A NEW LC-MS APPROACH FOR SYNTHETIC PEPTIDE CHARACTERIZATION AND IMPURITY PROFILING

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INTRODUCTION

- **Peptide therapeutics are an emerging group of pharmaceuticals applicable to a wide range of medical challenges.**
- **These peptide drugs exhibit relatively low toxicity and high biological activity compared to most conventional drugs.[1,2]**
- **Chemical synthesis is one of the most common method of production for synthetic peptides**
- **While the low toxicity of these drug products makes them more appealing as therapeutics, the impurities introduced during chemical synthesis of these peptides demand thorough characterization procedures to maintain efficacy and safety of the drug.**
- **The conventional synthetic peptide impurity profiling methods are mostly LC optical based assays relying on chromatographic separation of impurities.**
- **Even at optimal chromatographic performance, obtaining baseline resolved peaks for optical detection of low abundance impurities is a challenge. Adding HRMS to the analysis resolves this by improving the detection and identification of low level impurities based on the accurate mass and MS/MS information.**
- **In this study, we have developed a single LC-HRMS based analytical workflow for characterization and impurity profile monitoring of synthetic peptide drugs.**

METHODS

Sample preparation

The synthetic peptide *Eledoisin* (pEPSKDAFIGLM-NH_2) was purchased from New England Peptides Ins (Gardner, MS) and *Calcitonin (Salmon)* (CSNLTCVLGKLSQELHKLQTYPRTNTGSGTP-NH_2) was purchased from Bachem (Torrance, CA). A 2 µg/µL stock solution in water was further diluted to a final concentration of 0.2 µg/µL for the analysis.

LC condition

LC system: ACQUITY UPLC H-Class Bio System
Column: ACQUITY UPLC Peptide CSH C_{18} 130 Å, 1.7 µm, 2.1 mm x 100 mm
Column temperature: 65 °C
Mobile phase: A: 0.1% FA in H_2O and B: 0.1% FA in Acetonitrile
Gradient: Eledoisin: 16-24% B over 30 min, Calcitonin: 14-34% B over 20 min

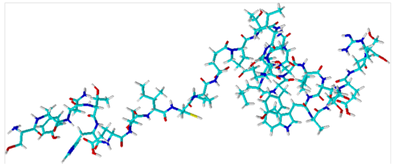
MS condition

MS instrumentation: Vion IMS QTof mass spectrometer
Capillary voltage: 2.8 kV
Cone voltage: 50 °C, Source offset: 50 °C, Source temperature: 80 °C
Desolvation Temp 300 °C, Cone gas: 20 L/hr, Desolvation gas: 500 L/hr

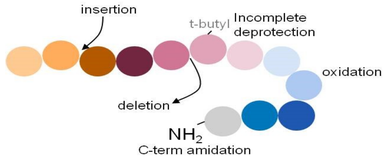
Informatics

UNIFI Scientific Information System 1.9.2 (Peptide mapping, screening workflows)

METHODS



API + Impurity



UNIFI Workflow Method

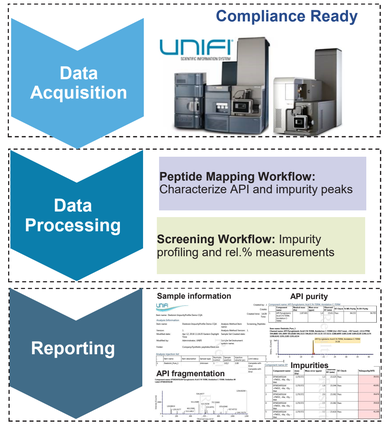


Figure 1. The schematic shows the analytical workflow used in synthetic peptide impurity characterization and profiling. The UNIFI software platform controls data acquisition, processing and reporting.

