH, ion pairing effect, polarity

In this section, several properties of the tested additives will be compared and associated to the resulting effect on the peptides' retention and peak shape.

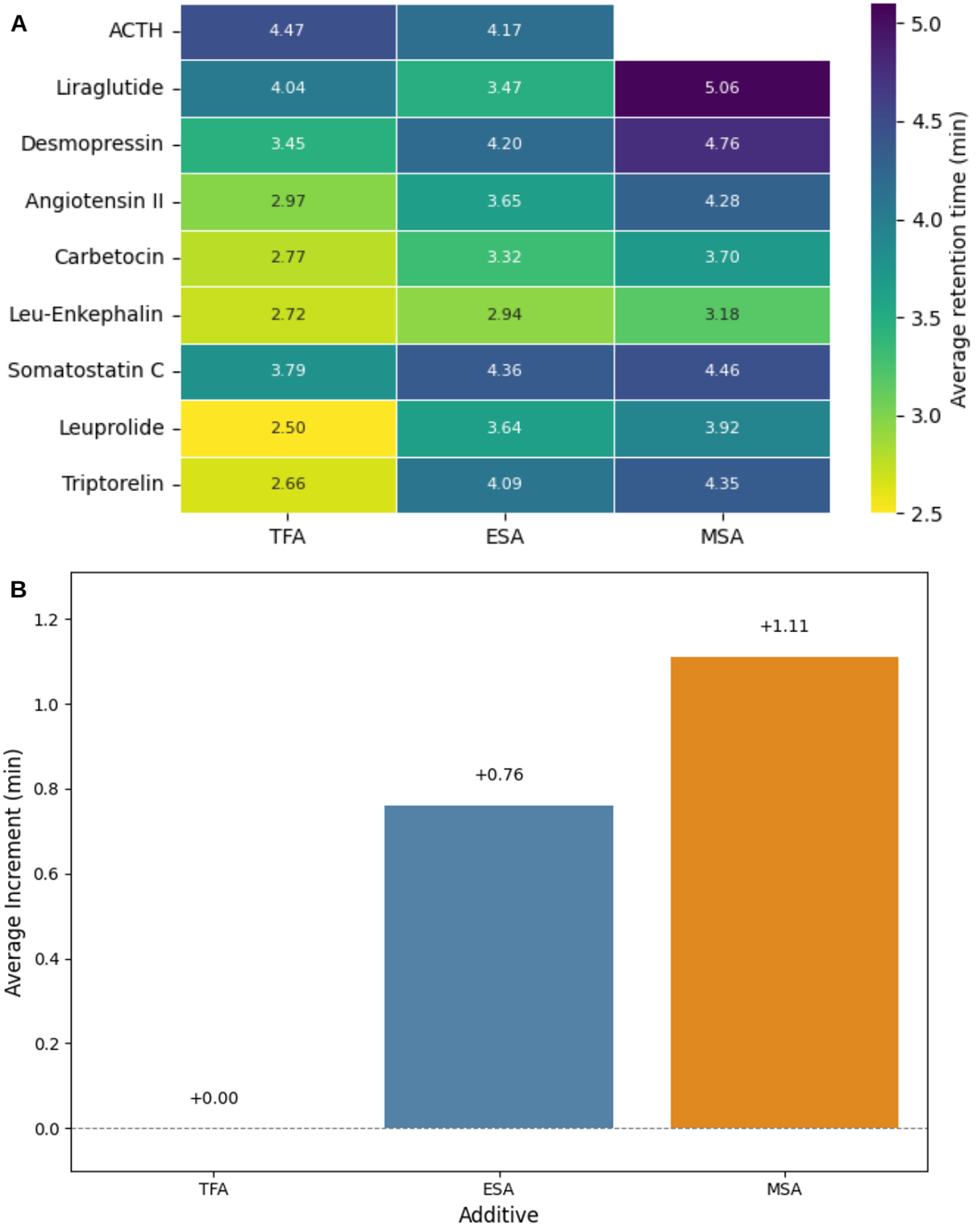


Figure 9. **A:** Heat map of average peptide retention times with colour-scaled ranking. The table summarises retention time (minutes) for a panel of peptides. Cells display the observed average retention time; blank cells indicate absent or non-integrable peaks under the tested condition. **B:** Overall average retention time increment by additive (relative to TFA)

Based on **Figure 9**, a generic pattern in the elution of the peptides is identifiable: in 7 out of the 9 peptides (ACTH and Liraglutide excluded) the average retention time across columns

increases in this order: TFA < ESA < MSA. The observed changes in retention can be attributed to differences in ion-pair complexation constants. In addition, the higher polarity of MSA may influence the partitioning of the ion-paired peptide into the apolar CO_2-rich mobile phase; once the ion pair forms, this effect would increase retention relative to the slightly more hydrophobic ESA.[18] The same reasoning is applicable to the comparison between ESA and TFA.

Despite their contrast in polarity and pKa, the apparent pH of the mobile phase is seemingly equally affected by the acidic additives examined. In fact, if acidic additives such as TFA and MSA are added on top of the organic modifiers, it has been demonstrated that the apparent pH of the mobile phase is close to aqueous pH 1 or even below.[13] As both TFA and MSA cluster around the same pH value, it´s reasonable to assume that the same result would be obtained using ESA (see pKa values in Table A2 in 7.5 Supplemental material). Thus, since TFA, MSA and ESA at similar concentration can create a comparable strong acidic microenvironment, a matching control of the analyte protonation might be expected; meaning that the different pKa values of the additives might have negligible effect on the retention behaviour of the peptides at the tested conditions.

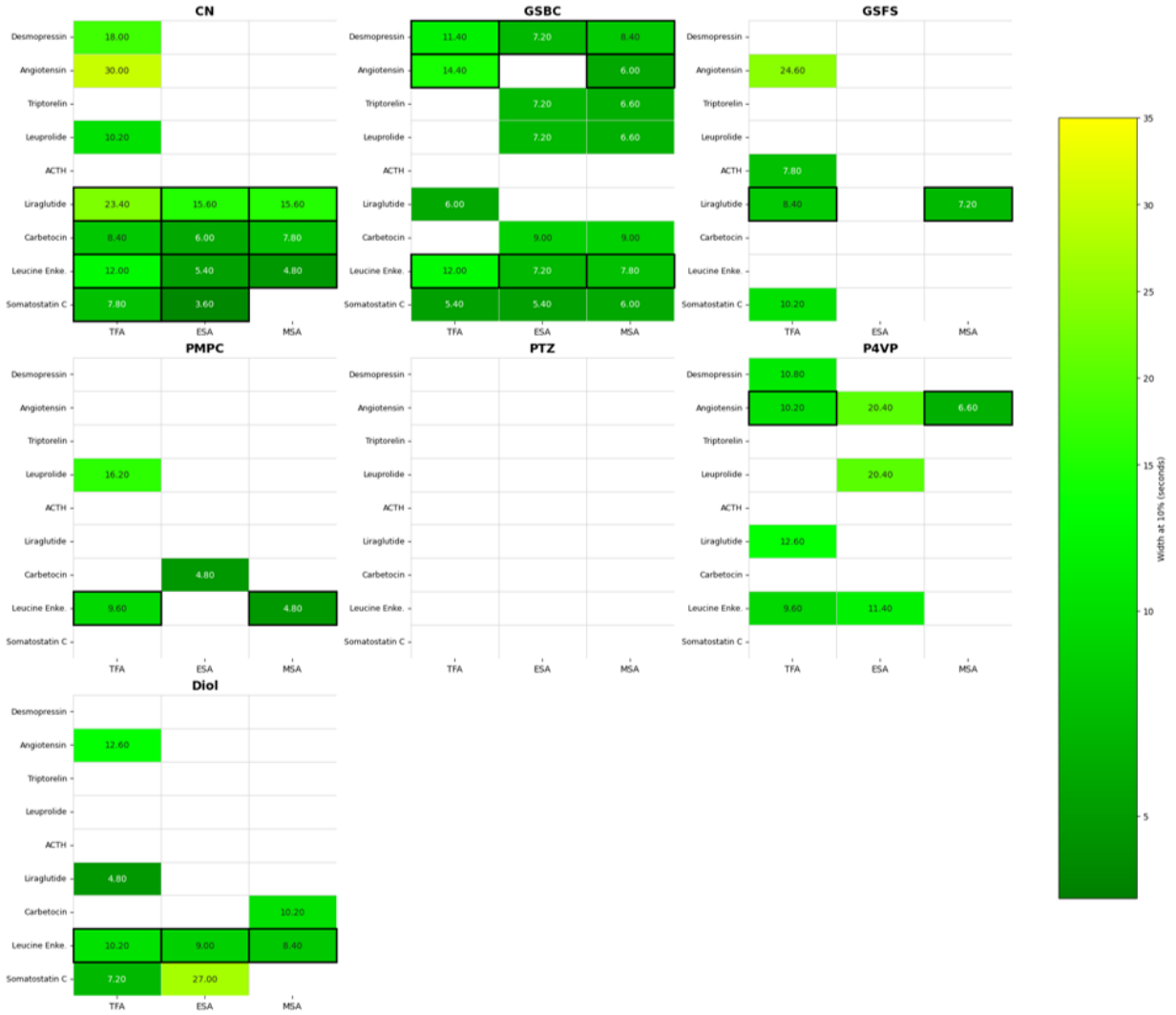


Figure 10. Peak width at 10% height across additives in CN/GSBC/GSFS (Heat Map). Black frames highlight cells where TFA > ESA/MSA. Blank cells indicate absent or non-integrable peaks under the tested condition.

Figure 11. Peak asymmetry at 10% height across additives in CN/GSBC. Blank cells indicate absent or non-integrable peaks under the tested condition.

The impact of additives on peak shape was investigated (see Figure 10-11).Across all conditions, peak asymmetry variation was partially consistent with the peak width: improvements in asymmetry often coincided with narrower peaks, especially on neutral polar phases (CN, Diol) with ESA/MSA. Overall, moving from TFA to ESA and MSA, peak shape was generally improved for many peptide-columns pairs. TFA showed more cases of broader peaks, possibly due to its strong ion-pairing nature: it could increase apparent hydrophobicity and alter charge distribution, as mentioned earlier. On polar/cationic phases this can introduce mixed-mode retention (partition + adsorption + electrostatics), which often yields tailing due to a distribution of interaction strengths and slow desorption kinetics.[13]

These results indicate that additive selection can affect peak shape in SFC; however, the present dataset is insufficient to establish a general conclusion across peptides and stationary phases. Ultimately, the choice of the acidic additive seems to impact the retention time of peptides by means of ion pairing and differences in polarity, while pKa variation might be negligible among the tested strong acids.

4.2.2 Peptides chemistry: isoelectric point and amino acidic sequence

Next, I examined how peptide chemistry influences retention time and peak shape. Several physicochemical properties (see Figure 12) were analysed relative to the number of successful elution conditions for each peptide, using Pearson and Spearman correlations. Successful elution conditions were defined as the chromatographic conditions having a certain peptide eluted within the gradient window without peak splitting and excessive broadening. The similar value calculated for Pearson and Spearman coefficients indicates that these relationships are robust across the peptide set.

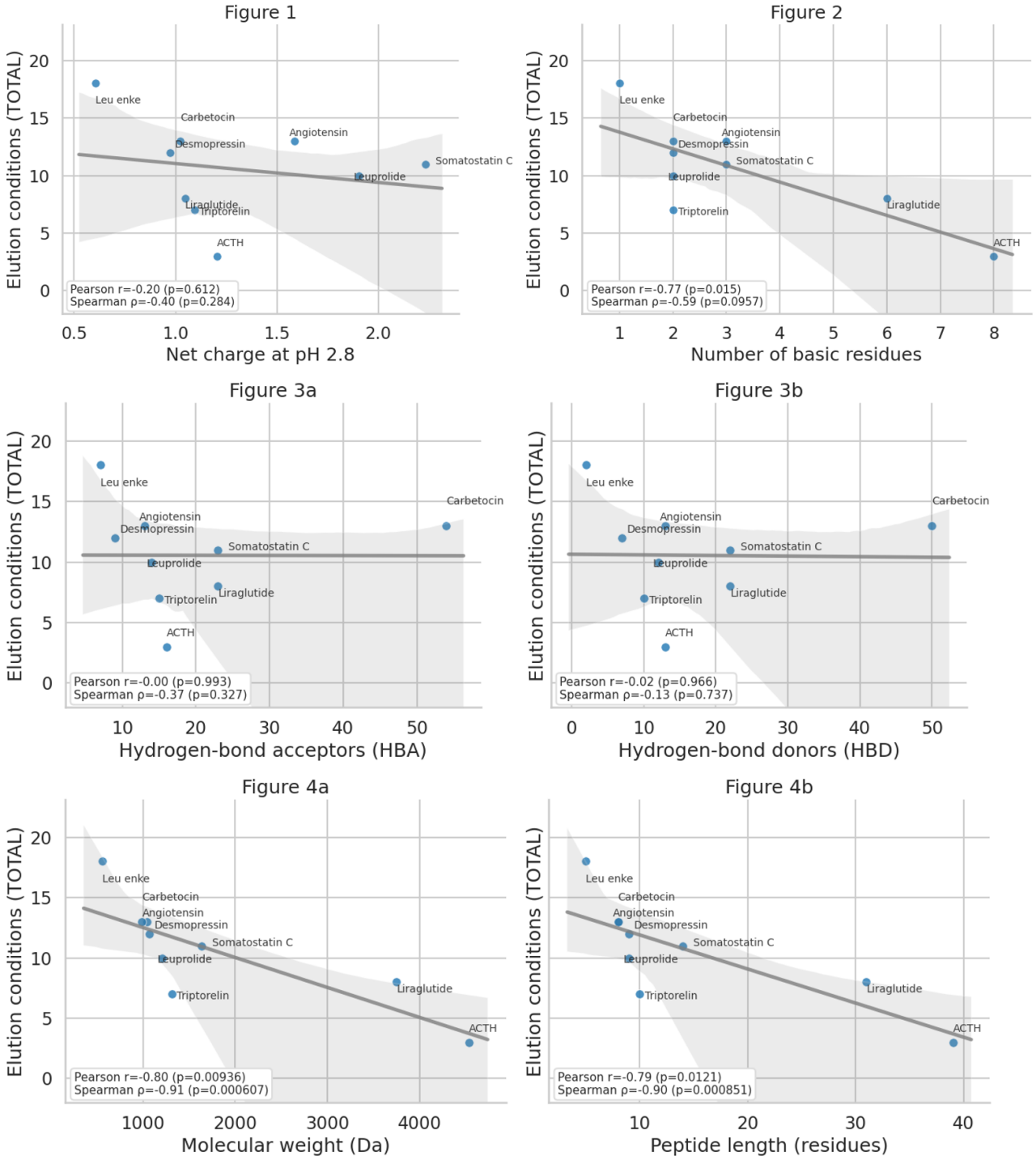


Figure 12. Pearson and Spearman correlation analyses between peptide physicochemical properties and elution time.

Among the evaluated descriptors, peptide size demonstrated the strongest relationship with the total number of successful elution conditions. Both molecular weight and peptide length showed significant negative correlations with elution performance, indicating that larger and longer peptides tended to elute under fewer conditions. Larger peptides such as ACTH and Liraglutide were associated with fewer elution conditions; on the contrary, Leucine Enkephalin and Carbetocin - the smallest peptides in the panel - exhibited good elution profile in most conditions. This trend suggests that SFC is more promising in analysing smaller peptides, potentially due to large peptides could have stronger interactions with the stationary phase or reduced kinetic of adsorption within the chromatographic system.

A strong negative correlation was observed between the number of basic residues and the total number of successful elution conditions as well. Peptides containing a greater number of basic amino acids (e.g. ACTH, Liraglutide) generally exhibited reduced elution across the tested conditions. Basic residues are likely to present a positive charge under the tested acidic conditions (apparent pH ≤ 1) and might enhance detrimental electrostatic interactions with the SP, resulting in altered retention.

In contrast, net charge at pH 2.8 showed only a weak and non-significant correlation with elution behavior. Although charge is often considered a major determinant of chromatographic retention, the lack of a significant relationship suggests that overall peptide charge alone is insufficient to explain the observed differences in elution. Instead, specific structural and compositional characteristics, such as peptide length and the distribution of basic residues, might exert a stronger influence. A weak correlation was determined for hydrogen-bond donor and acceptor capabilities, indicating that hydrogen-bonding potential is not a primary factor governing peptide retention under the experimental conditions.

4.2.3 Columns chemistry: functional groups and charge state of the stationary phase ligands

Across seven SFC stationary phases (see Table 3), peptide retention potentially reflects the balance of overall protonation at apparent pH ≈ 1 or below, hydrophobic/aromatic content, and hydrogen-bonding capacity with the ligands. Despite potential electrostatic repulsion between protonated stationary phases and highly cationic peptides, ACTH and liraglutide display prolonged retention potentially because of extensive polar interactions (hydrogen bonding and dipolar) that effectively anchor them to both charged and neutral polar ligands; these interactions could outweigh simple charge–charge effects. The dominance of these polar interactions seems to be supported by the minimal TFA-dependent retention shifts for ACTH and liraglutide (Table 6), indicating that strong ion pairing might not readily disrupt the interaction network.

In contrast, close to neutral peptides (e.g. Leucine enkephalin, Carbetocin) display consistent elution across phases, possibly indicating limited electrostatic interactions and a greater reliance on hydrophobic and π interactions.

Among neutral SPs, CN performs better than diol across MPs. On Diol, strong retention of protonated peptides is observed when using ESA and MSA. This elution profile might be due to strong interactions between the Diol column and the peptides. The diol ligand can engage in ion–dipole interactions with protonated peptide residues. In addition, its multiple hydrogen-bonding sites promote extensive polar binding, which may hinder mass transfer and cause band

broadening and smearing. TFA reduces this tendency (Figure 9A), likely by disrupting multi-site H-bonding and enhancing peptide solvation.[13] CN, being non-protic and more π/dipole-selective, might avoid this multiple interaction profile, eluting a larger fraction of peptides than Diol under the same conditions.

Overall, selectivity is likely governed by charge distribution and hydrogen-bond topology rather than nominal net charge alone. Neutral peptides elute across a wider range of conditions than peptides bearing multiple basic residues under these SFC conditions and apparent pH, potentially due to protonated residues of the peptides being involved in detrimental electrostatic interactions.

5. Conclusions

The present study evaluated HILIC and SFC as alternative and complementary chromatographic techniques to RP-LC for the analysis and purification of synthetic peptides. The practical implementation of these techniques presents a relevant knowledge gap in current research; through a systematic investigation of SP-chemistry, MP-composition, and modifier selection, this study attempted to address it.

In particular, the HILIC experiments confirmed orthogonal selectivity to RP-LC when analysing peptide crudes. Moreover, the inversion of elution order between degradation species and the target peptide was highlighted in this study. Among the evaluated conditions, the amide stationary phase combined with acetonitrile provided the broadest elution coverage and the highest impurity resolution, whereas methanol generally promoted earlier peptide elution.

On the other hand, the SFC screening showed that MP additives, SP chemistry, and peptide properties all influence retention, peak shape, and elution coverage. Sulfonic acid additives generally improved peak symmetry and reduced peak width, while TFA more frequently produced broader peaks. Kromasil CN and GSBC columns delivered the most consistent performance across the tested peptide panel, whereas PTZ performed the worst. Column phases bearing multiple protonated sites might lead to detrimental electrostatic interactions with the peptides, ultimately compromising their elution profile. Peptide size-related parameters and the number of basic residues impact peptides' elution the most: a significant negative correlation between them and the number of elution conditions was found.

From a scale-up standpoint, SFC's CO_2-rich mobile phase reduces liquid organic solvent consumption and offers environmental and safety benefits, [12] while the use of relatively "greener" solvents on HILIC columns offers a more sustainable option and downstream-processing advantages compared with modes such as NP-LC.[7]

Altogether, these findings underscore the potential of HILIC and SFC as complementary and more sustainable modes to RP-LC for the analysis and purification of synthetic peptides.

6. Future aspects

Building on these findings, future work could expand the peptide panel to include a broader range of physicochemical properties, explore additional SPs, and perform further

modifier/additive screens with controlled gradients. Since the final aim is the purification of synthesised peptides, further research could examine the scalability of the established techniques. Namely, studying the loadability of samples on HILIC and SFC columns, the effect of injection solvent on the process, as well as transferring the analytical techniques to preparative chromatography, would contribute significantly to understanding the applicability of such techniques. Additionally, testing downstream process operations such as fraction lyophilisation conditions and solvent reuse would allow comparison of the environmental and operational advantages of HILIC and SFC against RP-LC.

7. References

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8. Appendix

8.1 Evaluation of HILIC for peptide analysis: chromatograms

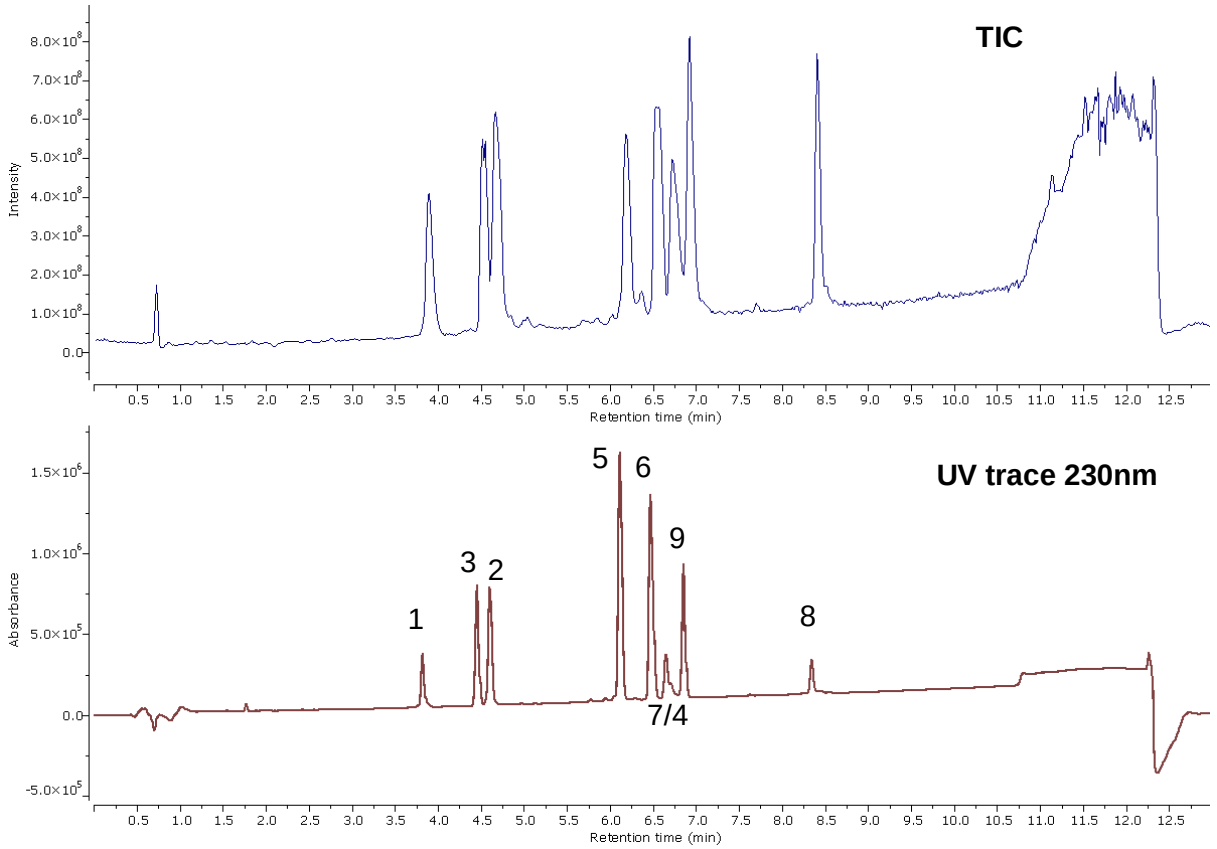

Figure A1. Peptide mixture elution profile on C18 column (RP-LC). MP1= A: 50 mM NH_4OAc in water + 0.5% AcOH (pH 4.45), B: MeOH + 0.5% AcOH. Linear gradient 30-80% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

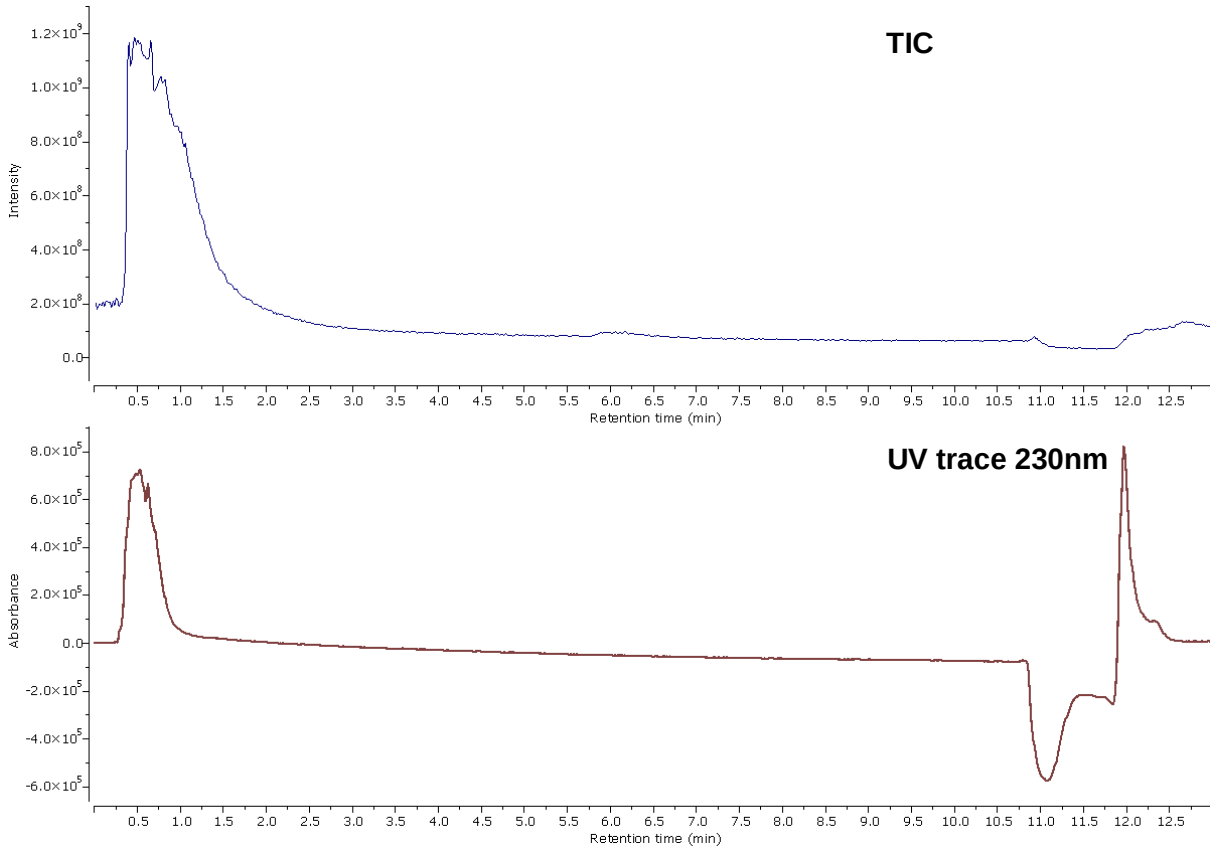

Figure A2. Peptide mixture elution profile on P4VP column. MP1= A: 50 mM NH_4OAc in water + 0.5% AcOH (pH 4.45), B: MeOH + 0.5% AcOH. Isocratic 100% for 1 min; then, linear gradient 100-80% B in 9 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1)