RESULTS

Peptide Mapping Workflow: Synthetic Peptide/Impurity Characterization

Method editor impurity identification

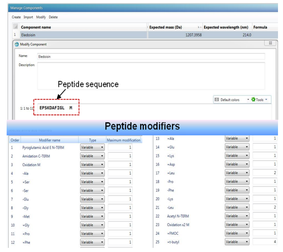


Figure 2. The modifications for impurity identification are selected from the method editor. The figure shows the modifiers of synthetic impurities used in Eledoisin impurity analysis such as: pyroglutamic acid modification (1), insertion and deletion of amino acids (4-21), addition of FOMC, and t-butyl groups due to incomplete deprotection (24, 25) and peptide degradation-related products (3,23)

LC-UV-HRMS for synthetic peptide API/impurity identification

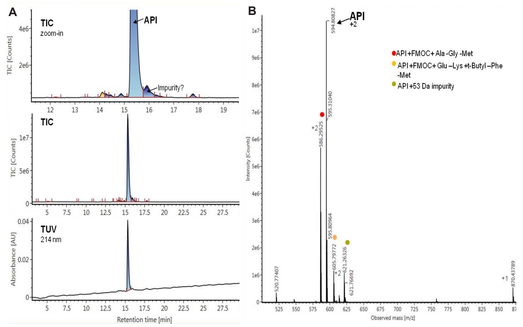


Figure 3. A) TUV, TIC and zoomed-in TIC spectra are shown for the Eledoisin API peak. B) The API peak has co-eluting impurity peptides detected by high-resolution mass spectrometry. The most abundant peaks are shown on the spectrum.

Validation of API/impurity using MS/MS

Eledoisin API: pEPSKDAFIGLM-NH_2 | Impurity: pEPSKDAFIGLM-NH_2 + Ox | Calcitonin API: CSNLSTCVLGKLSQELHKLQTYPRT NTGSGTP-NH_2

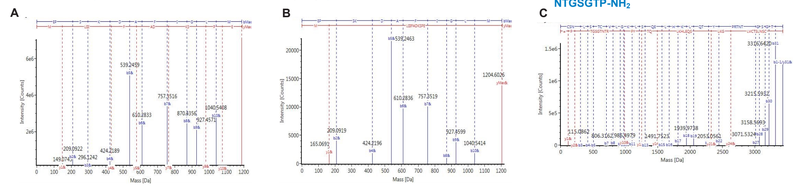
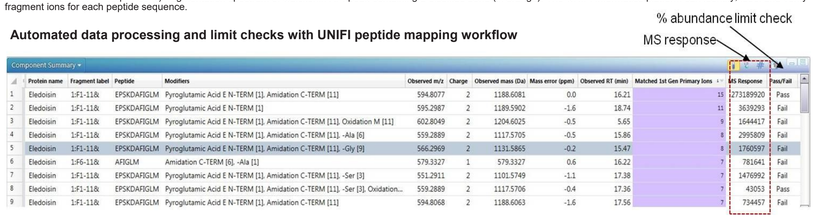


Figure 4. The fragmentation spectra for A) Eledoisin API sequence containing two modifications; N-terminal pyroglutamic acid and C-terminal amidation. B) fragmentation spectrum of oxidized Eledoisin API sequence C) fragmentation spectrum of Calcitonin API peak containing a disulfide bond (in orange). The UNIFI informatics platform can identify, label and verify fragment ions for each peptide sequence.