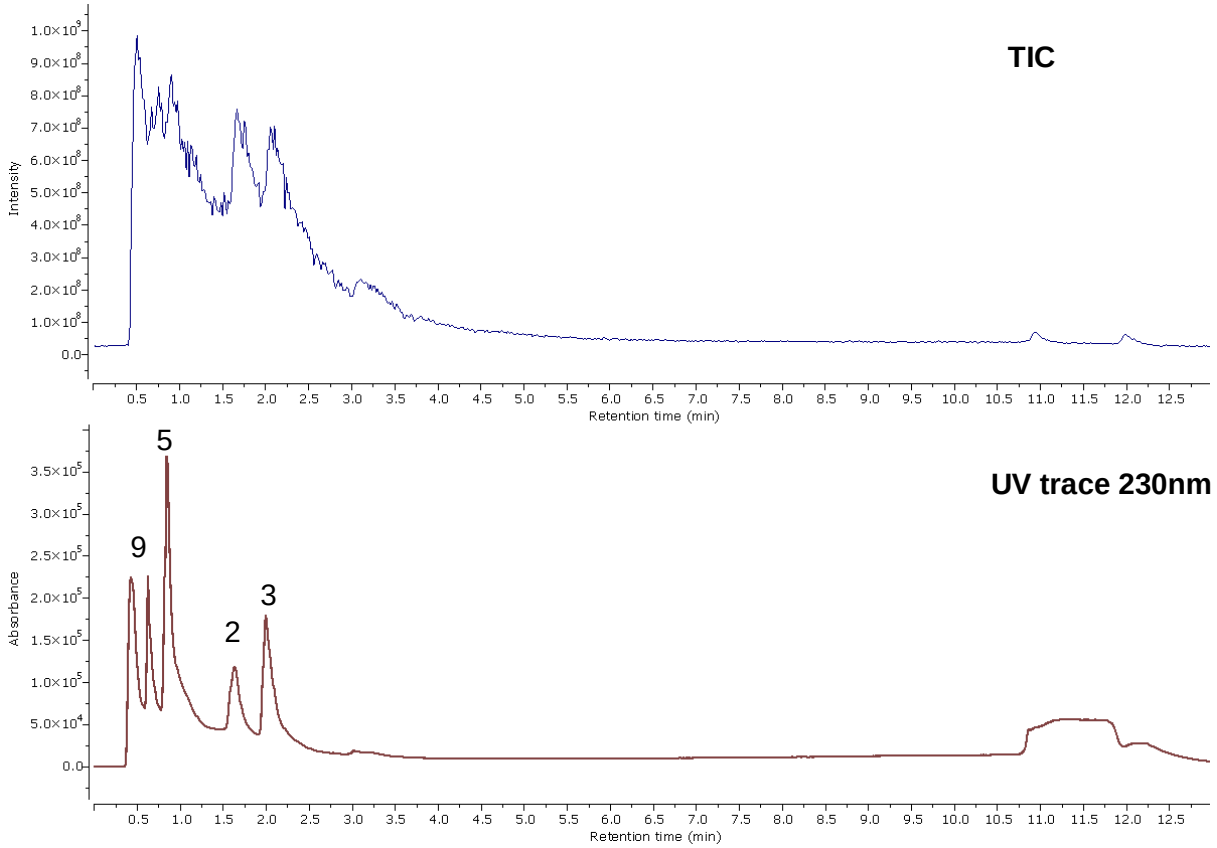

Figure A3. Peptide mixture elution profile on P4VP column. MP2= A: 50 mM NH_4OAc in water (pH 6.90), B: MeOH. Isocratic 100% for 1 min; then, linear gradient 100-80% B in 9 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

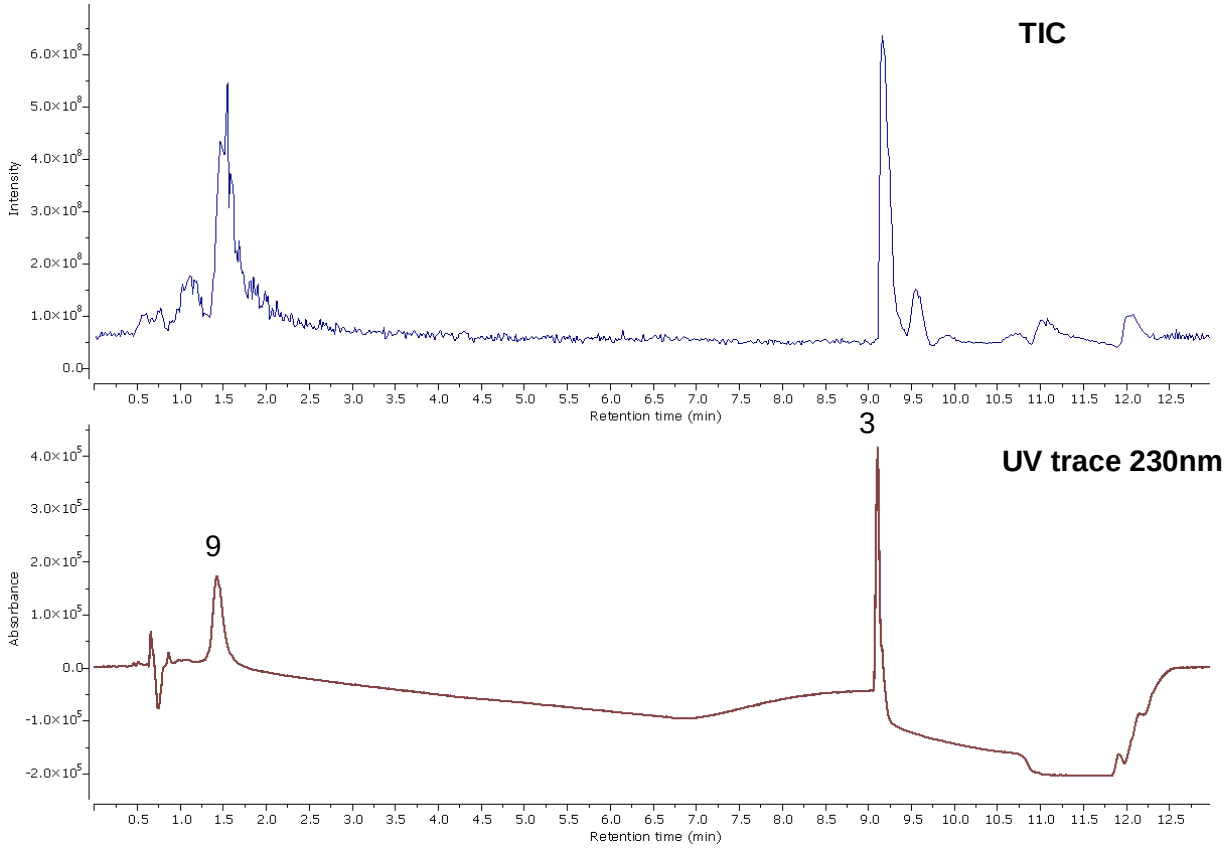

Figure A4. Peptide mixture elution profile on PTZ column. MP1= A: 50 mM NH_4OAc in water + 0.5% AcOH (pH 4.45), B: MeOH + 0.5% AcOH. Linear gradient 100-50% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

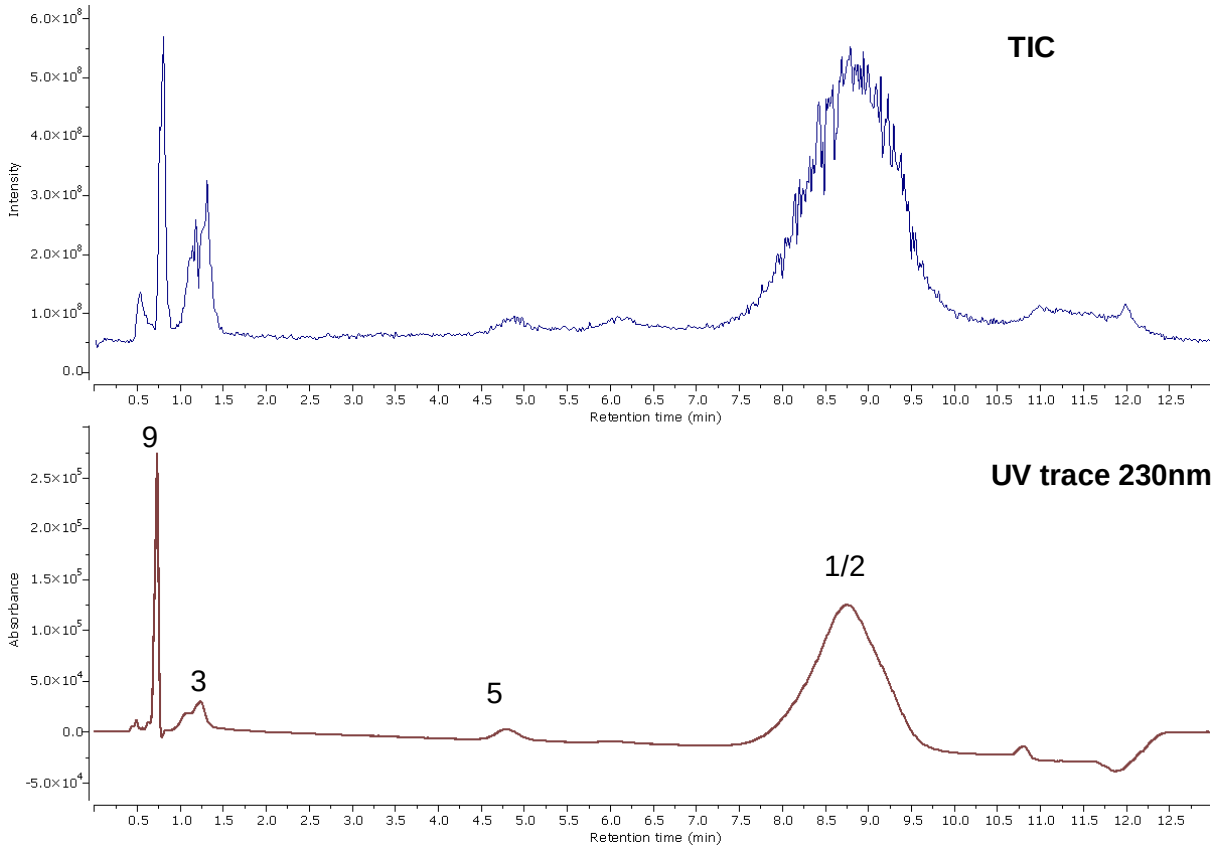

Figure A5. Peptide mixture elution profile on PTZ column. MP3= A: 20 mM NH_4OAc in water (pH 6.90), B: 20 mM NH_4OAc in MeOH. Linear gradient 100-70% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

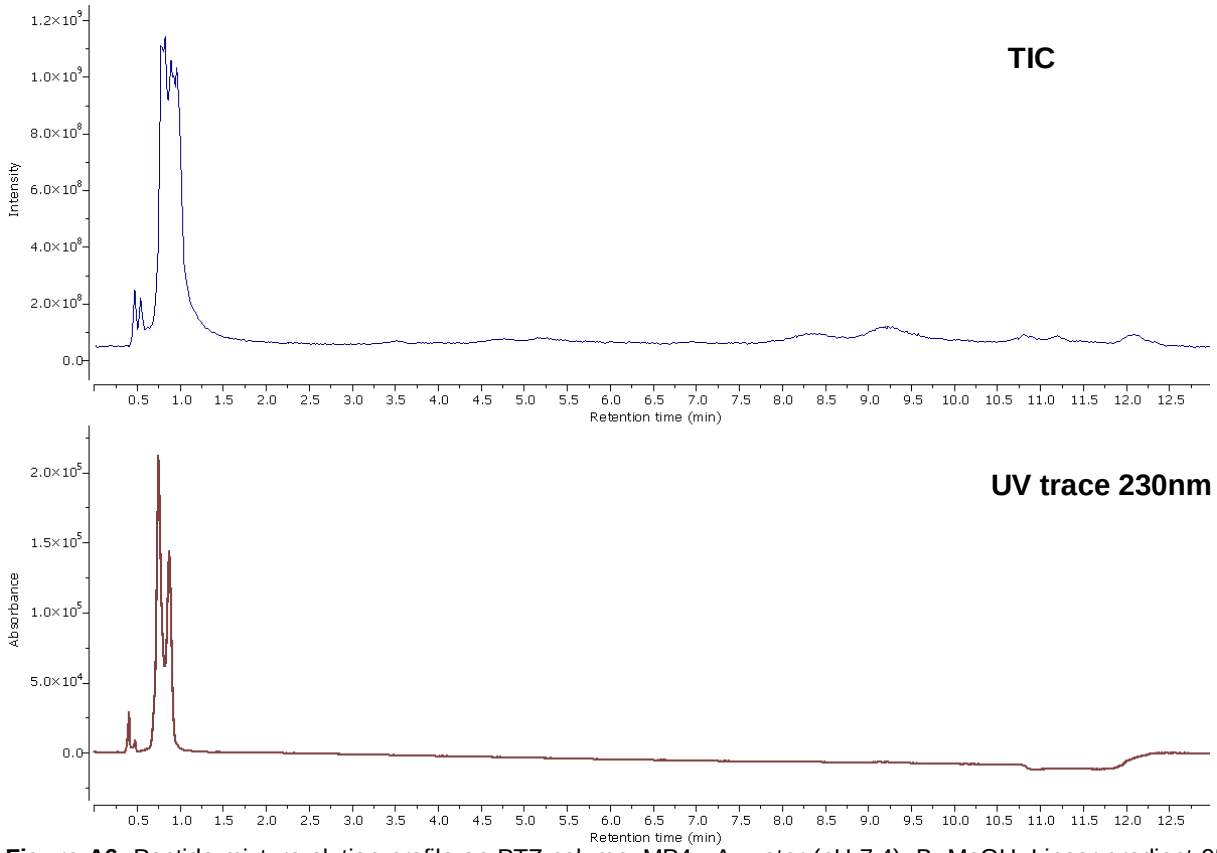

Figure A6. Peptide mixture elution profile on PTZ column. MP4= A: water (pH 7.4), B: MeOH. Linear gradient 95-70% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

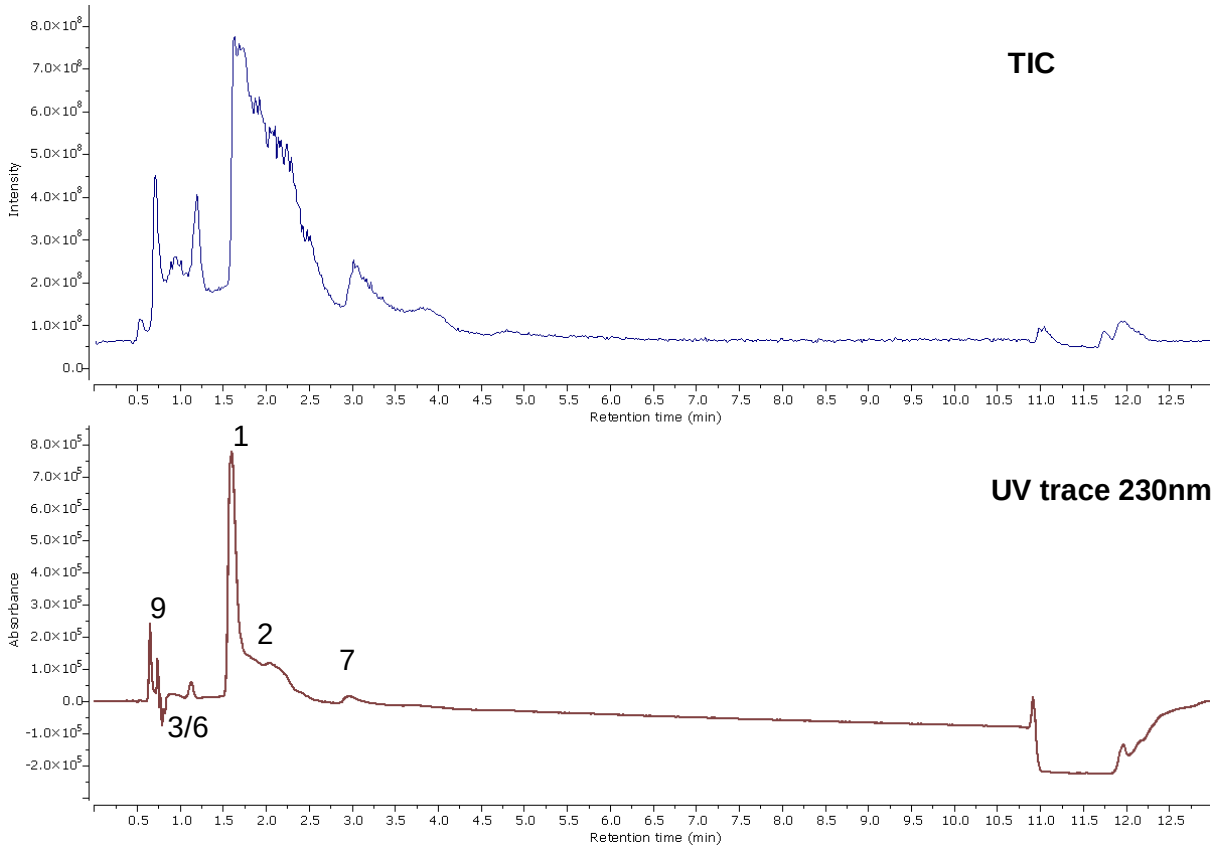

Figure A7. Peptide mixture elution profile on PMPC column. MP1= A: 50 mM NH_4OAc in water + 0.5% AcOH (pH 4.45), B: MeOH + 0.5% AcOH. Linear gradient 95-80% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

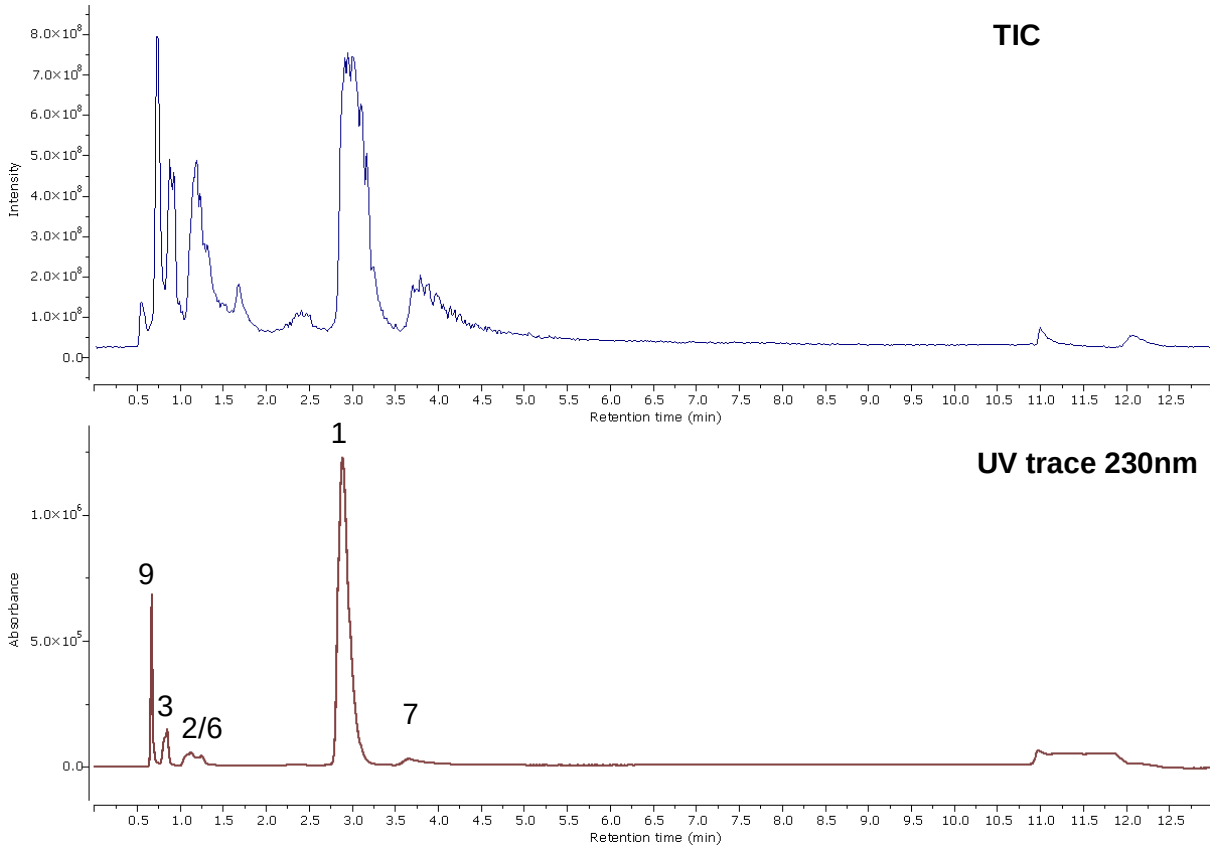

Figure A8. Peptide mixture elution profile on PMPC column. MP2= A: 50 mM NH_4OAc in water (pH 6.90), B: MeOH. Linear gradient 95-80% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

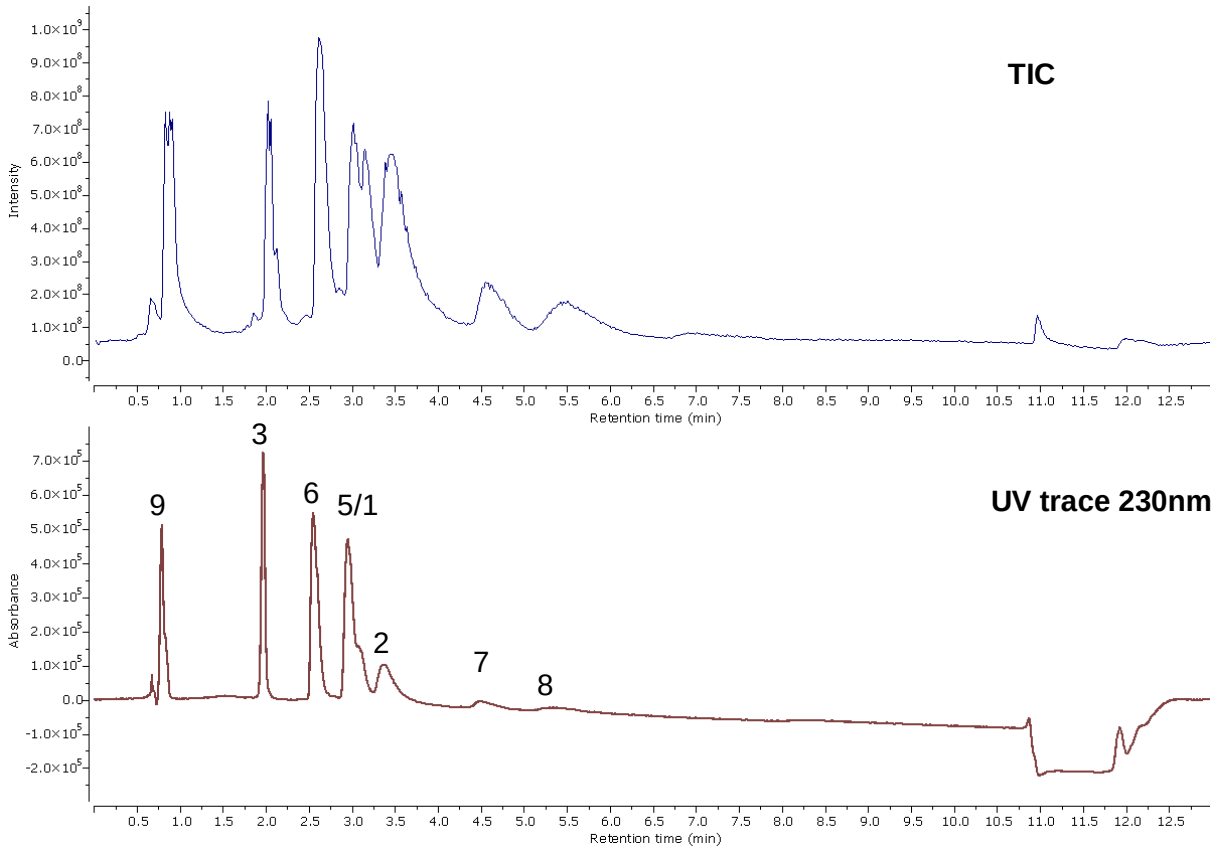

Figure A9. Peptide mixture elution profile on Amide column. MP1= A: 50 mM NH_4OAc in water + 0.5% AcOH (pH 4.45), B: MeOH + 0.5% AcOH. Linear gradient 100-80% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

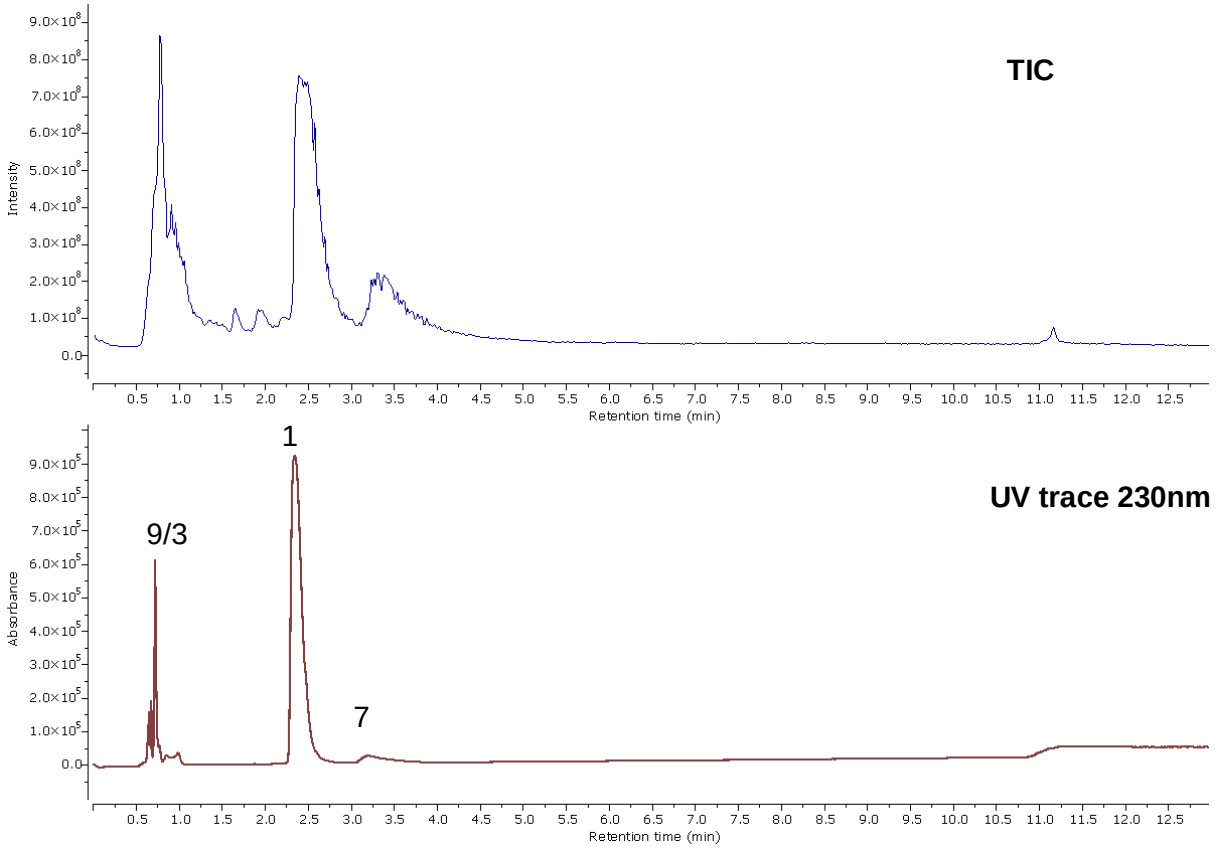

Figure A10. Peptide mixture elution profile on Amide column. MP2= A: 50 mM NH_4OAc in water (pH 6.90), B: MeOH. Linear gradient 95-70% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

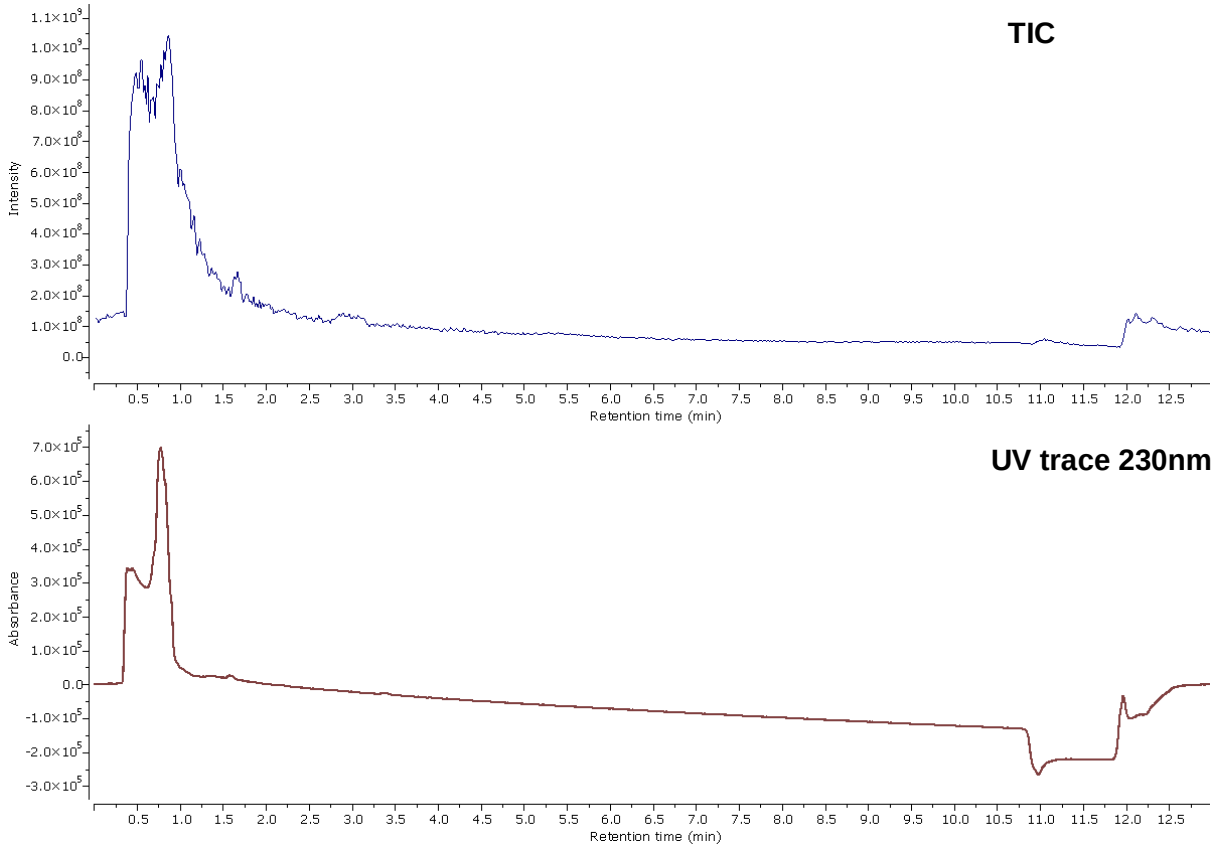

Figure A11. Peptide mixture elution profile on Luna Polar Pesticide column. MP1= A: 50 mM NH_4OAc in water + 0.5% AcOH (pH 4.45), B: MeOH + 0.5% AcOH. Linear gradient 100-80% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

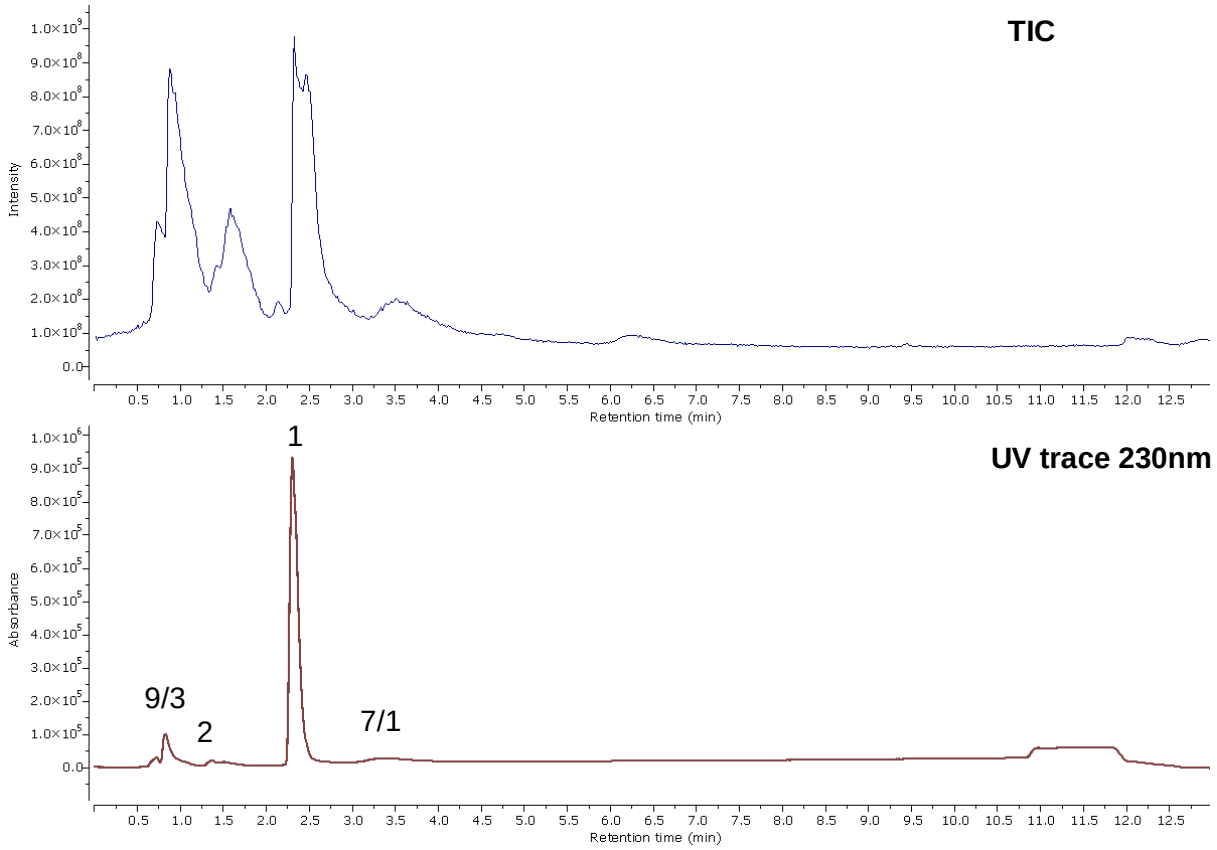

Figure A12. Peptide mixture elution profile on Luna Polar Pesticides column. MP2= A: 50 mM NH_4OAc in water (pH 6.90), B: MeOH. Linear gradient 100-70% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

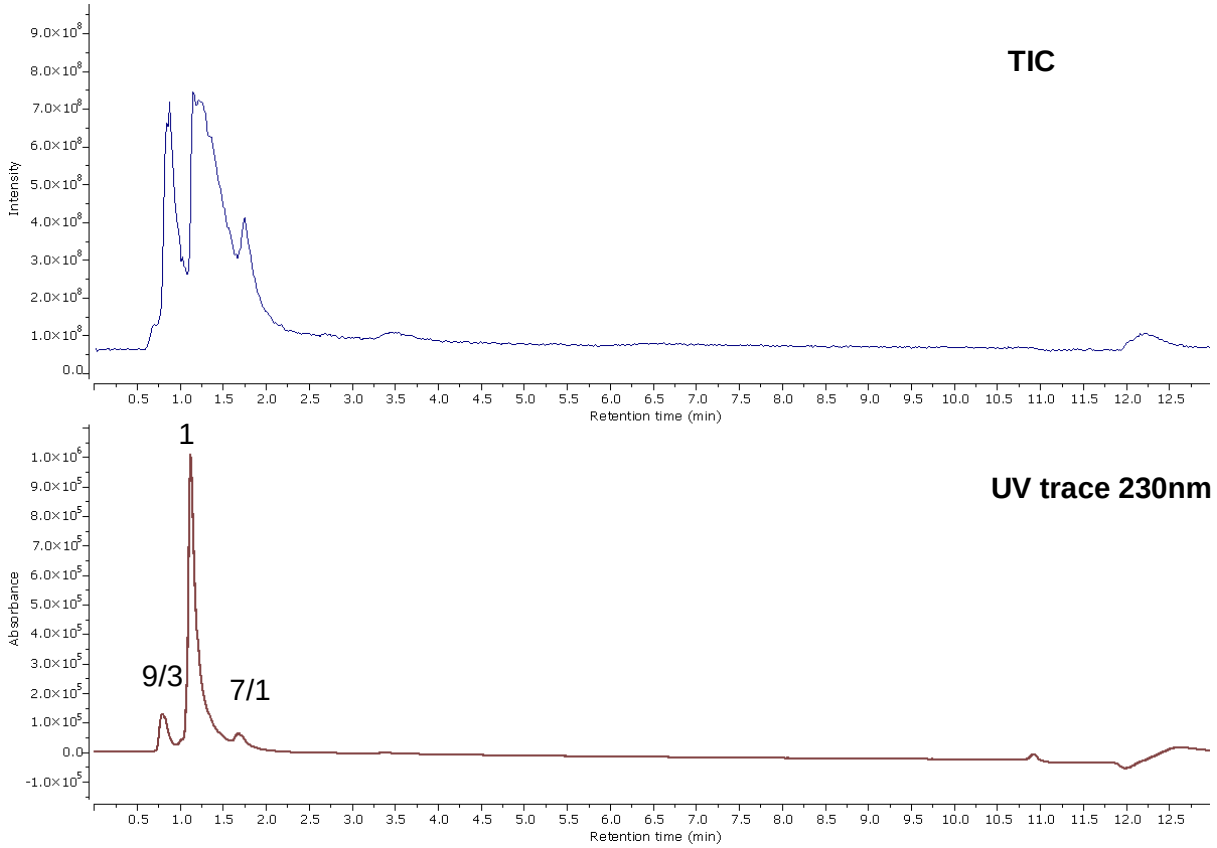

Figure A13. Peptide mixture elution profile on Luna Polar Pesticides column. MP3= A: 20 mM NH_4OAc in water (pH 6.90), B: 20 mM NH_4OAc in MeOH. Linear gradient 100-70% B in 10 min. The numbers correspond to the peptides from the reference peptide mixture (Table 1).

8.2 Evaluation of HILIC methods for the separation of peptide degradation impurities: Xevo G3 QTof HR-MS chromatograms

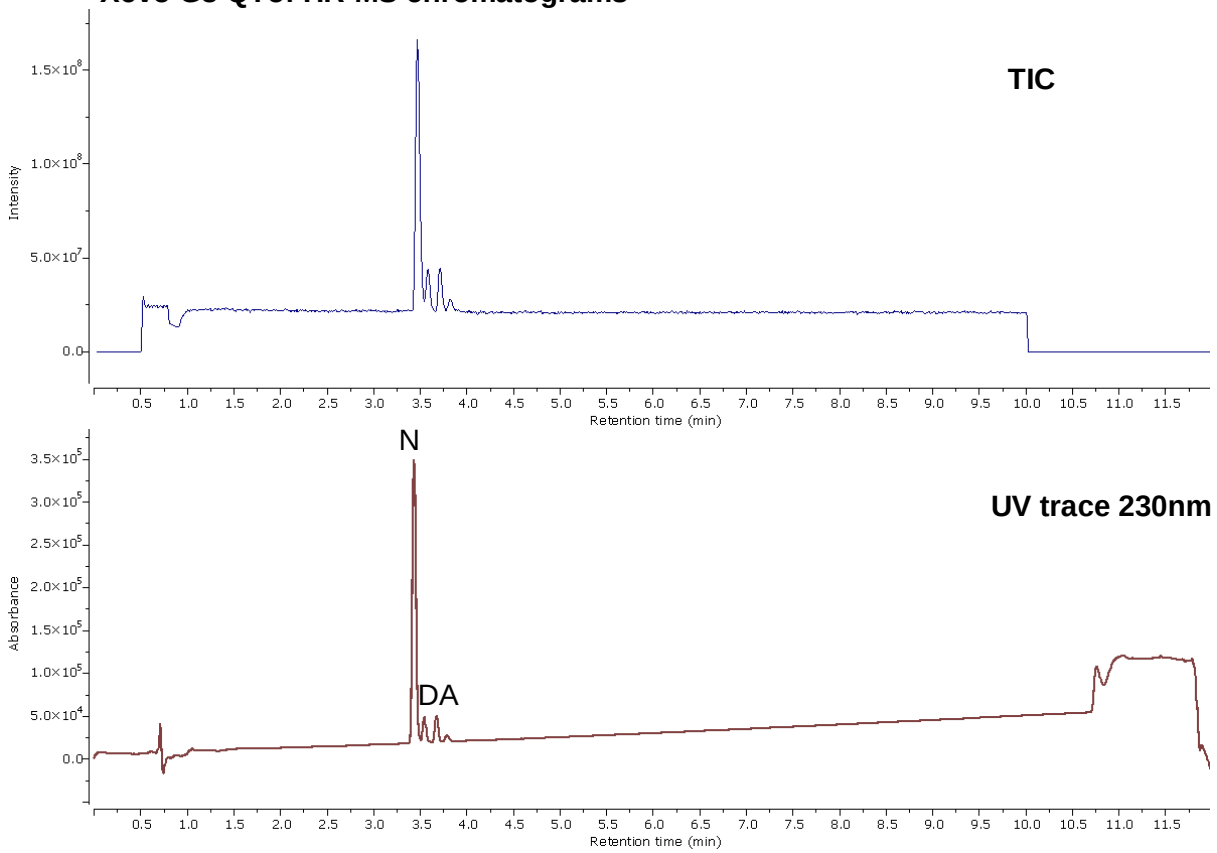

Figure A14. ACQUITY Premier Peptide CSH C18 RP. Acidic degradation of Desmopressin. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da). N (3.45 min); DA (3.69 min).

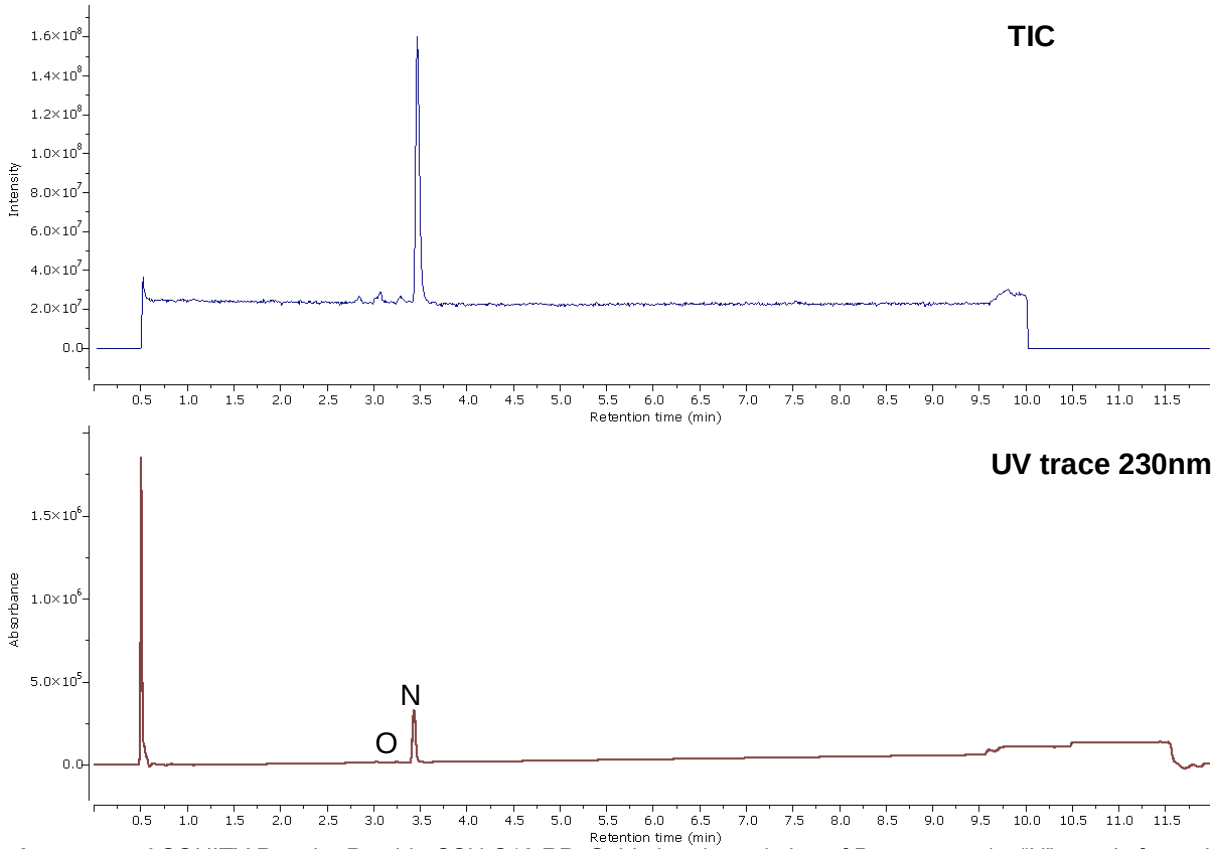

Figure A15. ACQUITY Premier Peptide CSH C18 RP. Oxidative degradation of Desmopressin. “N” stands for native peptide; “O” stands for mono-oxidated product; “O2” stands for di-oxidated product. N (3.45 min); O (3.08 min).

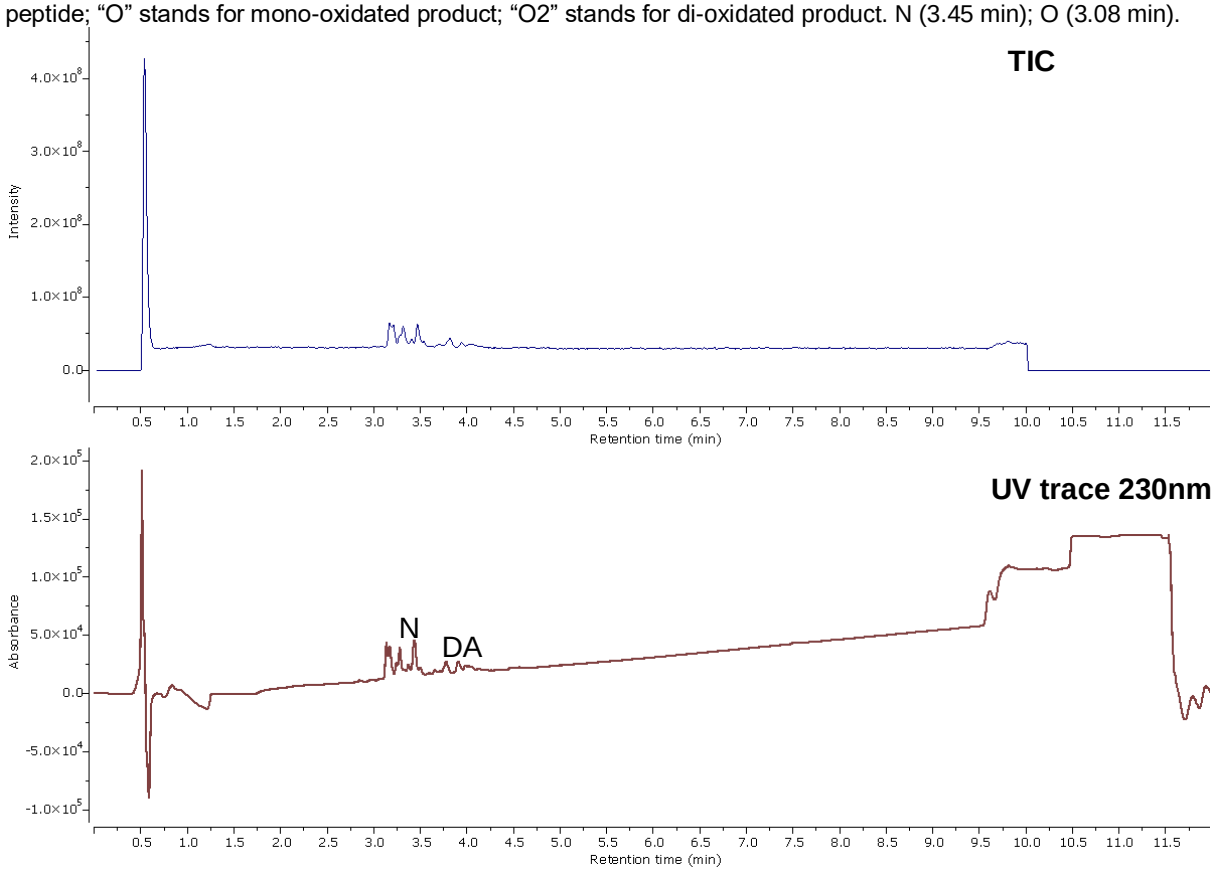

Figure A16. ACQUITY Premier Peptide CSH C18 RP. Basic degradation of Desmopressin. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da). N (3.45 min); DA (3.80 min).

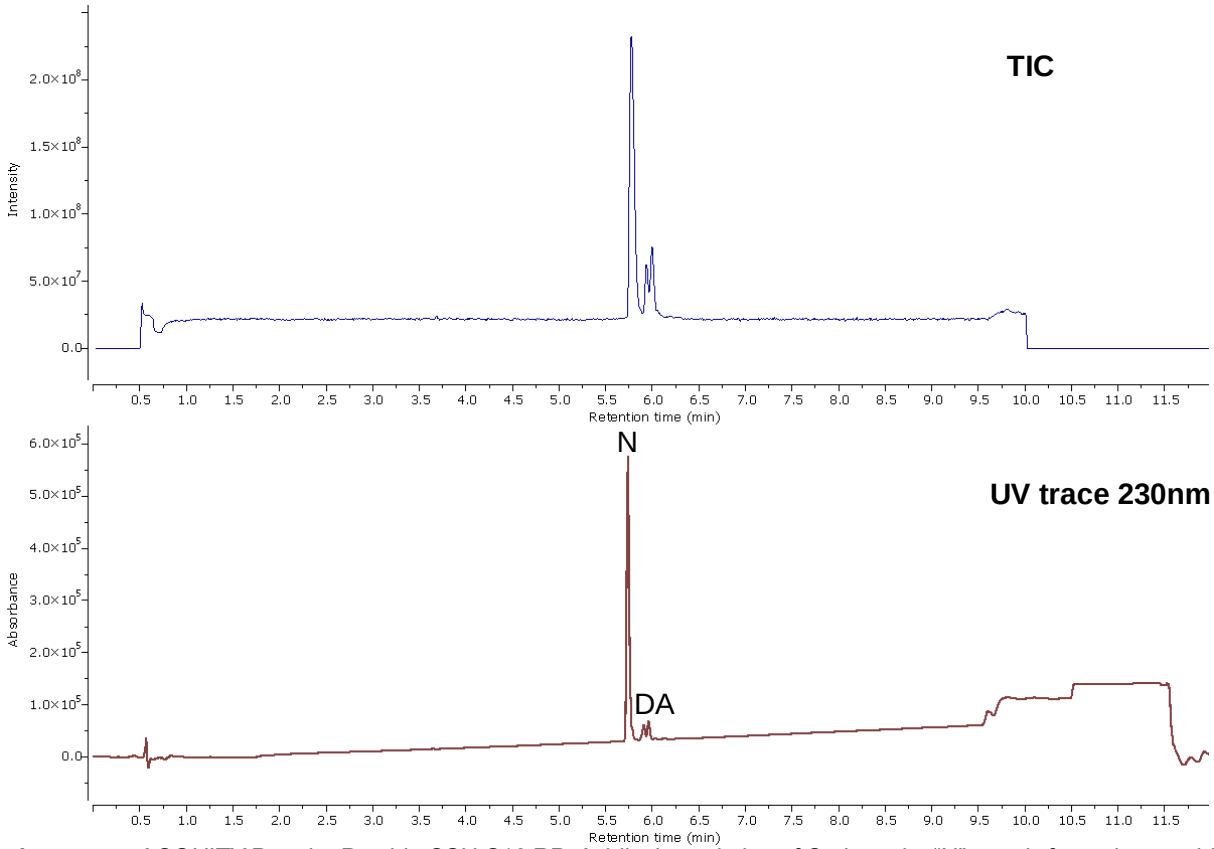

Figure A17. ACQUITY Premier Peptide CSH C18 RP. Acidic degradation of Carbetocin. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da). N (5.76 min); DA (5.98 min).

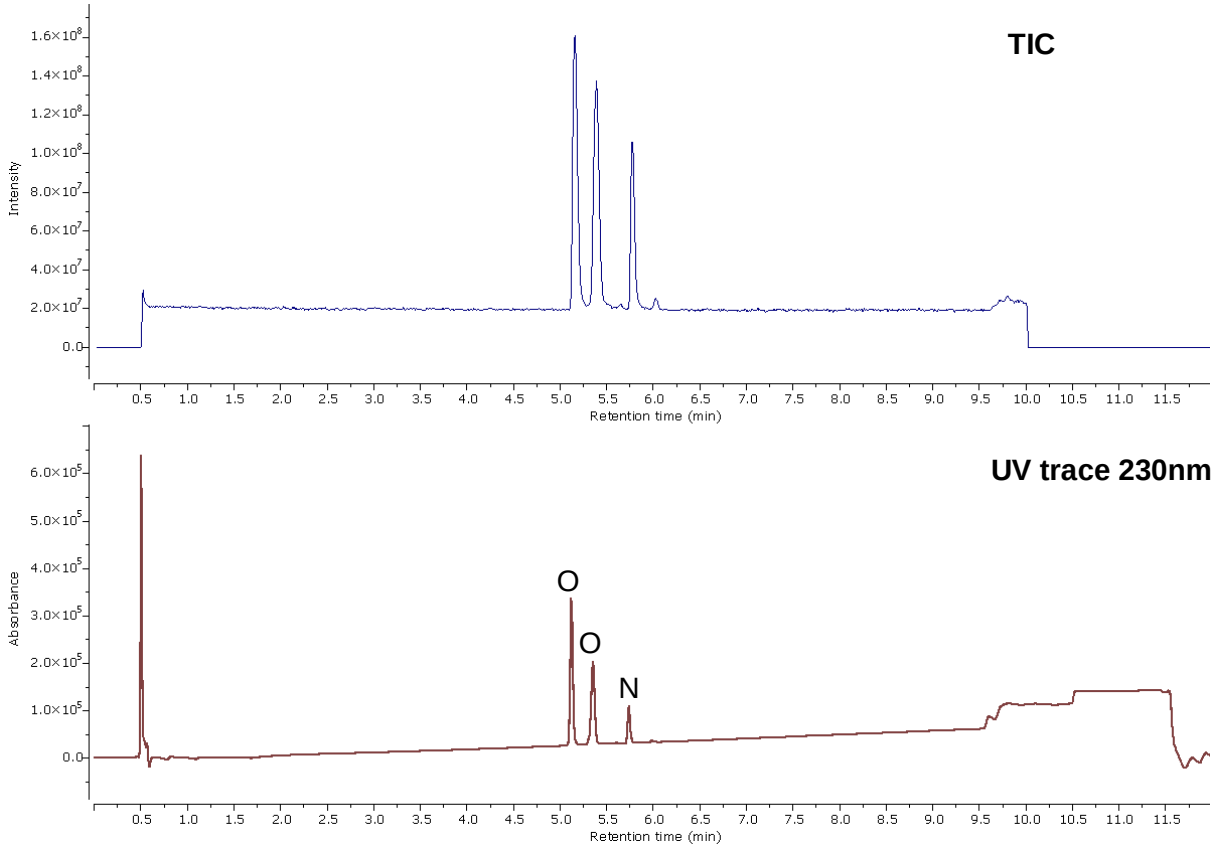

Figure A18. ACQUITY Premier Peptide CSH C18 RP. Oxidative degradation of Carbetocin. “N” stands for native peptide; “O” stands for mono-oxidated product; “O2” stands for di-oxidated product. N (5.76 min); O (5.14 and 5.37 min).