Automated data processing and limit checks with UNIFI peptide mapping workflow



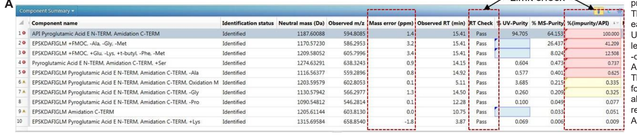
	Protein name	Fragment label	Peptide	Modifiers	Observed m/z	Charge	Observed mass (Da)	Mass error (ppm)	Observed RT (min)	Matched 1st Gen Primary Ions	MS Response	Pass/Fail
1	Eledoisin	1:F1-11:b	EPSKDAFIGLM	Pyroglutamic Acid E N-TERM [1], Amidation C-TERM [11]	594.8077	2	1188.6081	0.0	16.21	13	1273189920	Pass
2	Eledoisin	1:F1-11:b	EPSKDAFIGLM	Pyroglutamic Acid E N-TERM [1]	595.2987	2	1189.5902	-1.6	18.74	11	3639293	Fail
3	Eledoisin	1:F1-11:b	EPSKDAFIGLM	Pyroglutamic Acid E N-TERM [1], Amidation C-TERM [11], Oxidation M [11]	602.8049	2	1204.6025	-0.5	5.65	9	1644417	Fail
4	Eledoisin	1:F1-11:b	EPSKDAFIGLM	Pyroglutamic Acid E N-TERM [1], Amidation C-TERM [11], -Ala [6]	559.2889	2	1117.5705	-0.5	15.86	8	2995809	Fail
5	Eledoisin	1:F1-11:b	EPSKDAFIGLM	Pyroglutamic Acid E N-TERM [1], Amidation C-TERM [11], -Gly [9]	566.2969	2	1131.5865	-0.2	15.47	8	1760597	Fail
6	Eledoisin	1:F6-11:b	AFIGLM	Amidation C-TERM [6], -Ala [1]	579.3327	1	579.3327	0.6	16.22	7	781641	Fail
7	Eledoisin	1:F1-11:b	EPSKDAFIGLM	Pyroglutamic Acid E N-TERM [1], Amidation C-TERM [11], -Ser [3]	551.2911	2	1101.5749	-1.1	17.38	7	1476992	Fail
8	Eledoisin	1:F1-11:b	EPSKDAFIGLM	Pyroglutamic Acid E N-TERM [1], Amidation C-TERM [11], -Ser [3], Oxidation...	559.2889	2	1117.5706	-0.4	17.36	7	43053	Pass
9	Eledoisin	1:F1-11:b	EPSKDAFIGLM	Pyroglutamic Acid E N-TERM [1], Amidation C-TERM [11]	594.8068	2	1188.6063	-1.6	17.56	7	734457	Fail

Figure 5. The UNIFI review panel displays API and impurities identified. The MS response is used to determine the pass/fail status based on the relative% response compared to the MS response of the API.

Processed data Export to library → **UNIFI Scientific library: *Custom API & Impurity Library*** → *Import to screening workflow*

Screening Workflow: Synthetic Peptide/Impurity profiling

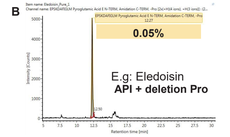
Impurity profiling using UNIFI screening workflow



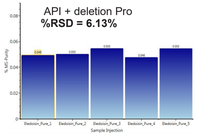
	Component name	Identification status	Neutral mass (Da)	Observed m/z	Mass error (ppm)	Observed RT (min)	RT Check	% UV-Purity	% MS-Purity	%(impurity/API)
1	API Pyroglutamic Acid E N-TERM, Amidation C-TERM	Identified	1187.60088	594.8085	1.4	15.41	Pass	94.705	64.153	100.000
2	EPSKDAFIGLM +FMOC, -Ala, -Gly, -Met	Identified	1170.57230	586.2953	3.2	15.41	Pass		26.437	41.209
3	EPSKDAFIGLM +FMOC, +Glu, -Lys, +t-butyl, -Phe, -Met	Identified	1209.58052	605.7996	3.4	15.41	Pass		8.024	12.508
4	Pyroglutamic Acid E N-TERM, Amidation C-TERM, +Ser	Identified	1274.63291	638.3243	0.9	14.15	Pass	0.604	0.473	0.737
5	EPSKDAFIGLM Pyroglutamic Acid E N-TERM, Amidation C-TERM, -Ala	Identified	1116.56777	559.2896	0.8	14.92	Pass	0.577	0.403	0.625
6	EPSKDAFIGLM Pyroglutamic Acid E N-TERM, Amidation C-TERM, Oxidation M	Identified	1203.59579	602.8053	0.1	5.12	Pass	3.685	0.215	0.335
7	EPSKDAFIGLM Pyroglutamic Acid E N-TERM, Amidation C-TERM, -Gly	Identified	1130.57942	566.2977	1.3	14.50	Pass	0.260	0.209	0.325
8	EPSKDAFIGLM Pyroglutamic Acid E N-TERM, Amidation C-TERM, -Pro	Identified	1090.54812	546.2814	0.1	12.28	Pass	0.100	0.049	0.077
9	EPSKDAFIGLM Amidation C-TERM	Identified	1205.61144	603.8130	0.0	10.79	Pass		0.034	0.051
10	EPSKDAFIGLM Pyroglutamic Acid E N-TERM, Amidation C-TERM, +Lys	Identified	1315.69584	658.8540	-1.8	3.87	Pass	0.069	0.006	0.009