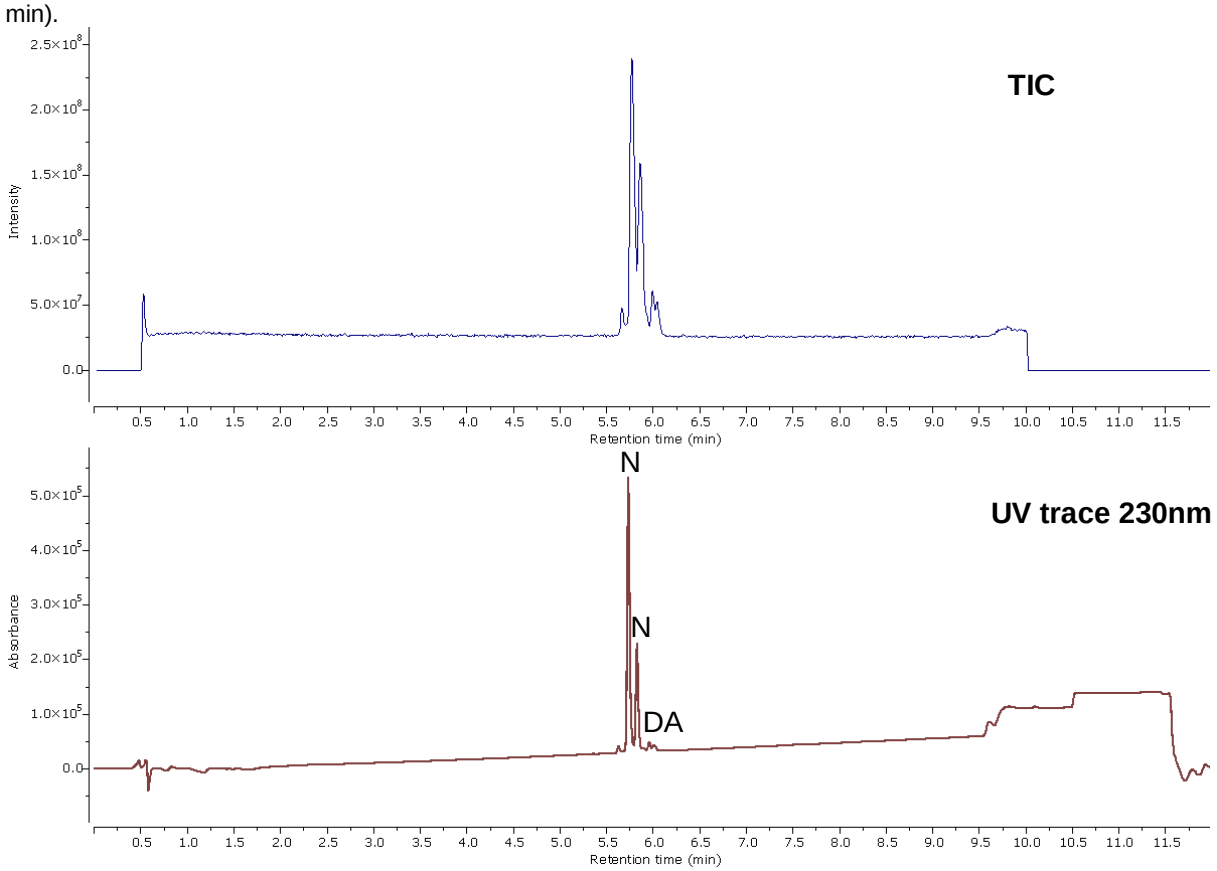

Figure A19. ACQUITY Premier Peptide CSH C18 RP. Basic degradation of Carbetocin. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da). N (5.75 and 5.84 min); DA (5.99 and 6.04 min).

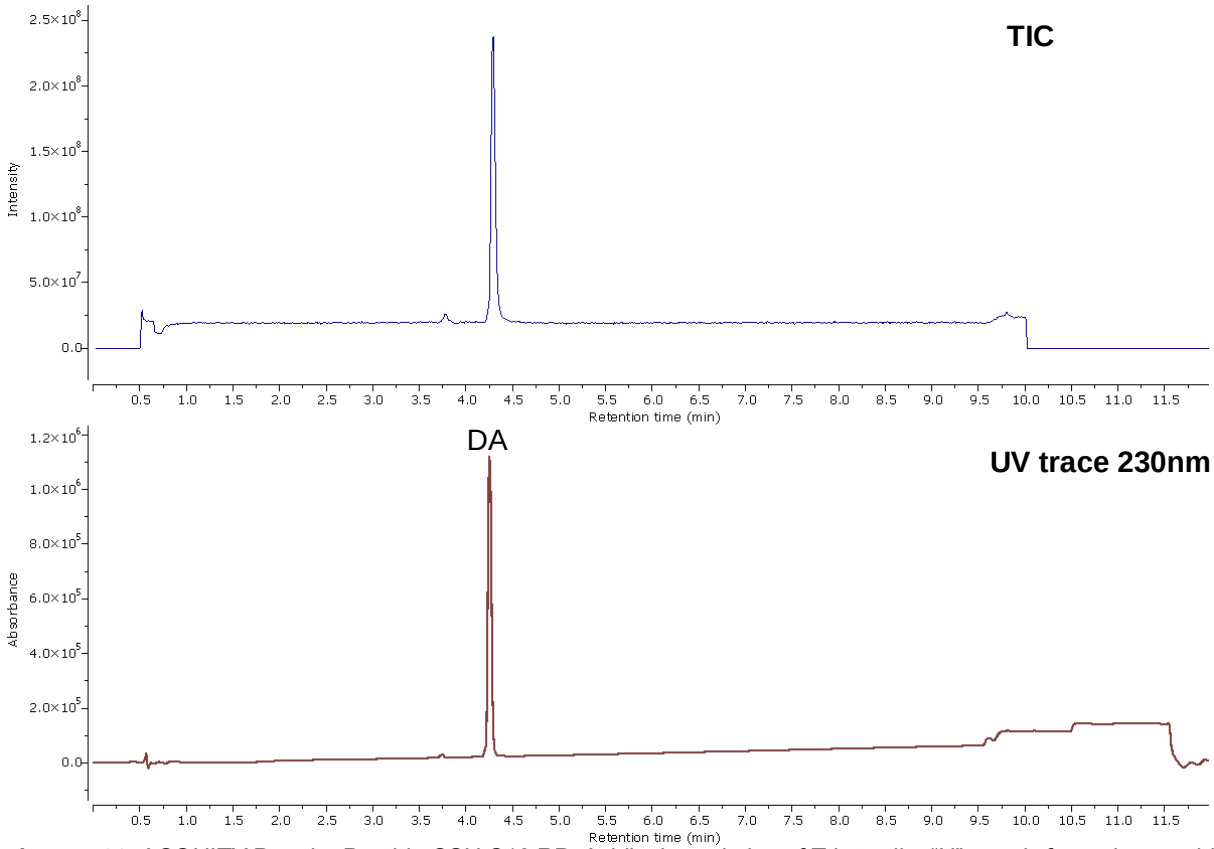

Figure A20. ACQUITY Premier Peptide CSH C18 RP. Acidic degradation of Triptorelin. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da). DA (4.27 min).

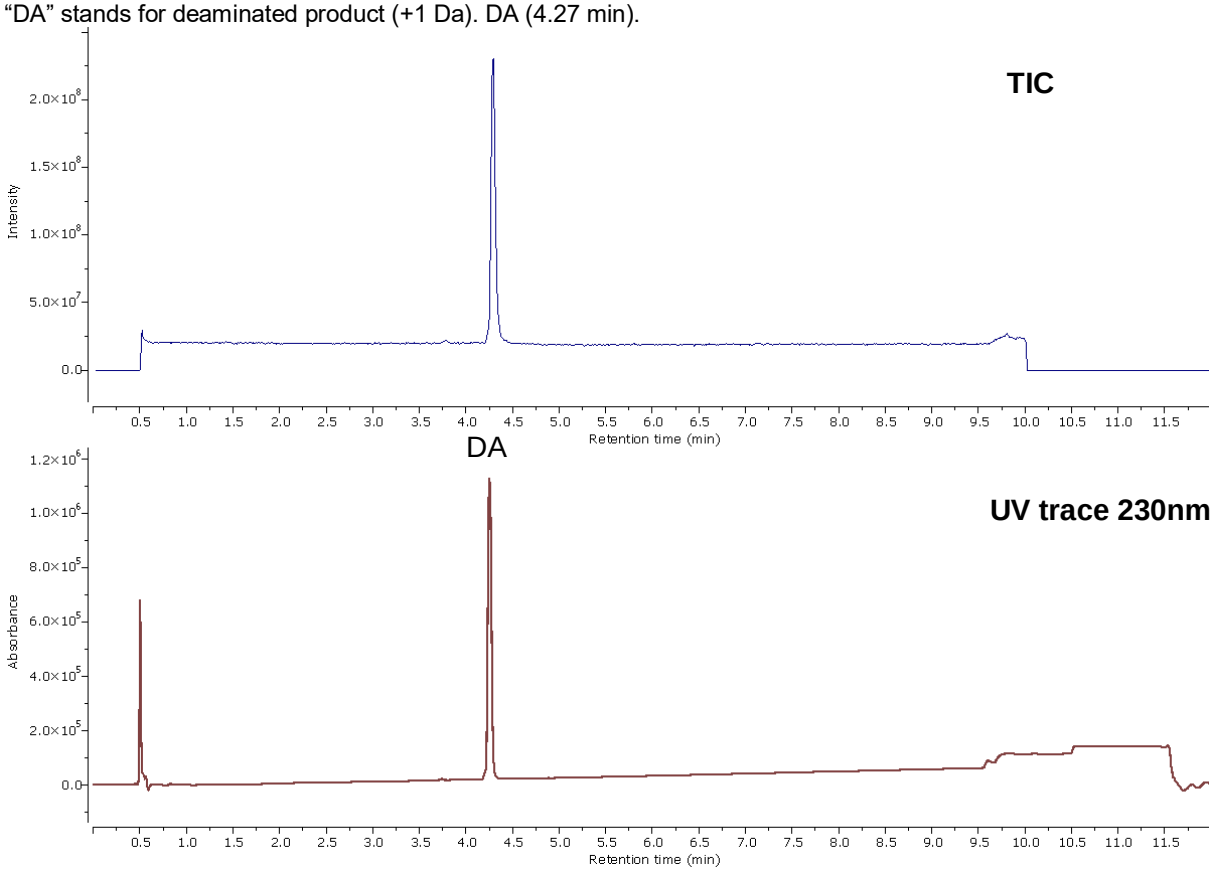

Figure A21. ACQUITY Premier Peptide CSH C18 RP. Oxidative degradation of Triptorelin. “N” stands for native peptide; “O” stands for mono-oxidated product; “O2” stands for di-oxidated product. DA (4.27 min).

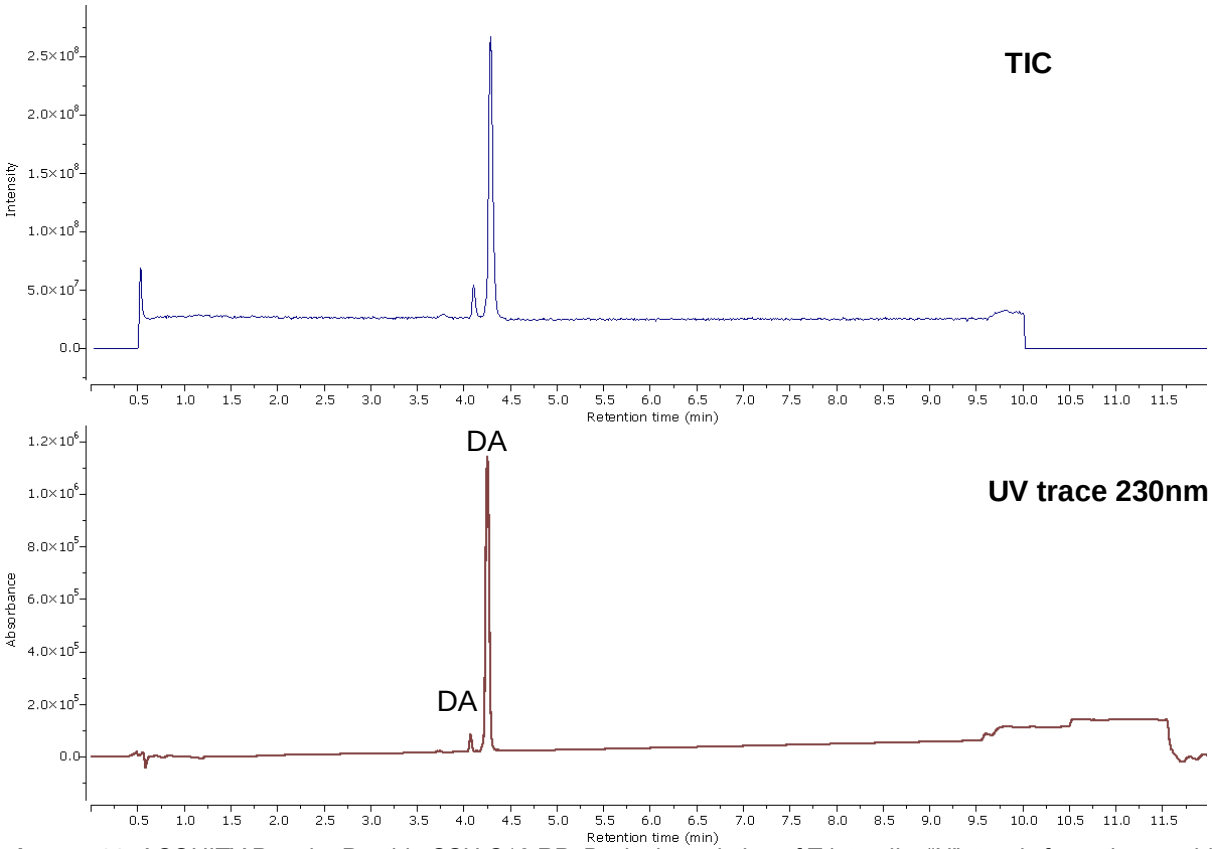

Figure A22. ACQUITY Premier Peptide CSH C18 RP. Basic degradation of Triptorelin. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da). DA (4.09 and 4.27 min).

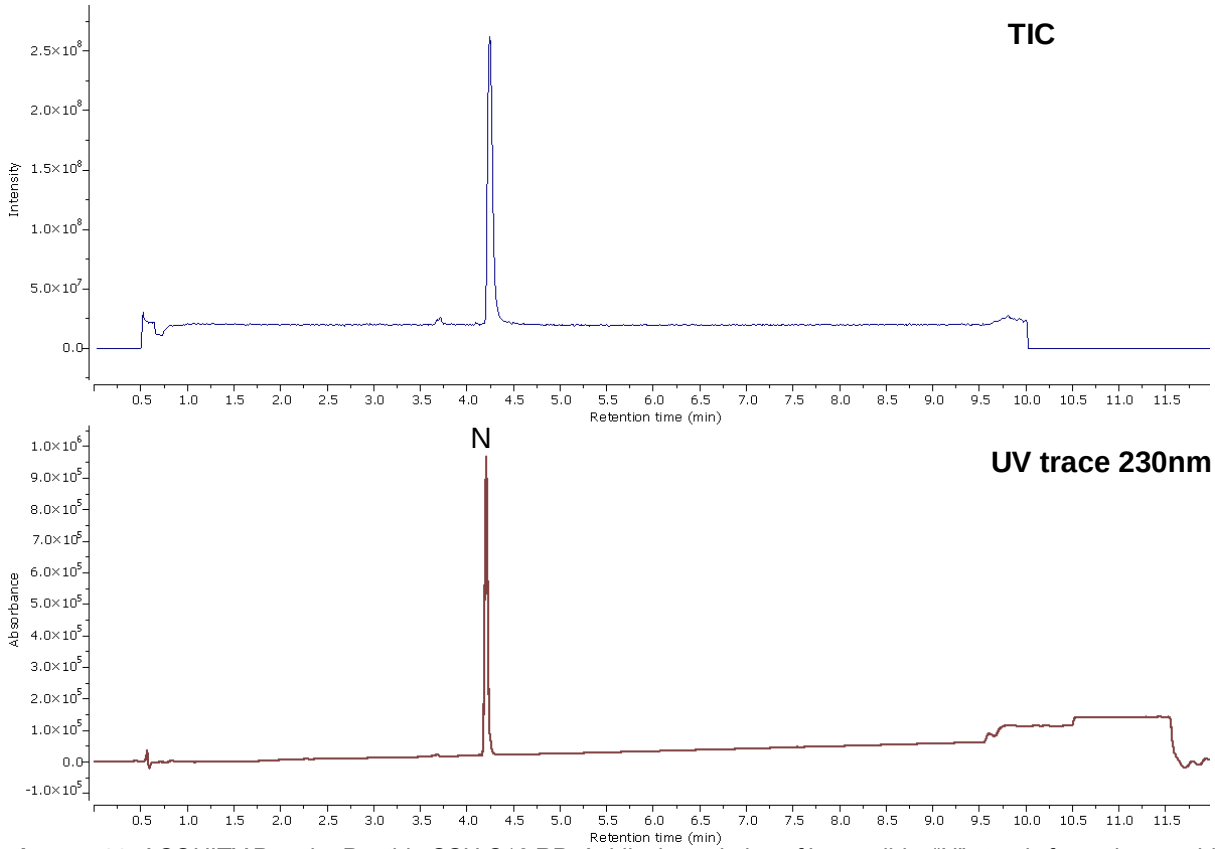

Figure A23. ACQUITY Premier Peptide CSH C18 RP. Acidic degradation of Leuprolide. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da). N (4.22 min).

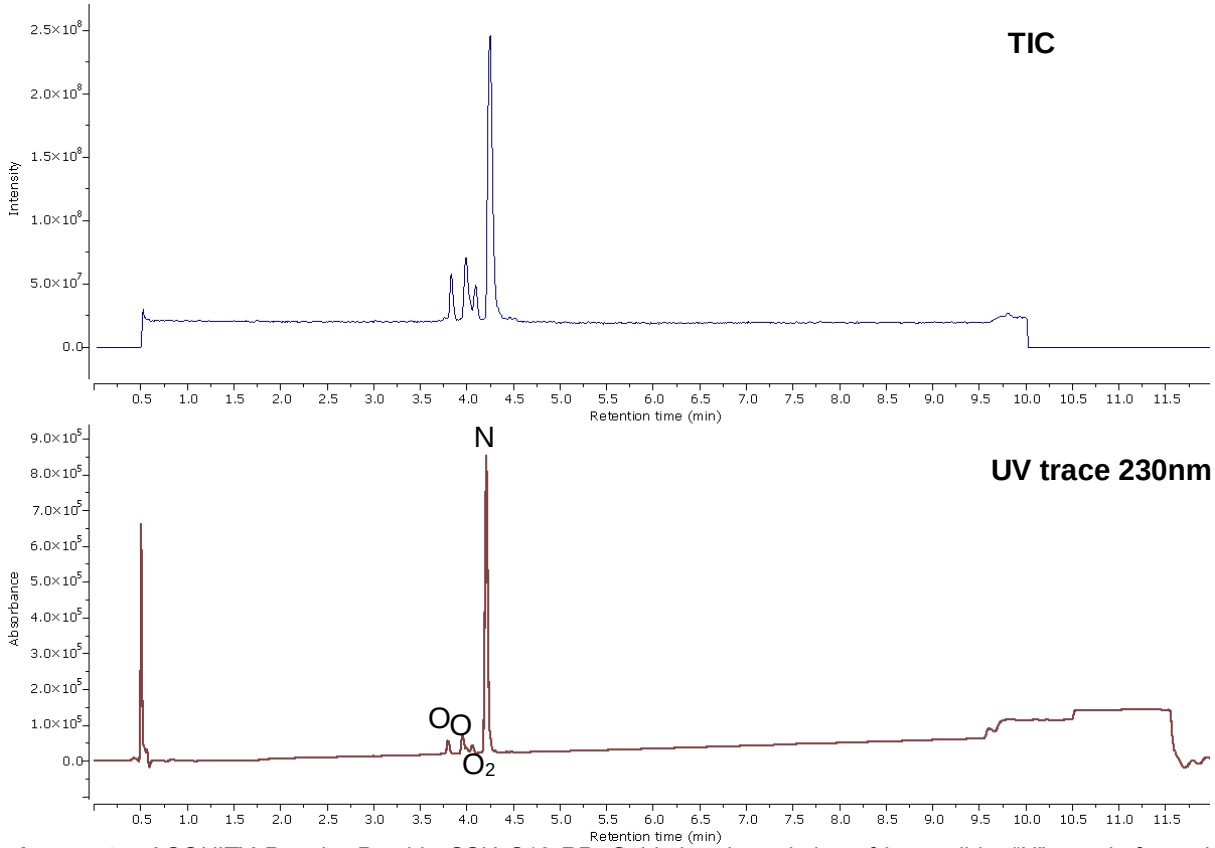

Figure A24. ACQUITY Premier Peptide CSH C18 RP. Oxidative degradation of Leuprolide. “N” stands for native

peptide; “O” stands for mono-oxidated product; “O2” stands for di-oxidated product. N (4.23 min); O (3.81 and 3.97 min); O2 (4.10 min).

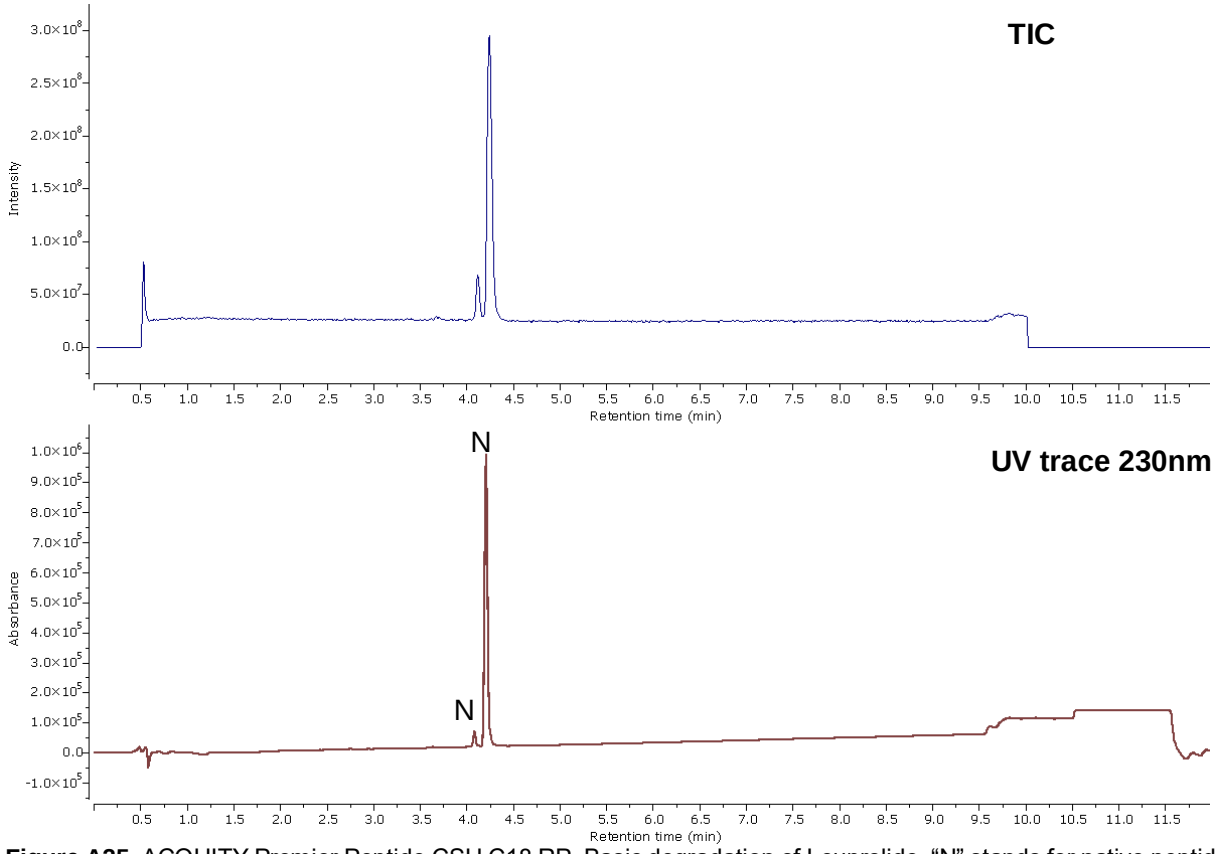

Figure A25. ACQUITY Premier Peptide CSH C18 RP. Basic degradation of Leuprolide. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da). N (4.10 and 4.22 min).

8.2.1 Evaluation of HILIC methods for the separation of peptide degradation-related impurities: evaluation of MeOH and ACN as organic solvents in HILIC chromatograms

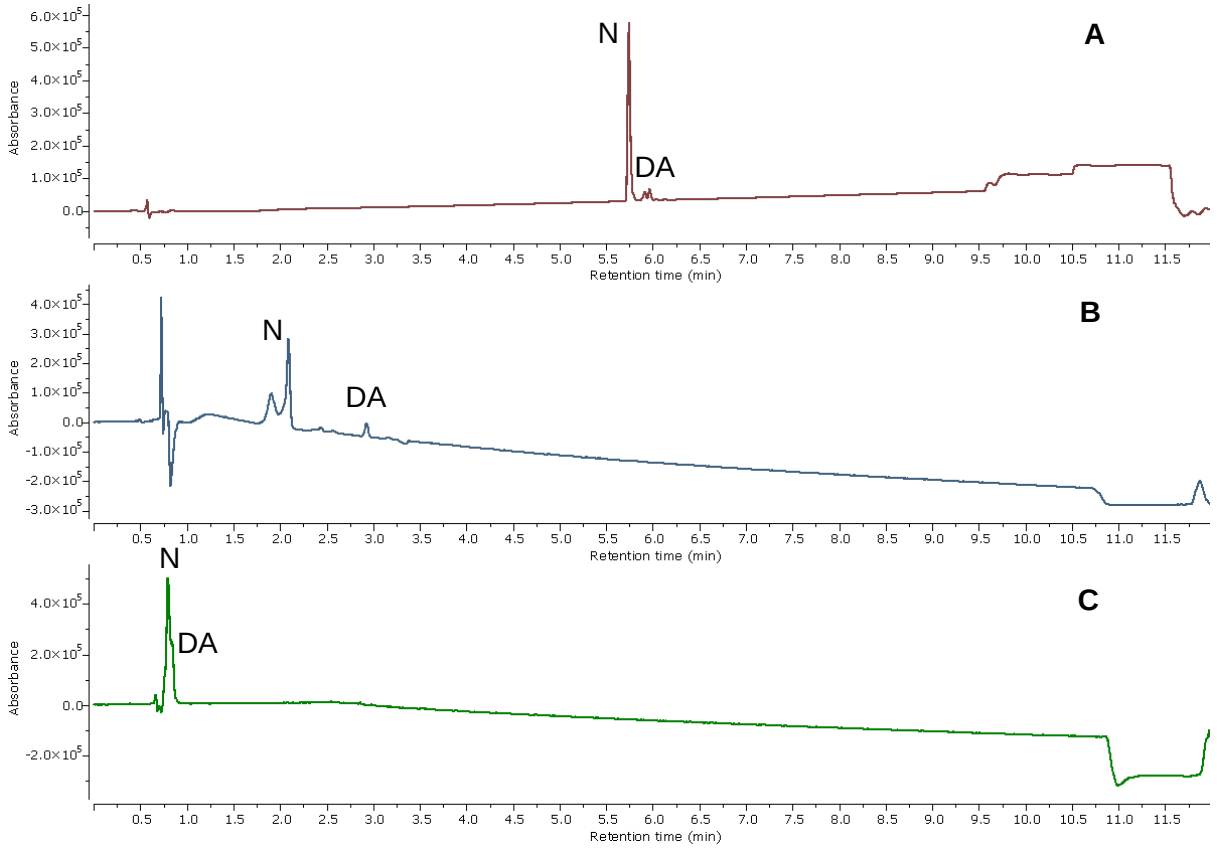

Figure A26. Comparative chromatograms at 230 nm for Carbetocin at acidic conditions. **A**: characterisation on an HR-MS-coupled UPLC system; **B**: analysis by HILIC using ACN as the organic modifier; **C**: analysis by HILIC using MeOH as the organic modifier. The figure illustrates differences in retention profile and peak shape attributable to the HILIC organic modifier (ACN vs MeOH) relative to the HR-MS/UPLC reference. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da).

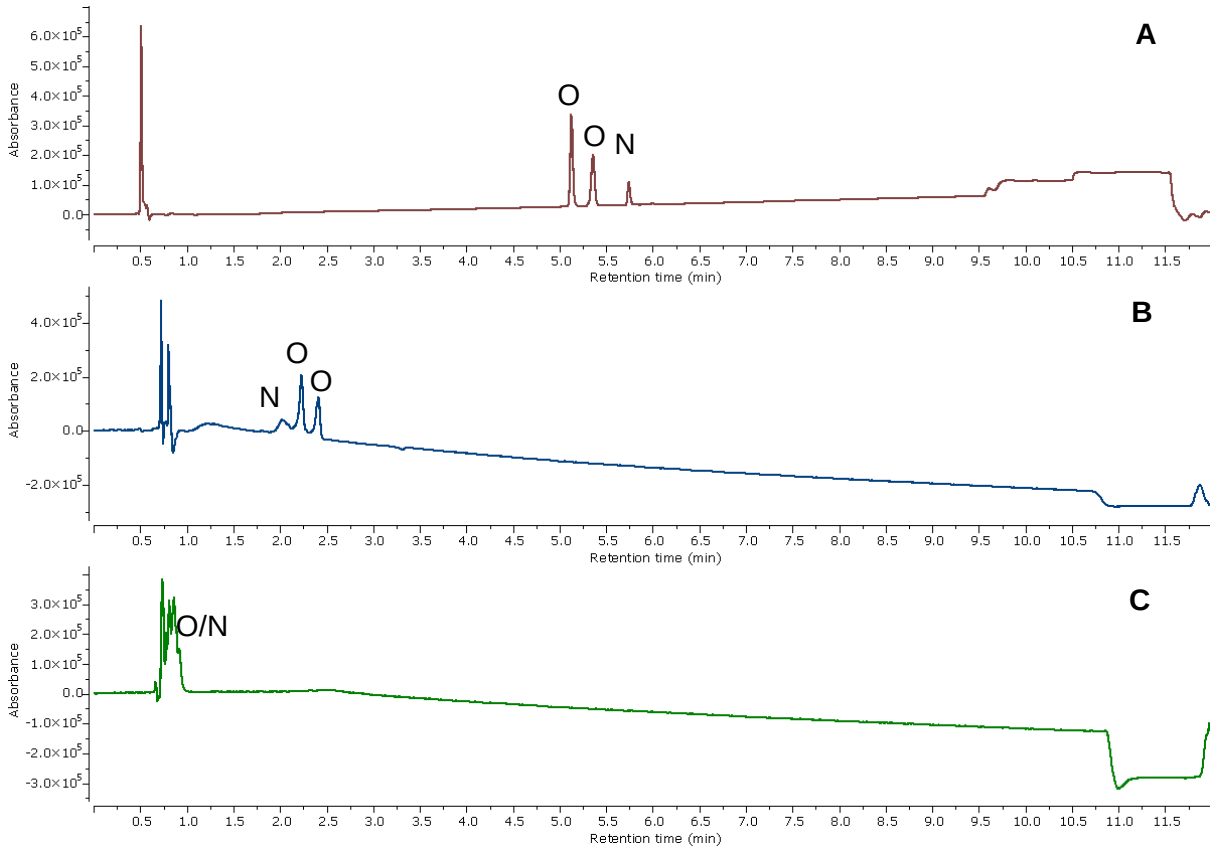

Figure A27. Comparative chromatograms at 230 nm for Carbetocin at oxidative conditions. **A**: characterisation on an HR-MS-coupled UPLC system; **B**: analysis by HILIC using ACN as the organic modifier; **C**: analysis by HILIC using MeOH as the organic modifier. The figure illustrates differences in retention profile and peak shape attributable to the HILIC organic modifier (ACN vs MeOH) relative to the HR-MS/UPLC reference. “N” stands for native peptide; “O” stands for mono-oxidated product (+16 Da).

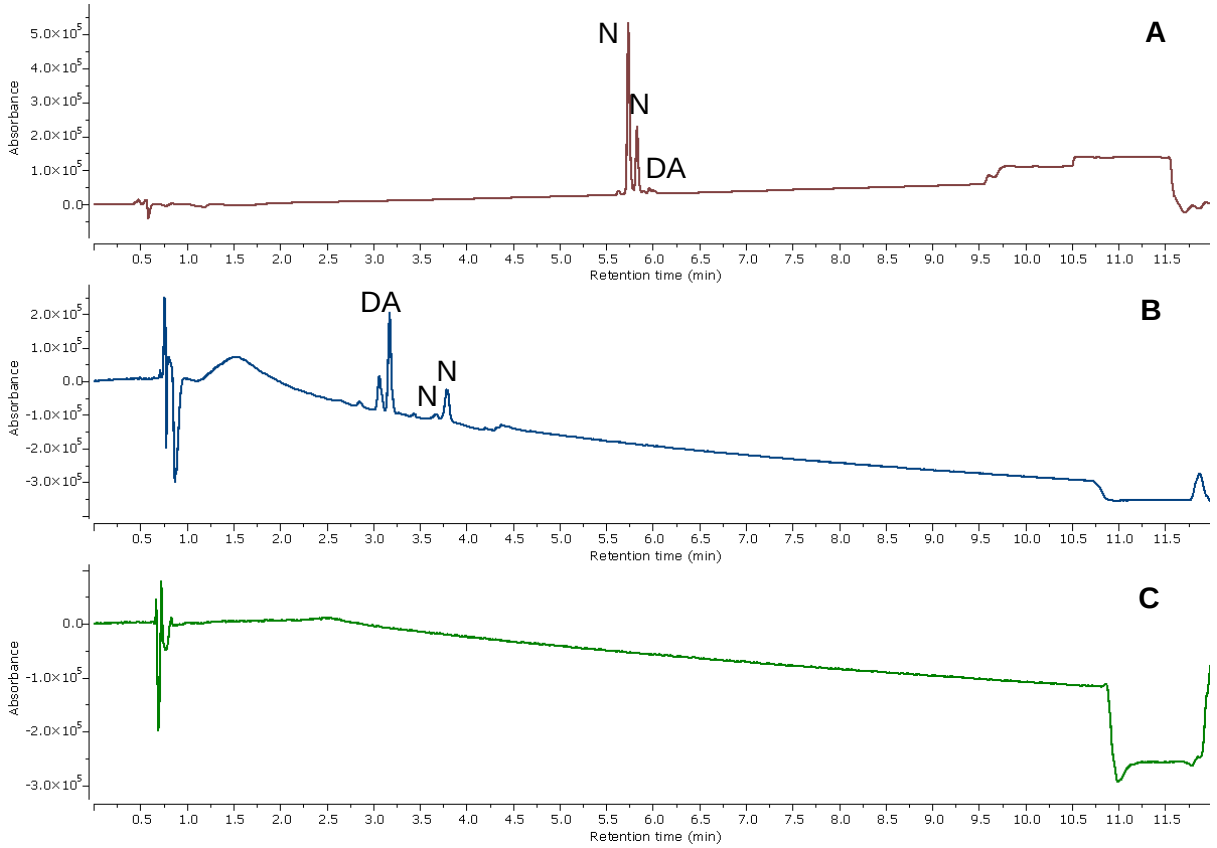

Figure A28. Comparative chromatograms at 230 nm for Carbetocin at basic conditions. **A**: characterisation on an HR-MS-coupled UPLC system; **B**: analysis by HILIC using ACN as the organic modifier; **C**: analysis by HILIC using MeOH as the organic modifier. The figure illustrates differences in retention profile and peak shape attributable to the HILIC organic modifier (ACN vs MeOH) relative to the HR-MS/UPLC reference. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da).

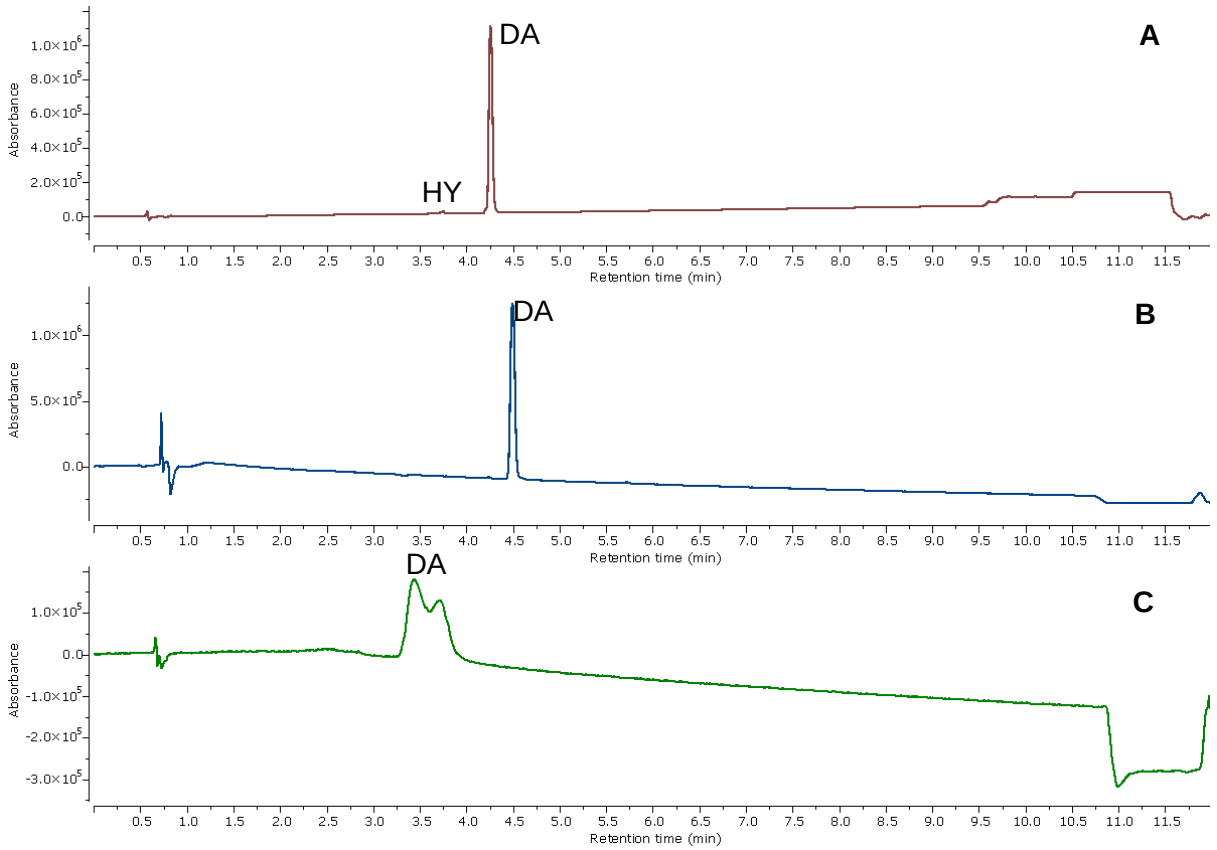

Figure A29. Comparative chromatograms at 230 nm for Triptorelin at acidic conditions. **A**: characterisation on an HR-MS-coupled UPLC system; **B**: analysis by HILIC using ACN as the organic modifier; **C**: analysis by HILIC using MeOH as the organic modifier. The figure illustrates differences in retention profile and peak shape attributable to the HILIC organic modifier (ACN vs MeOH) relative to the HR-MS/UPLC reference. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da); “HY” stands for hydrolysis product (-18 Da).

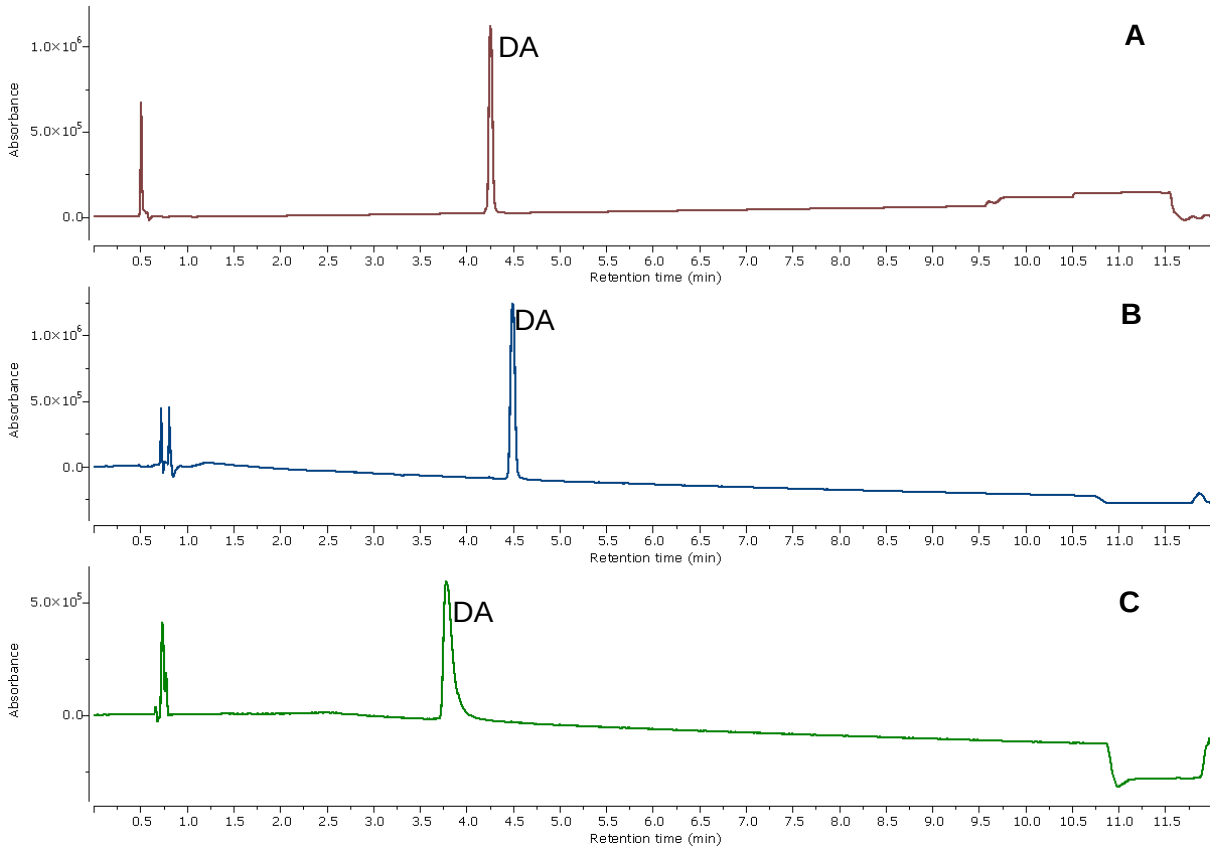

Figure A30. Comparative chromatograms at 230 nm for Triptorelin at oxidative conditions. **A**: characterisation on an HR-MS-coupled UPLC system; **B**: analysis by HILIC using ACN as the organic modifier; **C**: analysis by HILIC using MeOH as the organic modifier. The figure illustrates differences in retention profile and peak shape attributable to the HILIC organic modifier (ACN vs MeOH) relative to the HR-MS/UPLC reference. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da); “O” stands for mono-oxidated product (+16 Da).

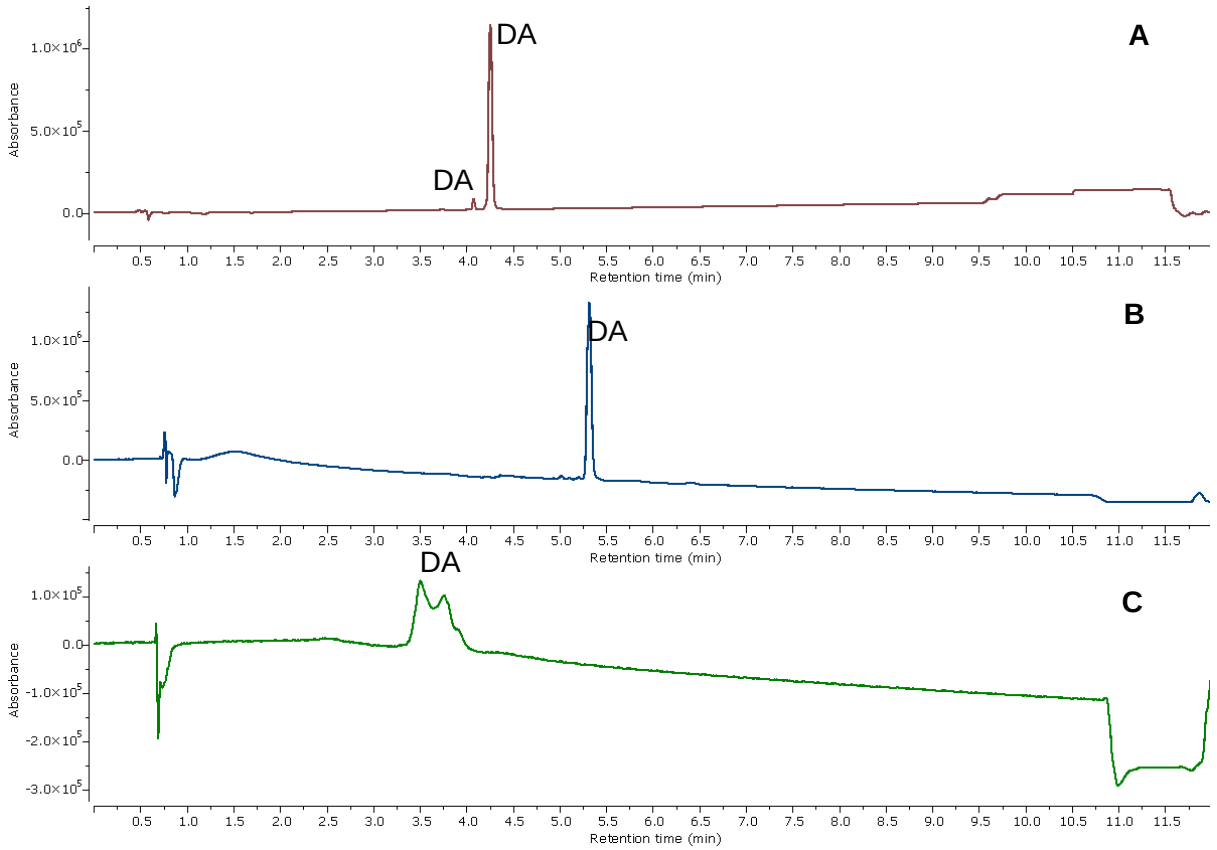

Figure A31. Comparative chromatograms at 230 nm for Triptorelin at basic conditions. **A**: characterisation on an HR-MS-coupled UPLC system; **B**: analysis by HILIC using ACN as the organic modifier; **C**: analysis by HILIC using MeOH as the organic modifier. The figure illustrates differences in retention profile and peak shape attributable to the HILIC organic modifier (ACN vs MeOH) relative to the HR-MS/UPLC reference. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da).

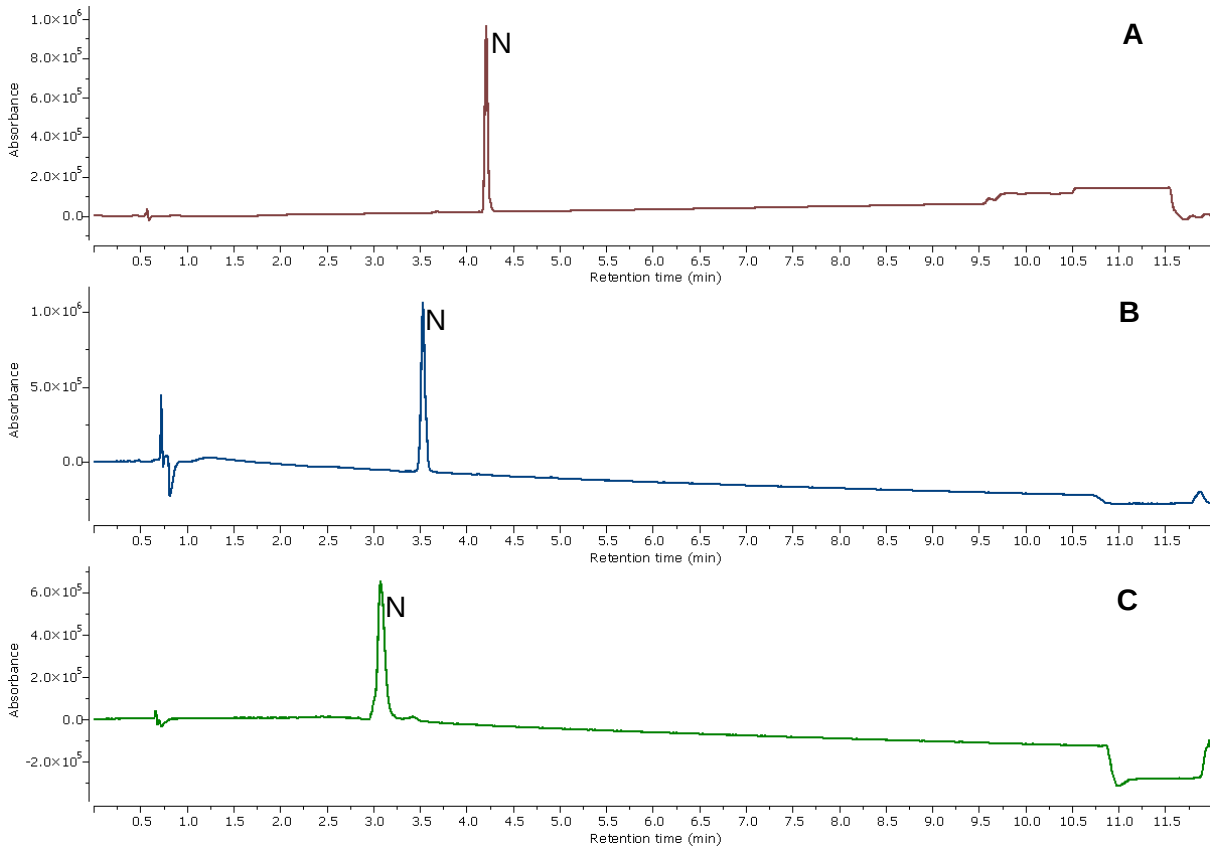

Figure A32. Comparative chromatograms at 230 nm for Leuprolide at acidic conditions. **A**: characterisation on an HR-MS-coupled UPLC system; **B**: analysis by HILIC using ACN as the organic modifier; **C**: analysis by HILIC using MeOH as the organic modifier. The figure illustrates differences in retention profile and peak shape attributable to the HILIC organic modifier (ACN vs MeOH) relative to the HR-MS/UPLC reference. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da).

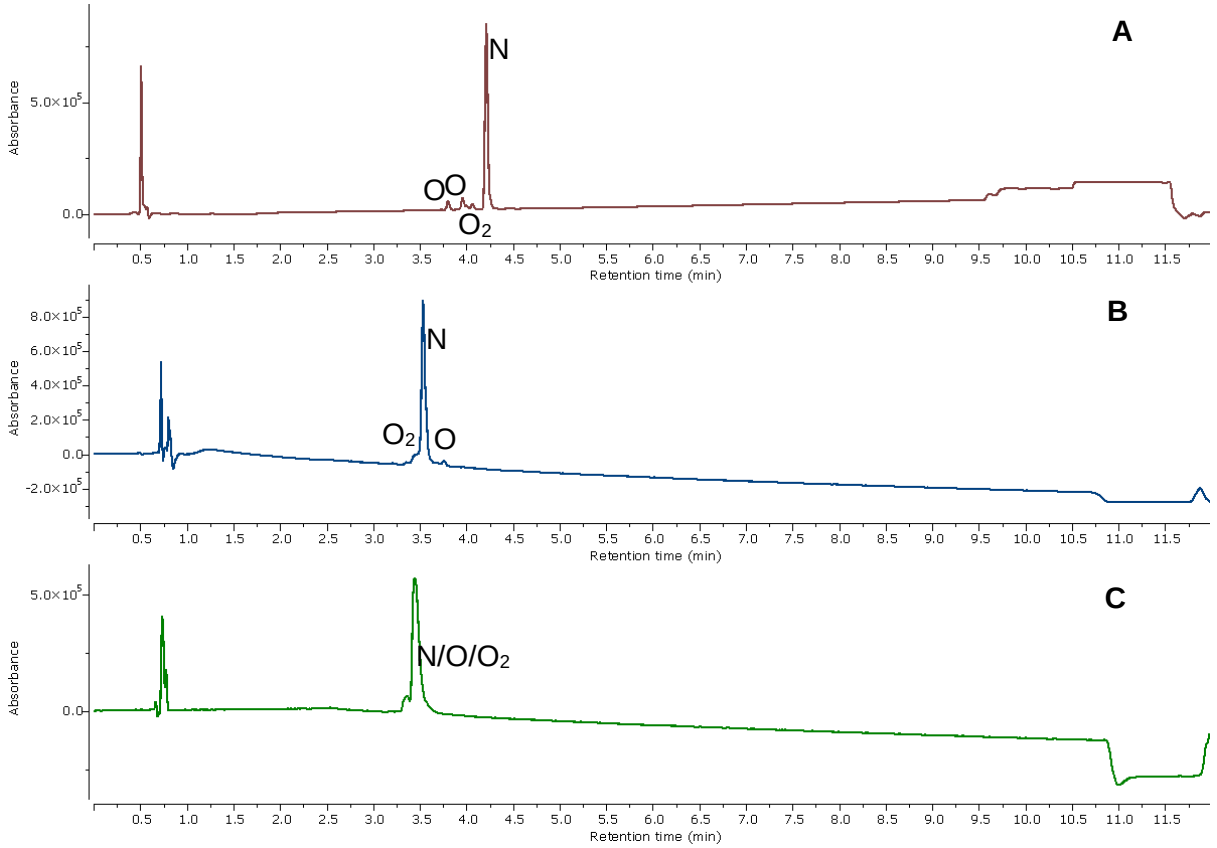

Figure A33. Comparative chromatograms at 230 nm for Leuprolide at oxidative conditions. **A**: characterisation on an HR-MS-coupled UPLC system; **B**: analysis by HILIC using ACN as the organic modifier; **C**: analysis by HILIC using MeOH as the organic modifier. The figure illustrates differences in retention profile and peak shape attributable to the HILIC organic modifier (ACN vs MeOH) relative to the HR-MS/UPLC reference. “N” stands for native peptide; “O” stands for mono-oxidated product (+16 Da); “O_2” stands for di-oxidated product (+32 Da).