Figure 6. This shows the impurity profiling in the screening workflow. A) The panel presents the purity levels of each modification using both MS and UV responses. The %(impurity/API) levels are flagged in color based on pre -defined limits. B) The XIC for Eledoisin API-Proline (sequence deletion). C) The summary plot displays the %RSD for the respective modification at 0.05% abundance level. D) The sample repeatability statistics for a given list of API and impurities is presented here.

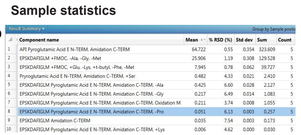
XIC for impurity abundance calculations



Summary plots



Sample statistics



	Component name	Mean	% RSD (%)	Std dev	Sum	Count
1	API Pyroglutamic Acid E N-TERM, Amidation C-TERM	64.722	0.55	0.354	323.609	5
2	EPSKDAFIGLM +FMOC, -Ala, -Gly, -Met	25.906	1.19	0.308	129.528	5
3	EPSKDAFIGLM +FMOC, +Glu, -Lys, +t-butyl, -Phe, -Met	7.945	0.78	0.062	39.727	5
4	Pyroglutamic Acid E N-TERM, Amidation C-TERM, +Ser	0.482	4.33	0.021	2.410	5
5	EPSKDAFIGLM Pyroglutamic Acid E N-TERM, Amidation C-TERM, -Ala	0.425	6.60	0.028	2.127	5
6	EPSKDAFIGLM Pyroglutamic Acid E N-TERM, Amidation C-TERM, -Gly	0.217	6.49	0.014	1.083	5
7	EPSKDAFIGLM Pyroglutamic Acid E N-TERM, Amidation C-TERM, Oxidation M	0.211	3.74	0.008	1.055	5
8	EPSKDAFIGLM Pyroglutamic Acid E N-TERM, Amidation C-TERM, -Pro	0.051	6.13	0.003	0.257	5
9	EPSKDAFIGLM Amidation C-TERM	0.015	7.54	0.003	0.173	5
10	EPSKDAFIGLM Pyroglutamic Acid E N-TERM, Amidation C-TERM, +Lys	0.006	4.62	0.000	0.030	5

CONCLUSION

The analytical workflow here demonstrates how a UPLC-QTof platform controlled by UNIFI Scientific Information System can be used to identify the API and it's impurities of a synthetic therapeutic peptide sample. The key benefits of the workflow are:

- **The instrument-informatics platform is a compliant-ready system that can be validated and implemented in regulated laboratory environments.**
- **The automated peptide mapping workflow identifies impurities based on accurate mass and validates the assignments using MS/MS data.**
- **The workflow can be used to characterize API and impurity sequences that are linear or cyclic.**
- **The scientific library can be used to a built custom impurity library for any selected peptide sequence.**
- **The screening workflow is utilized in robust high throughput profiling therapeutic peptide impurities.**

References:

1. Mason, J. M., Design and development of peptides and peptide mimetics as antagonists for therapeutic intervention. *Future Medicinal Chemistry* **2010,** *2* (12), 1813-1822.
2. 2. Pernot, M.; Vanderesse, R.; Frochot, C.; Guillemin, F.; Barberi-Heyob, M., Stability of peptides and therapeutic success in cancer. *Expert Opinion on Drug Metabolism & Toxicology* **2011,** *7* (7), 793-802.

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- The conventional synthetic peptide impurity profiling methods are mostly LC-optical-based assays relying on chromatographic separation of impurities.
- Even at optimal chromatographic performance, obtaining baseline resolved peaks for optical detection of low abundance impurities is a challenge. Adding HRMS to the analysis resolves this by improving the detection and identification of low level impurities based on the accurate mass and MS/MS information.
- LC-MS based monitoring of impurity profile provides both accurate mass and relative abundance information that benefits process development and quality control.
- In this study, we have developed LC-HRMS-based analytical workflows for characterization and impurity profile monitoring of synthetic peptide drugs.