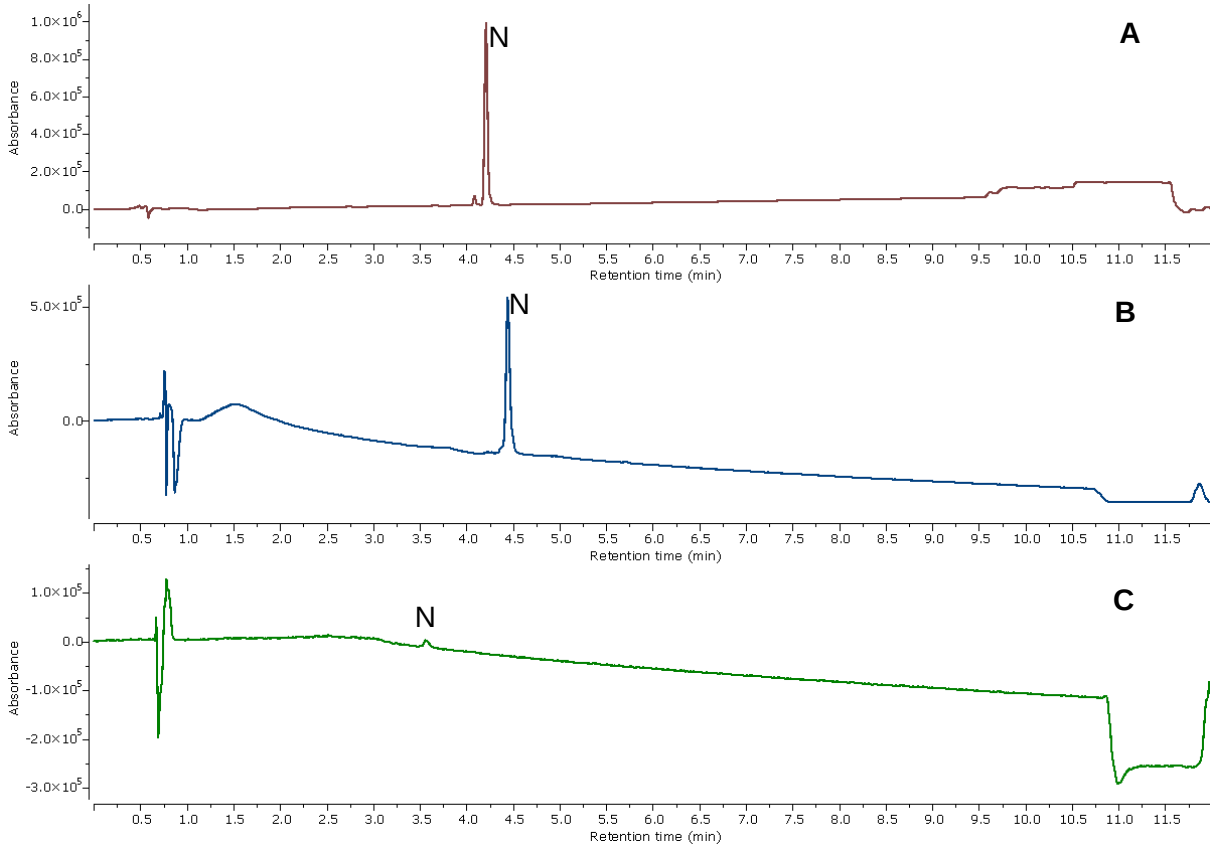

Figure A34. Comparative chromatograms at 230 nm for Leuprolide at basic conditions. **A**: characterisation on an HR-MS-coupled UPLC system; **B**: analysis by HILIC using ACN as the organic modifier; **C**: analysis by HILIC using MeOH as the organic modifier. The figure illustrates differences in retention profile and peak shape attributable to the HILIC organic modifier (ACN vs MeOH) relative to the HR-MS/UPLC reference. “N” stands for native peptide; “DA” stands for deaminated product (+1 Da).

8.3 Evaluation of HILIC methods for the separation of peptide synthesis-related impurities: chromatograms

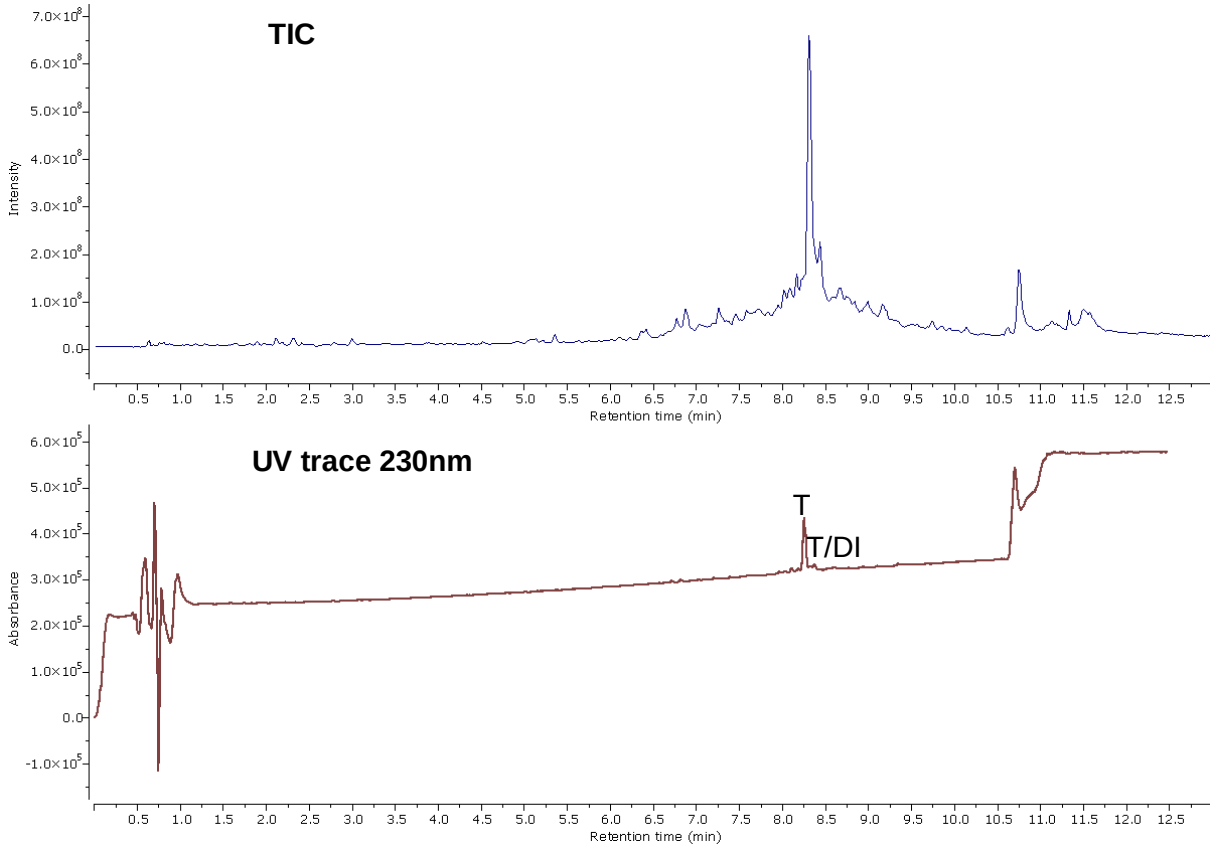

Figure A35. Crude A, C18 RP-LC; ACN. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

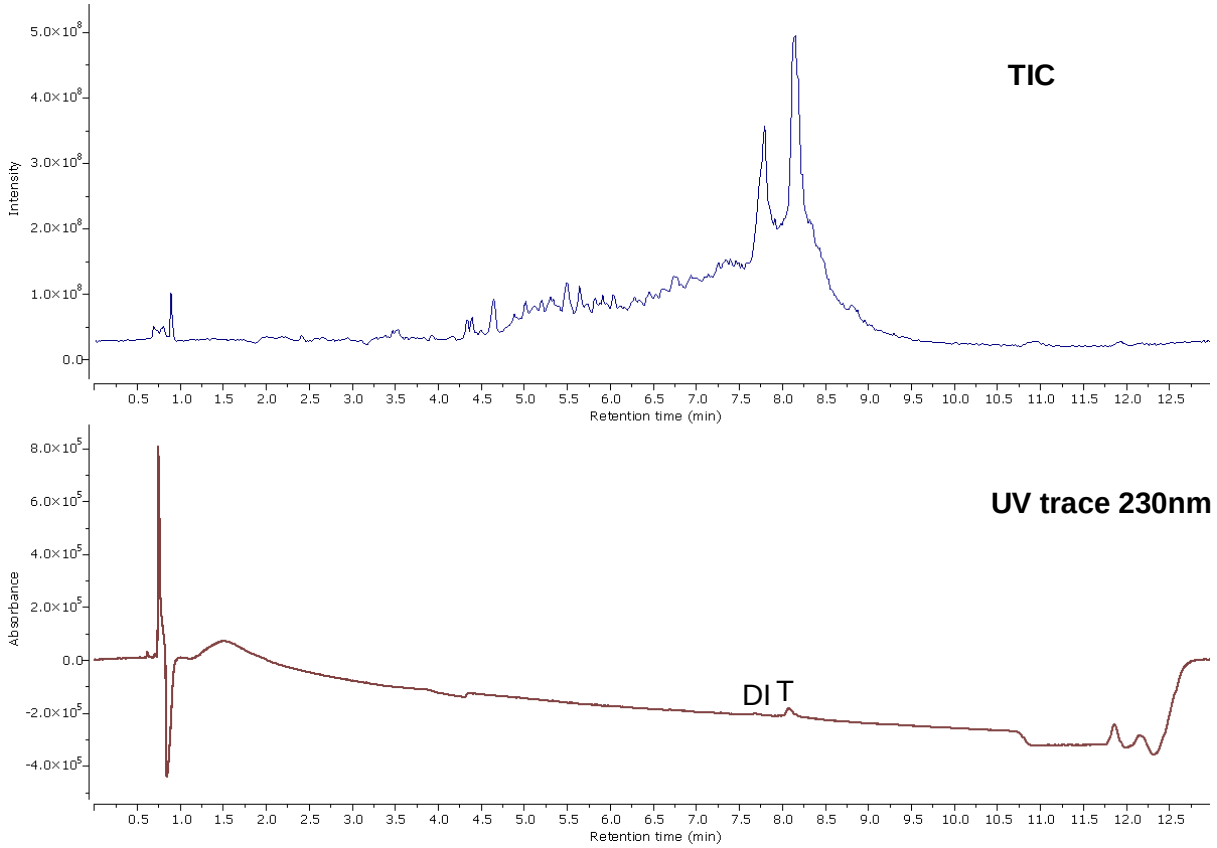

Figure A36. Crude A, Amide HILIC; ACN. "T" stands for target peptide; "DI" stands for deletion impurity; "T/D" indicates co-elution of the fractions.

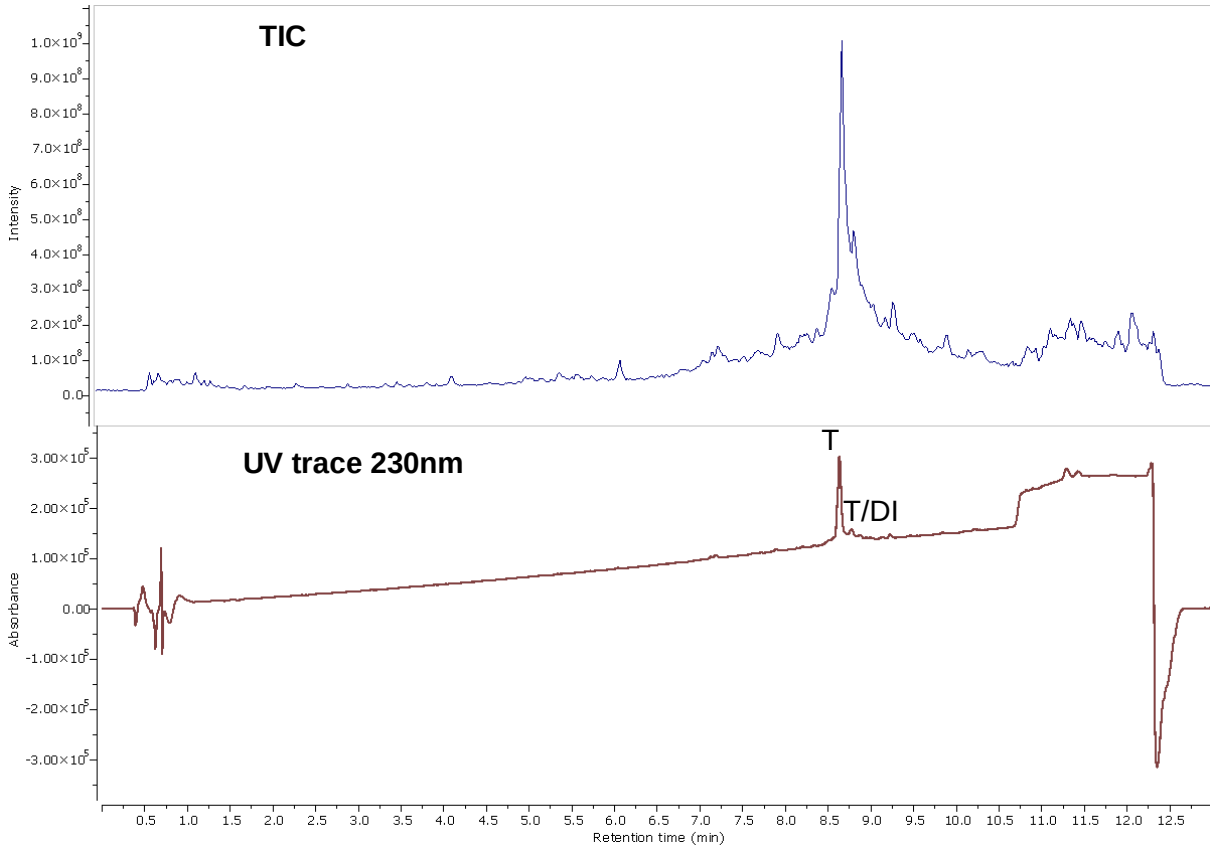

Figure A37. Crude A, C18 RP-LC; MeOH. "T" stands for target peptide; "DI" stands for deletion impurity; "T/D" indicates co-elution of the fractions.

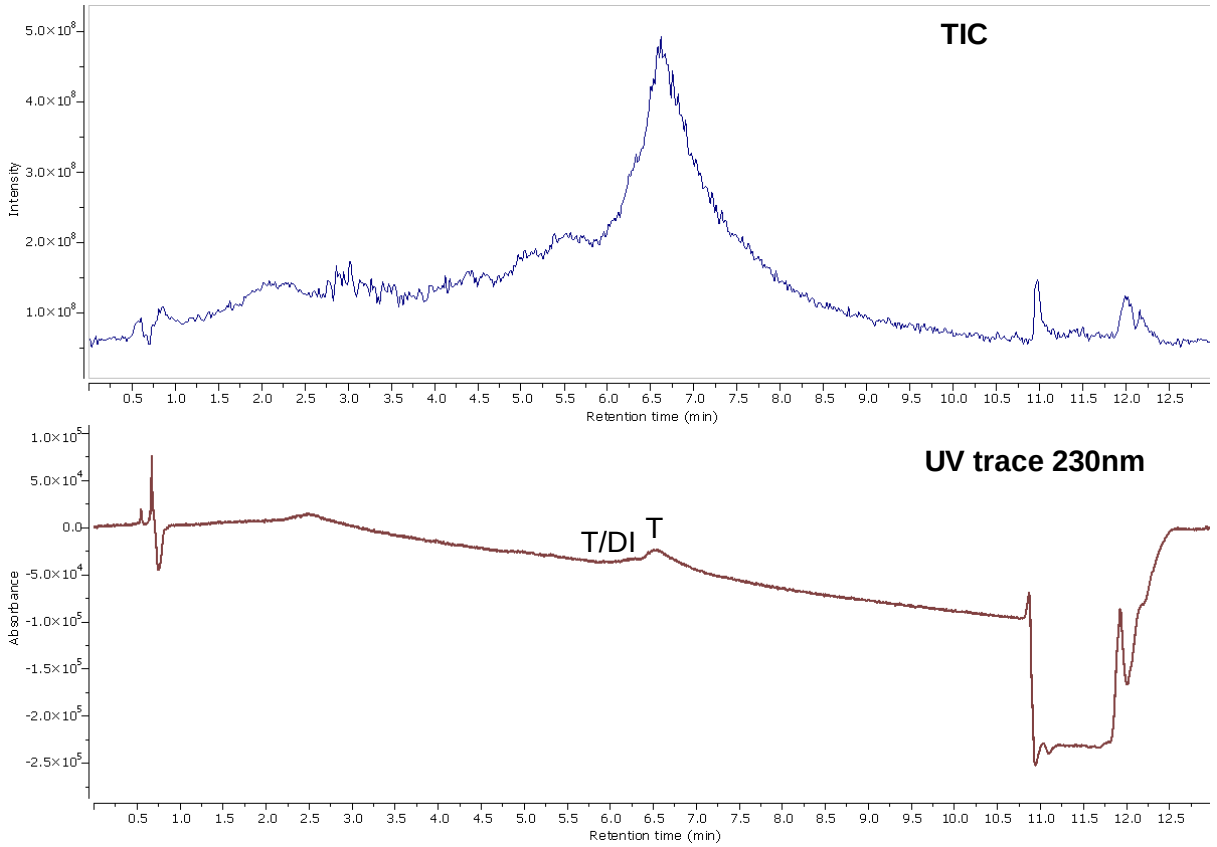

Figure A38. Crude A, Amide HILIC; MeOH. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

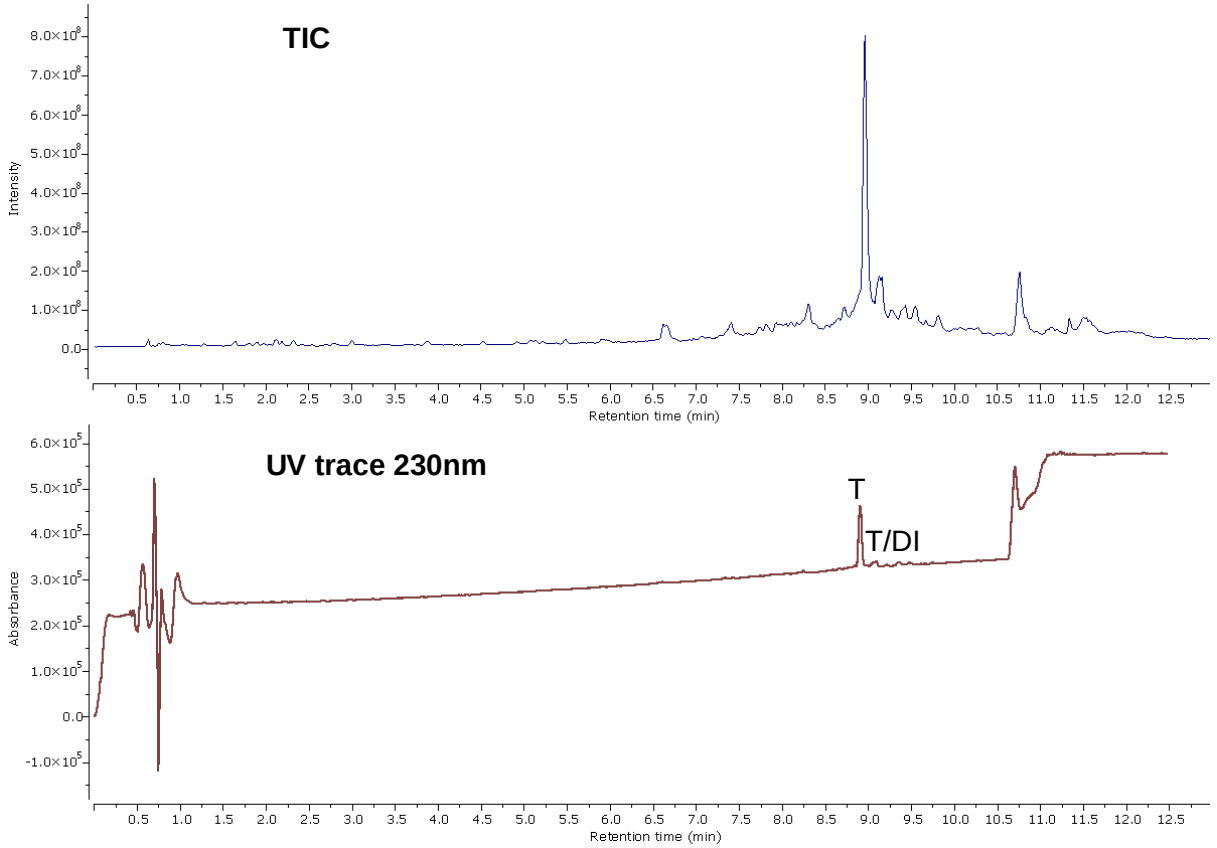

Figure A39. Crude B, C18 RP-LC; ACN. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

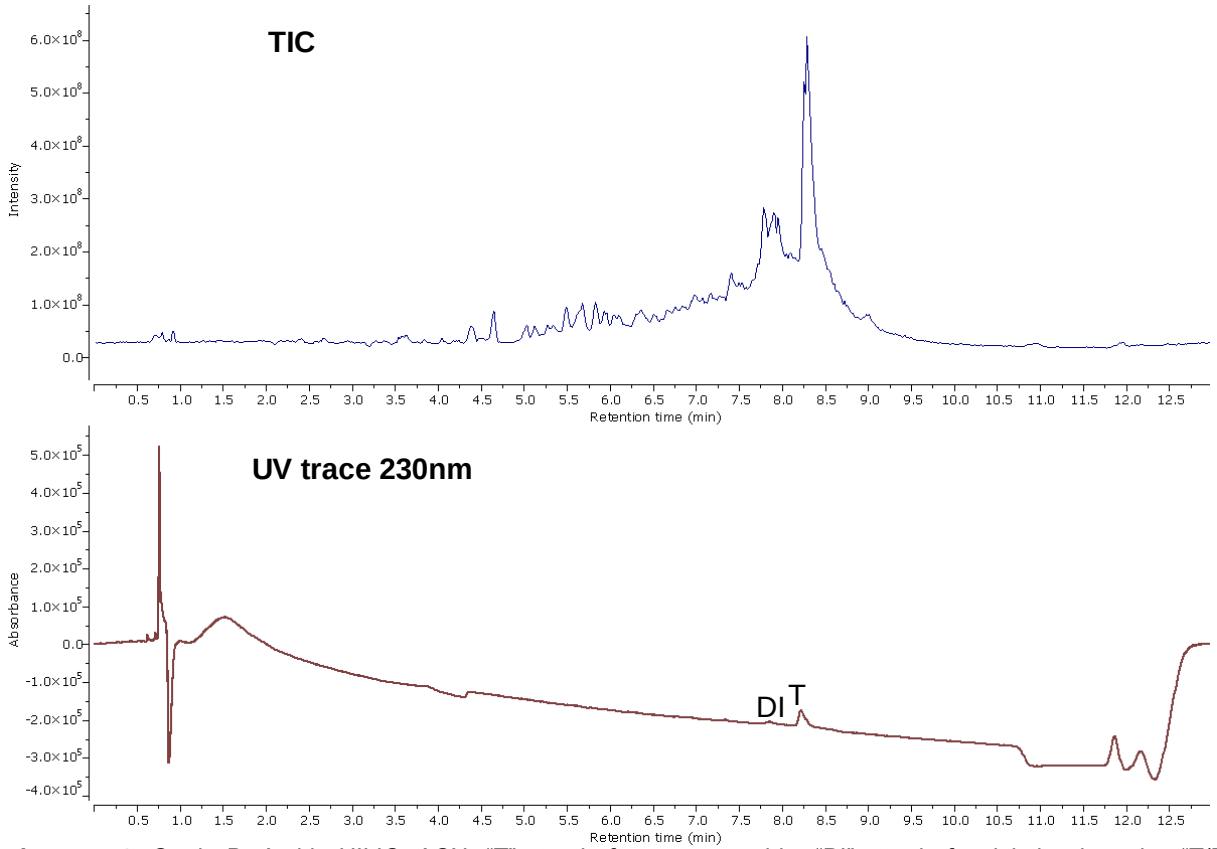

Figure A40. Crude B, Amide HILIC; ACN. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

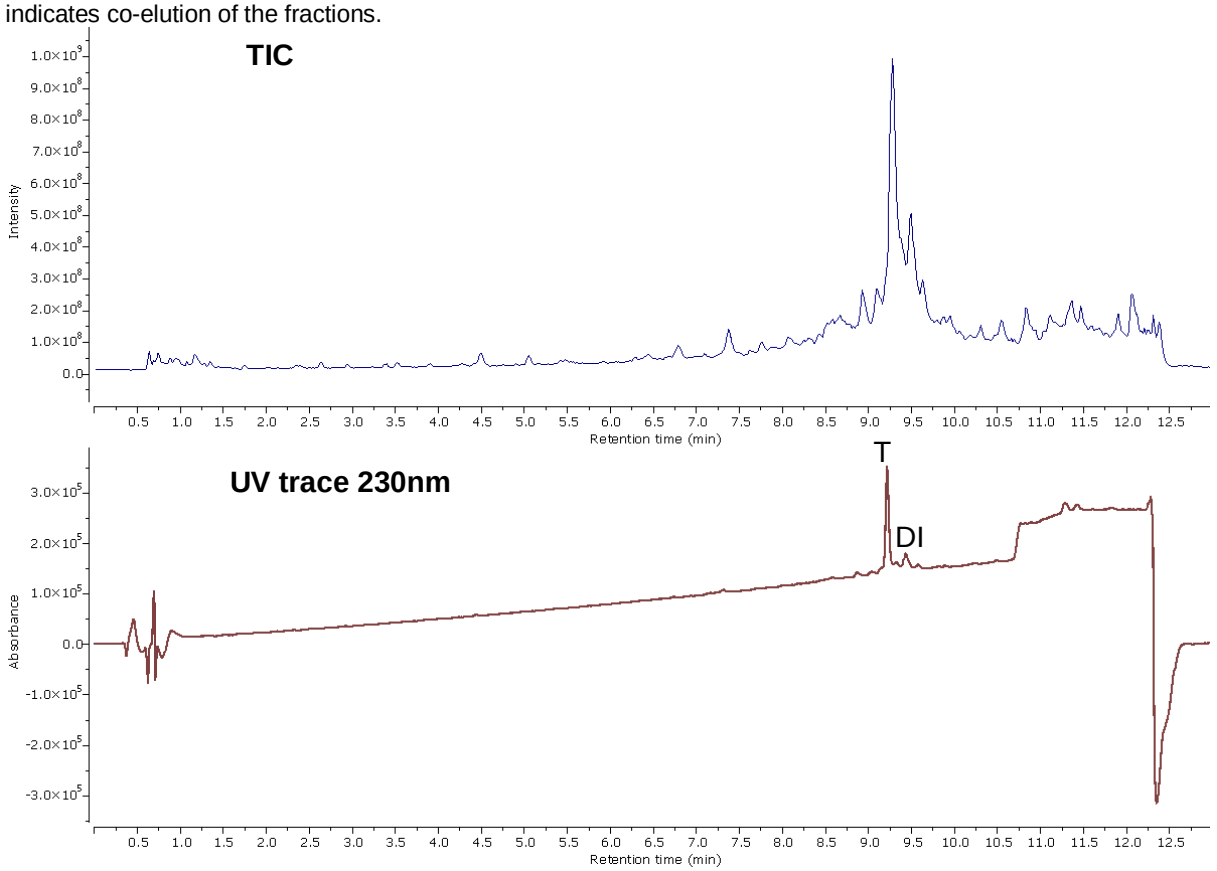

Figure A41. Crude B, C18 RP; MeOH. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

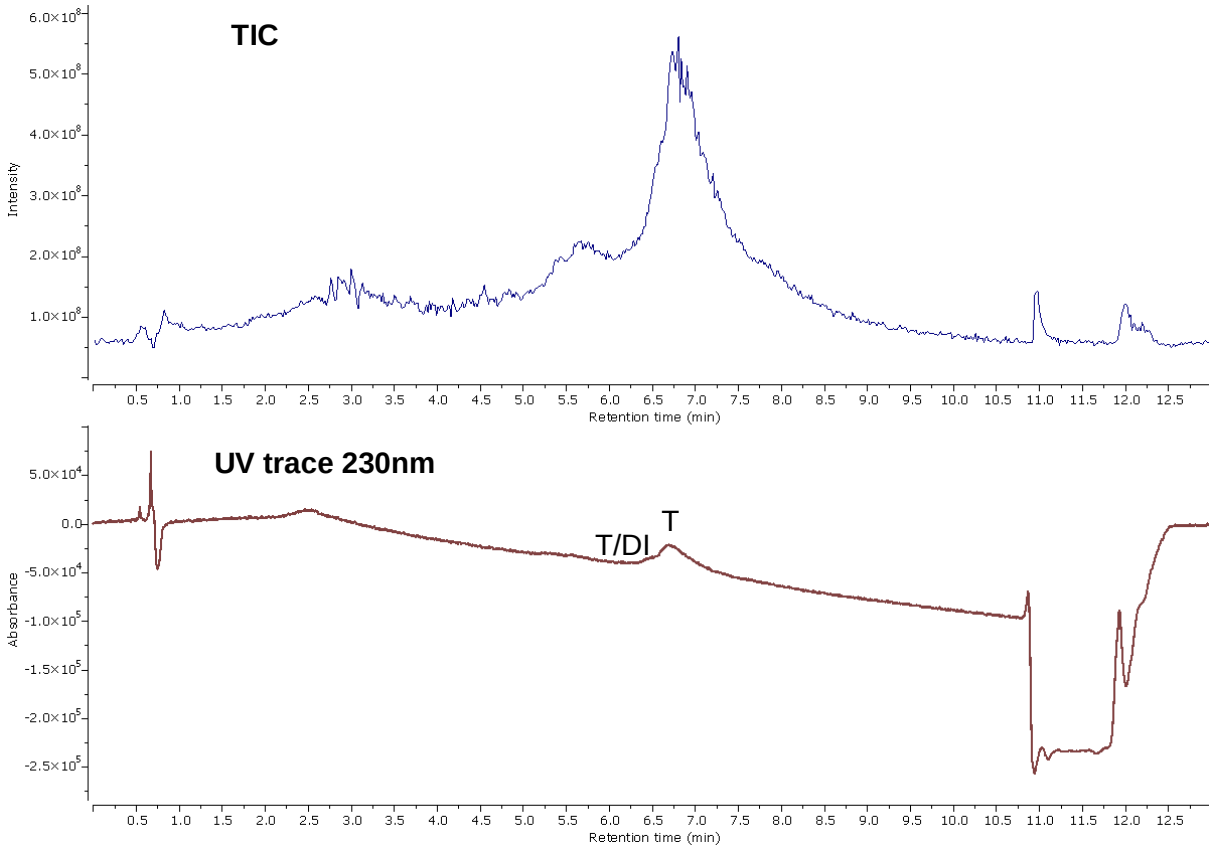

Figure A42. Crude B, Amide HILIC; MeOH. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

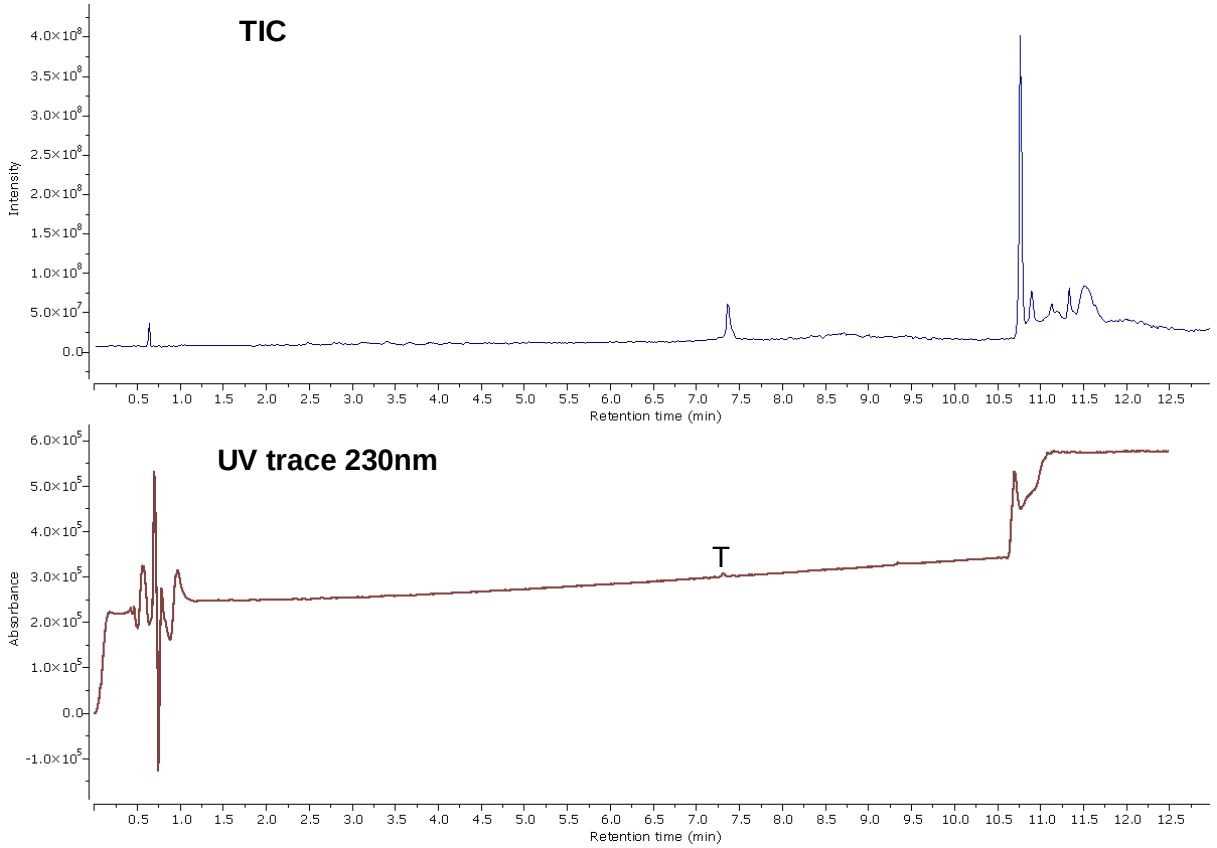

Figure A43. Crude C, C18 RP; ACN. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

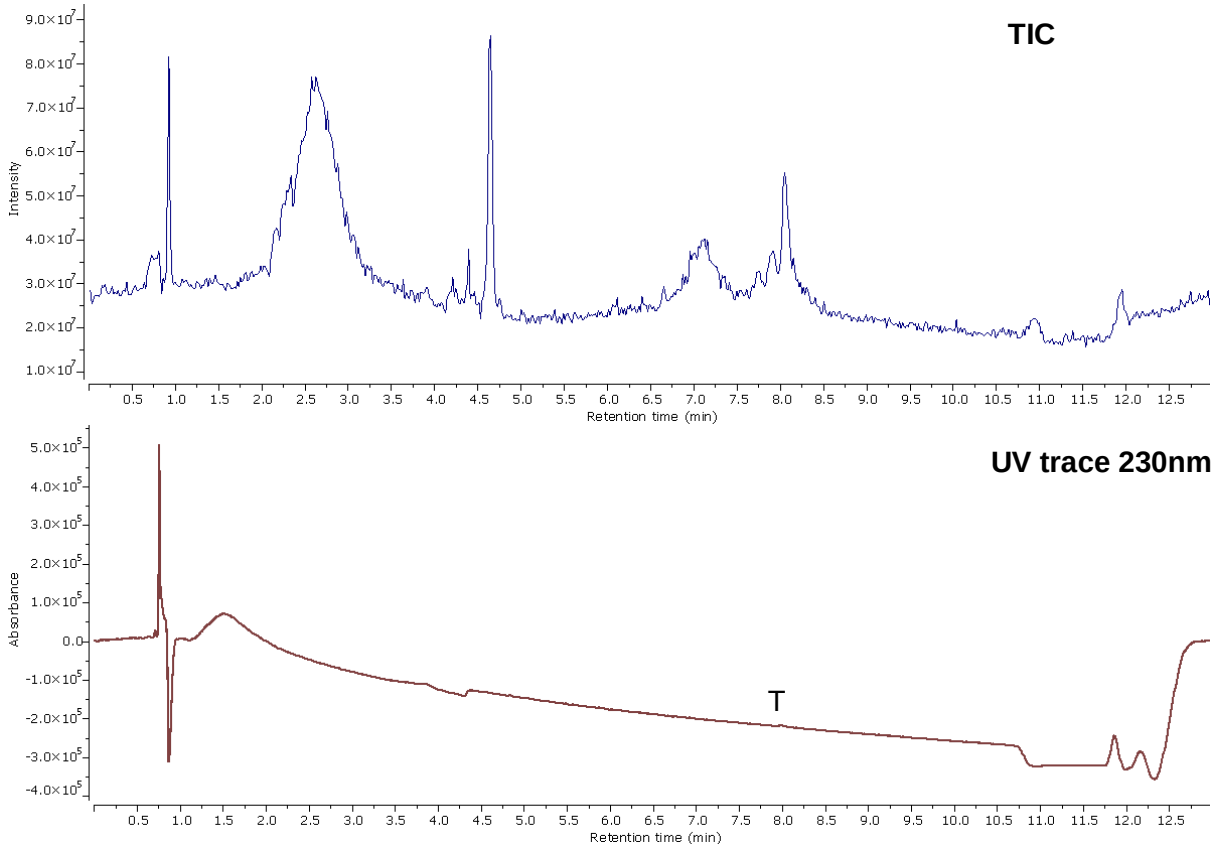

Figure A44. Crude C, Amide HILIC; ACN. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

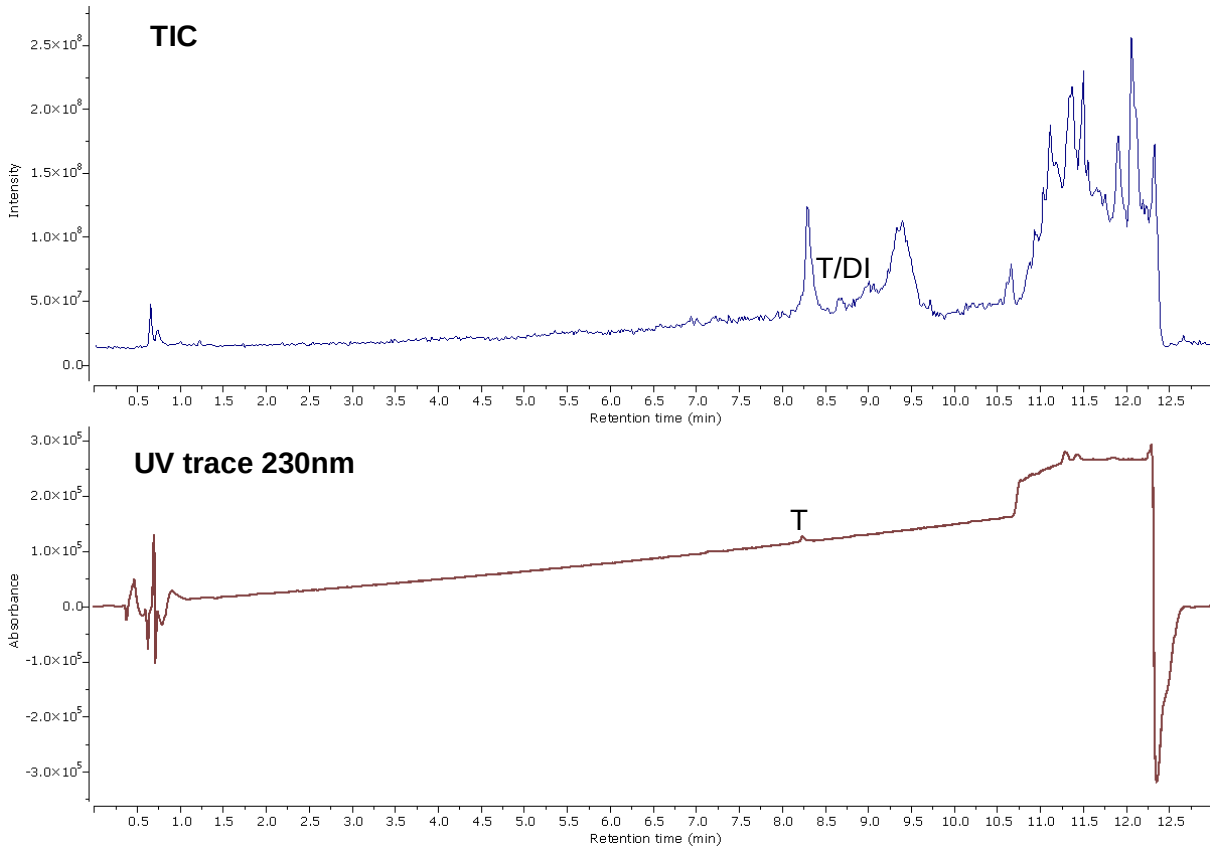

Figure A45. Crude C, C18 RP; MeOH. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

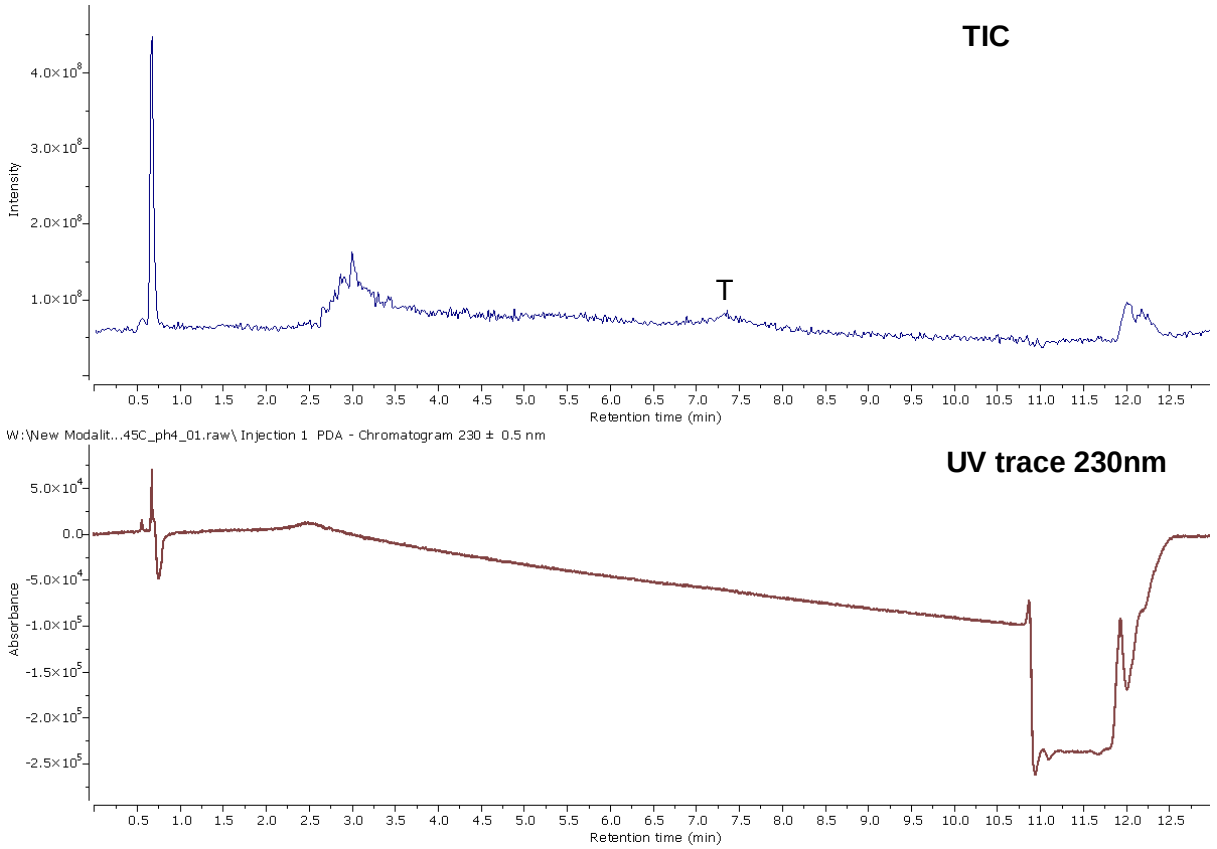

Figure A46. Crude C, Amide HILIC; MeOH. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

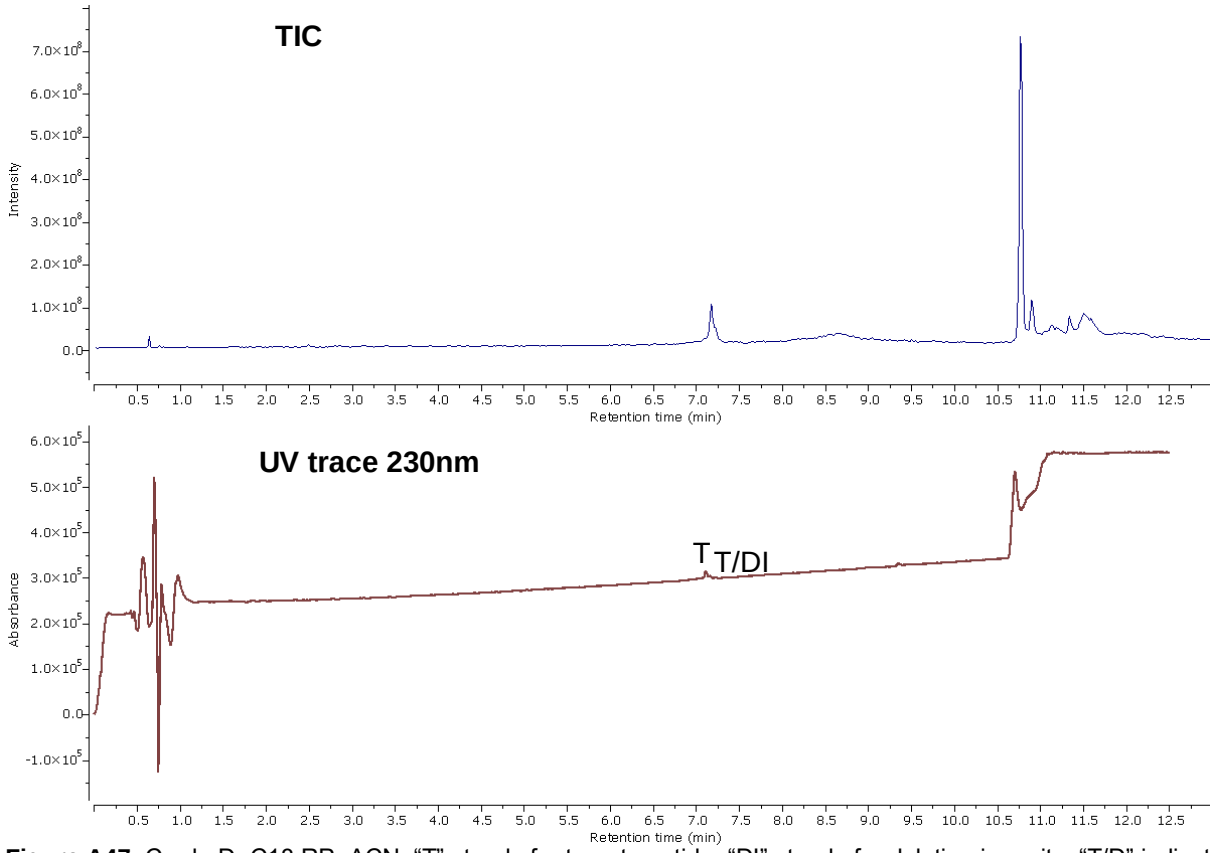

Figure A47. Crude D, C18 RP; ACN. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

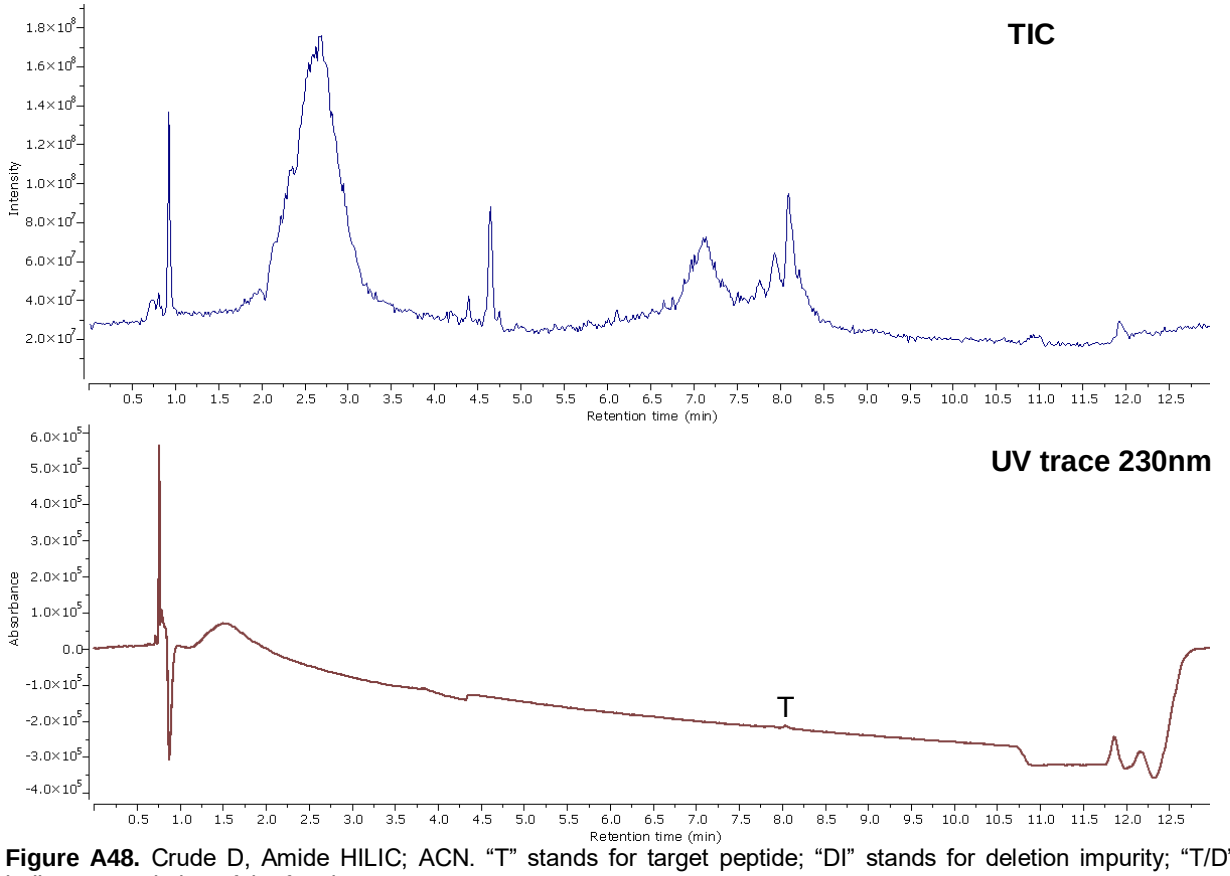

Figure A48. Crude D, Amide HILIC; ACN. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

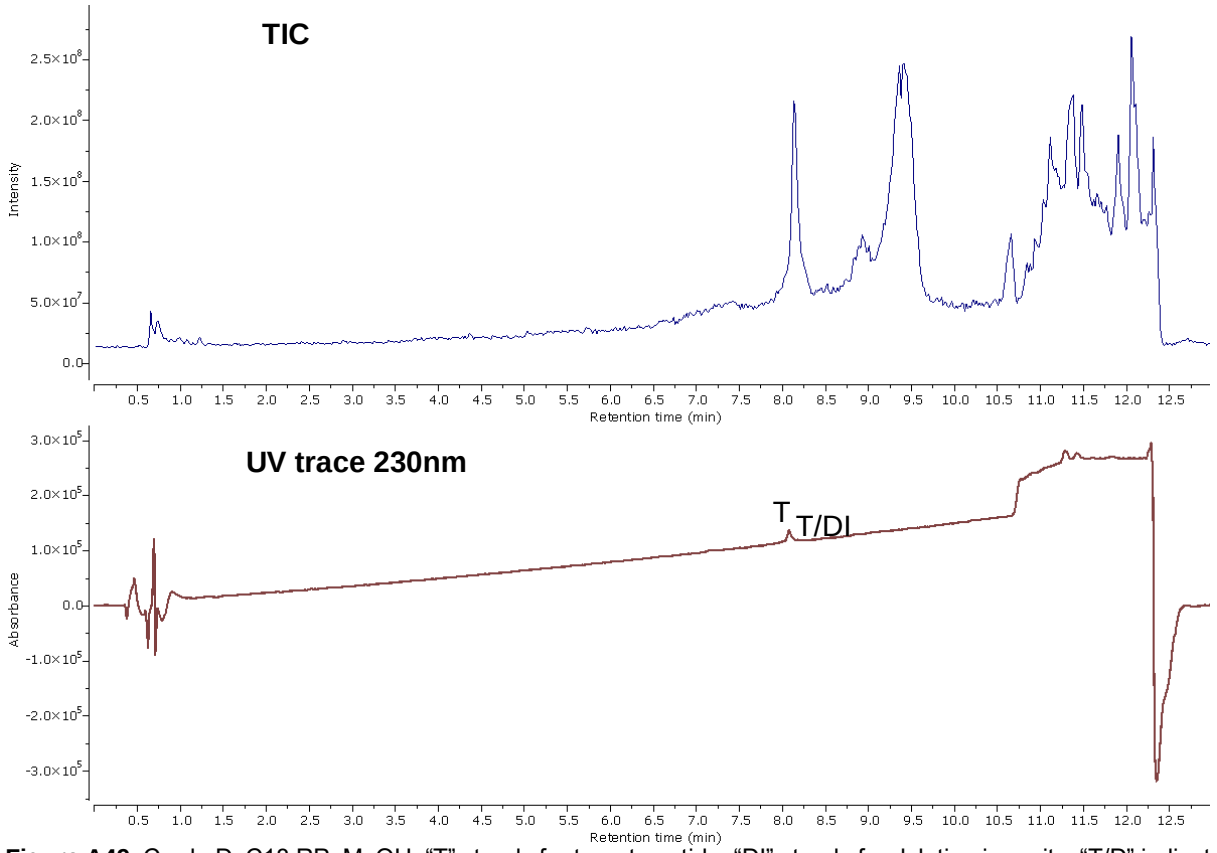

Figure A49. Crude D, C18 RP; MeOH. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

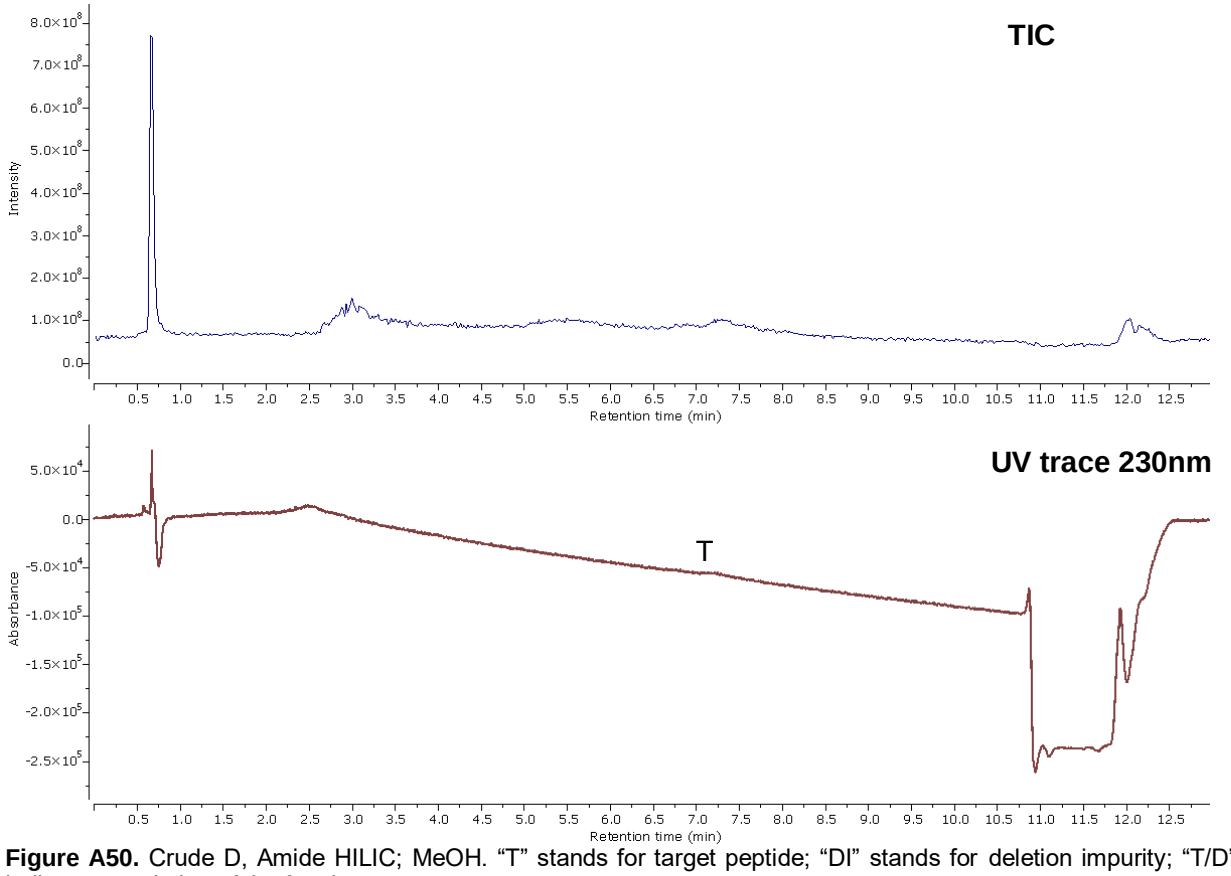

Figure A50. Crude D, Amide HILIC; MeOH. “T” stands for target peptide; “DI” stands for deletion impurity; “T/D” indicates co-elution of the fractions.

8.4 Evaluation of SFC methods for peptide analysis: chromatograms

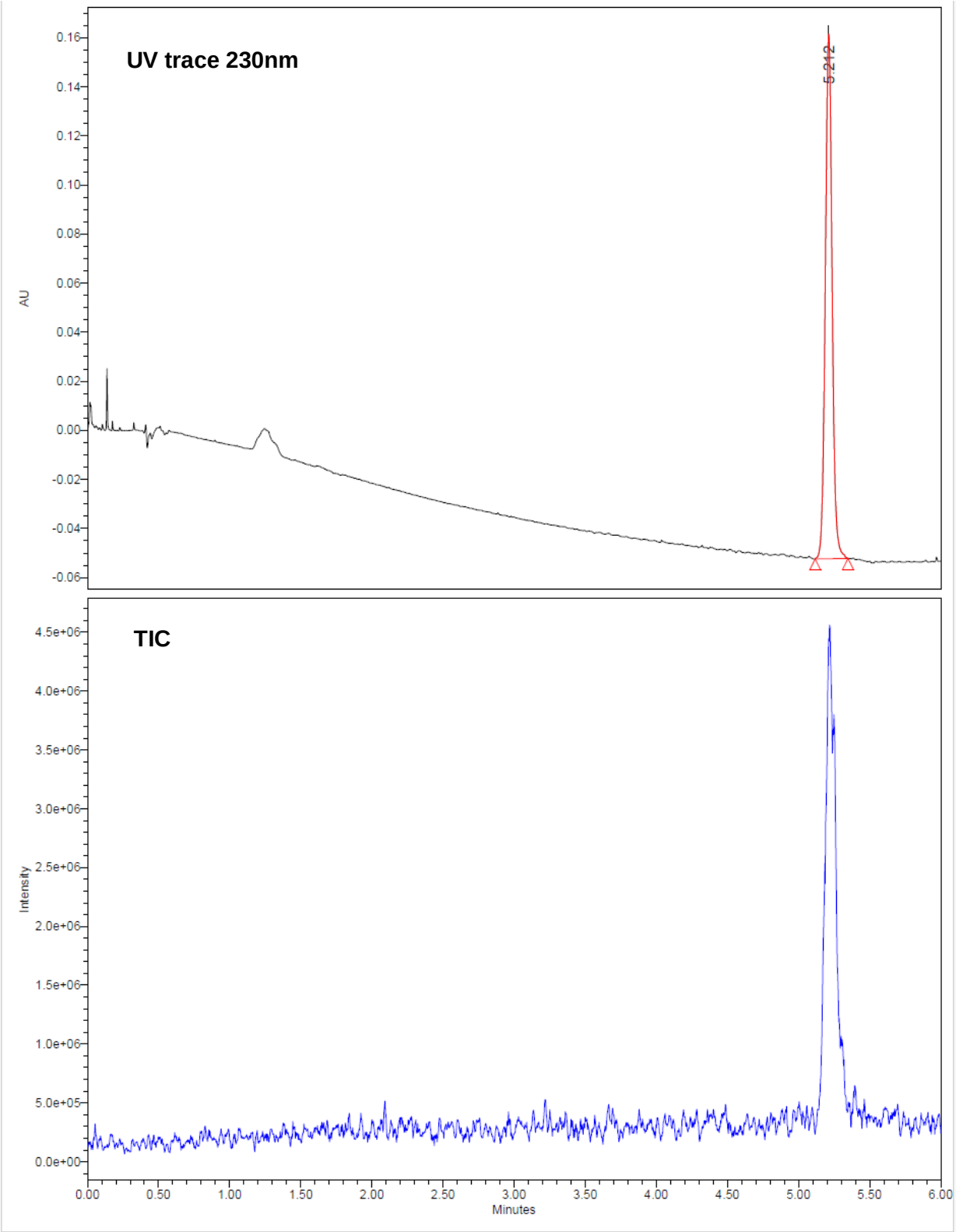

Figure A51. SFC separation of somatostatin (cyclic) on a GreenSep Basic (GSBC) column using MSA.

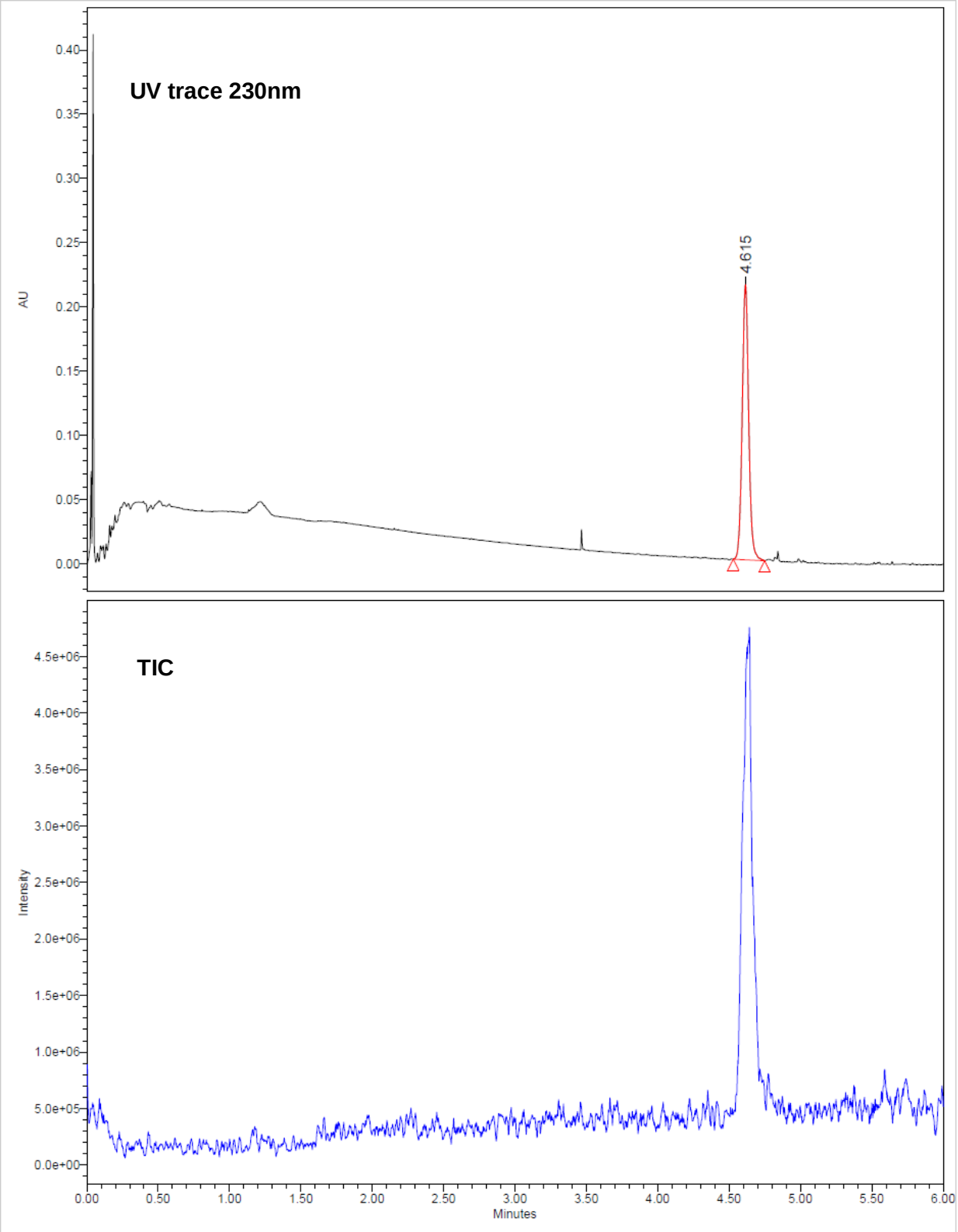

Figure A52. SFC separation of somatostatin (cyclic) on a GreenSep Basic (GSBC) column using ESA.

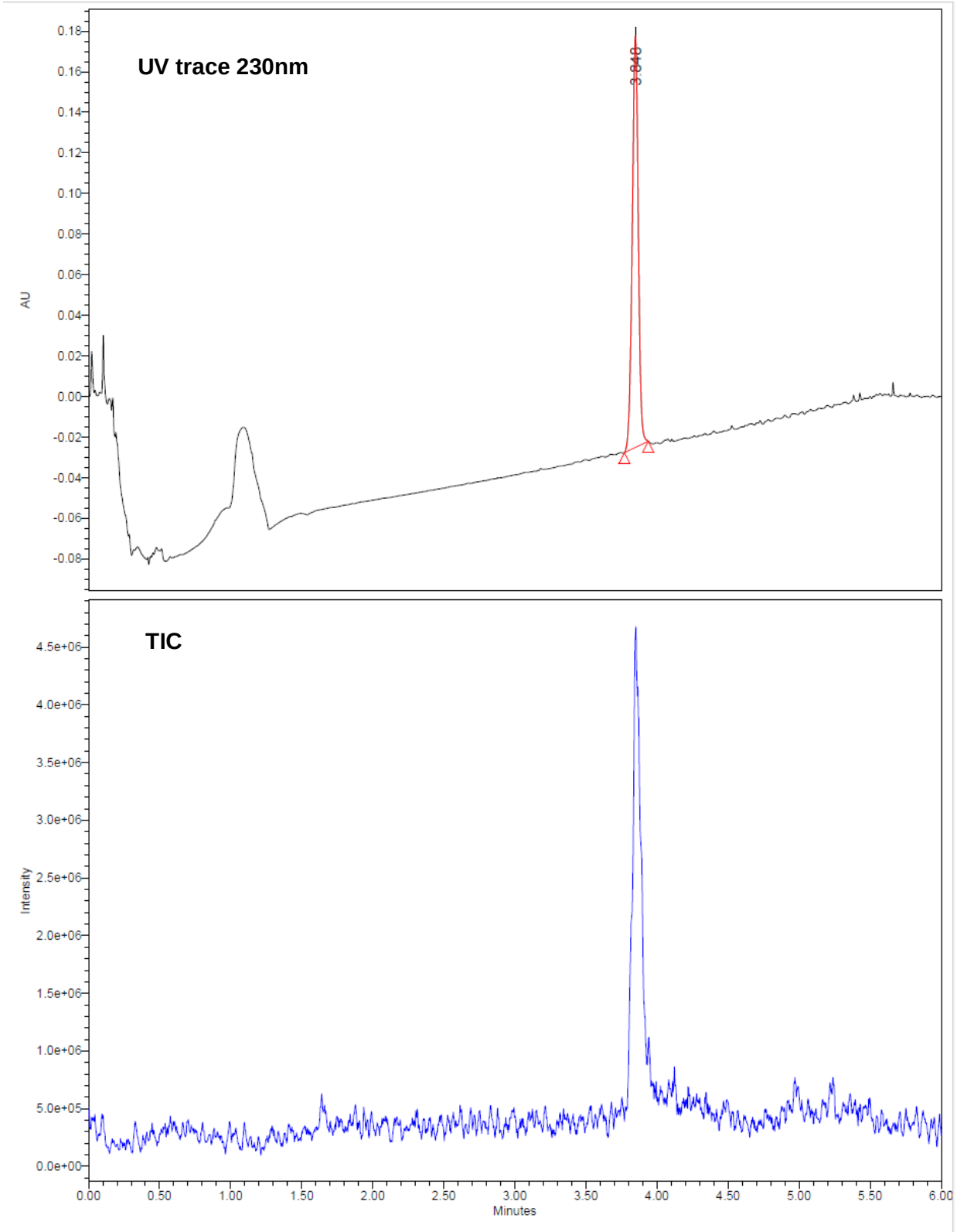

Figure A53. SFC separation of somatostatin (cyclic) on a GreenSep Basic (GSBC) column using TFA.

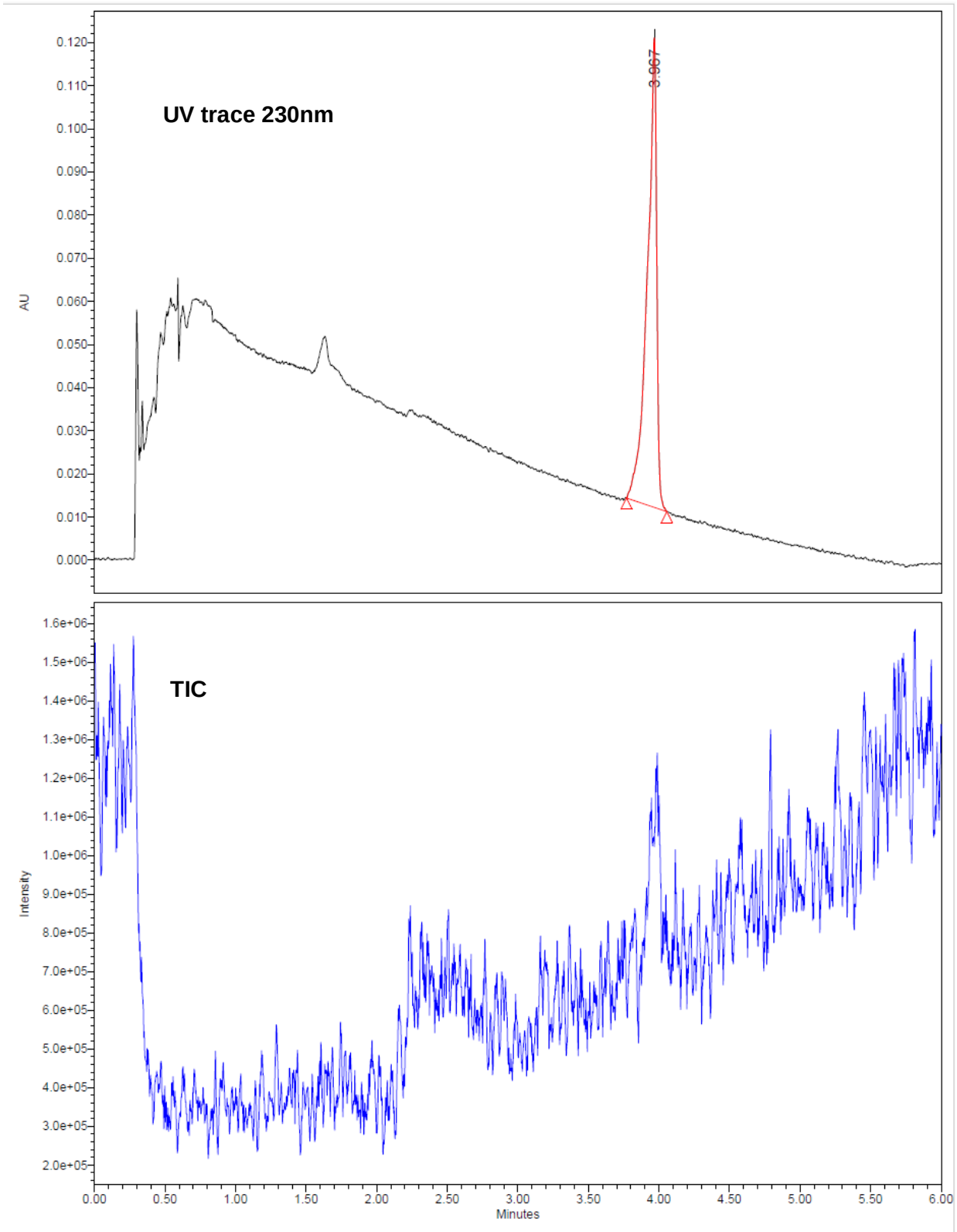

Figure A54. SFC separation of leucine enkephaline on a Diol column using MSA.

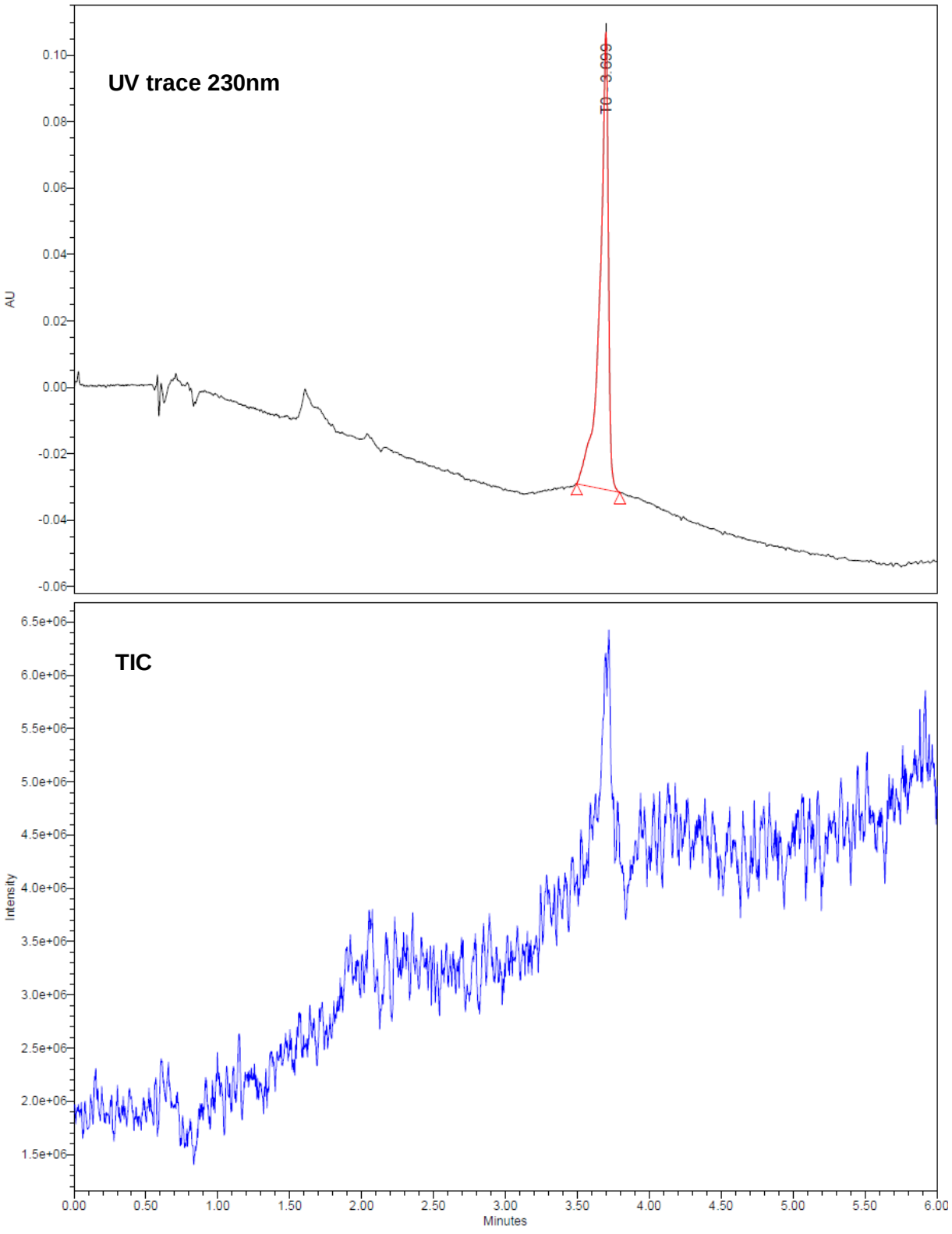

Figure A55. SFC separation of leucine enkephaline on a Diol column using ESA.

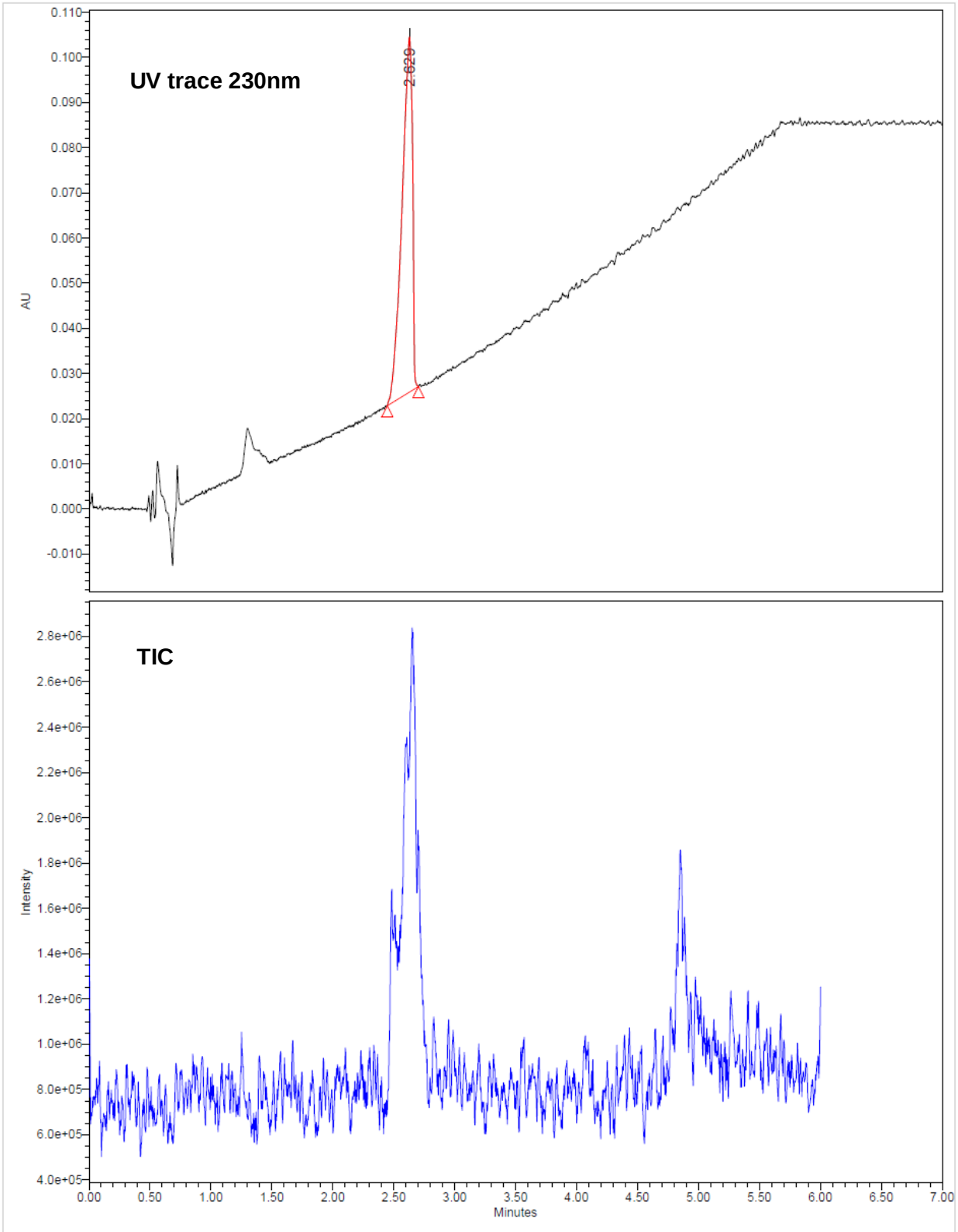

Figure A56. SFC separation of leucine enkephaline on a Diol column using TFA.

8.5 Supplemental material

Table A1. Tested columns and conditions

	Mobile phases			
Columns	MP1	MP2	MP3	MP4
Amide BEH	✓	✓		
PMPC	✓	✓		
PTZ	✓		✓	✓
P4VP	✓	✓		
Luna Polar P.	✓	✓	✓	

Table A2 – pKa values of acidic additives in aqueous solution at room temperature

Additive	pKA
TFA	0.3
ESA	-1.9
MSA	-1.68

Table A3. Heat map of peptide retention times across stationary phases and mobile phases. The table displays retention time (minutes) for a panel of peptides (see Table 1) measured on multiple columns and mobile-phase modifiers. Cell colours encode relative retention within the displayed range: green denotes mid-range values (30–80%), yellow denotes low retention (<30%), and blue denotes high retention (>80%). The symbol "X" marks conditions under which the peptide was too strongly retained (no elution within the run window).

	CN			GSBC			GSFS		
Peptides	TFA	ESA	MSA	TFA	ESA	MSA	TFA	ESA	MSA
Desmopressin	3.42	3.07	3.36	2.68	5.07	5.78	2.41	5.06	5.13
Angiotensin	3.54	2.63	2.81	2.11	x	4.16	1.81	3.61	3.66
Triptorelin	2.66	3.05	3.25	x	4.65	5.21	x	4.57	4.59
Leuprolide	2.50	2.75	3.03	x	3.63	4.14	x	3.47	3.54
ACTH	X	4.17	x	x	x	x	3.47	x	x
Liraglutide	4.48	3.47	3.73	3.15	x	5.92	2.98	x	5.52
Carbetocin	2.55	2.36	2.48	x	3.21	3.81	x	2.89	2.98
Leucine enkephaline	2.85	1.86	1.94	1.59	2.55	2.89	1.21	2.09	2.11
Somatostatin Cyclic	2.58	3.44	3.41	3.91	4.62	5.21	3.72	4.07	4.77

	PMPC			PTZ			P4VP		
Peptides	TFA	ESA	MSA	TFA	ESA	MSA	TFA	ESA	MSA
Desmopressin	x	3.61	x	x	x	x	4.20	x	x
Angiotensin	x	x	5.57	x	x	x	3.75	4.71	5.22
Triptorelin	x	x	x	x	x	x	x	x	X
Leuprolide	x	4.59	x	x	x	x	x	3.77	4.98
ACTH	x	X	x	x	x	x	x	x	x
Liraglutide	X	x	x	x	x	x	4.80	x	x
Carbetocin	3.00	3.77	3.67	x	x	x	x	x	5.10
Leucine enkephaline	4.92	3.73	4.17	x	x	x	3.15	3.73	4.00
Somatostatin Cyclic	x	x	x	x	x	x	x	x	x

	Diol		
Peptides	TFA	ESA	MSA
Desmopressin	4.53	x	x
Angiotensin	3.65	x	x
Triptorelin	X	x	x
Leuprolide	X	x	x
ACTH	5.47	x	x
Liraglutide	4.81	x	x
Carbetocin	x	3.95	4.15
Leucine enkephaline	2.63	3.70	3.97
Somatostatin Cyclic	4.96	5.33	x