

# Impurity profiling of the synthetic peptide LL-37 using high-performance liquid chromatography with combined UV and single quadrupole mass spectrometric detection

## Author

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## Keywords

Impurity profiling, synthetic peptide, ISQ EM single quadrupole mass spectrometer, high-performance liquid chromatography, Vanquish Flex Binary UHPLC, LL-37, quality control, LC-MS, UHPLC

## Goal

Leveraging UV detection for the determination of the impurity levels and mass detection for identification of the active pharmaceutical ingredient (API) and other product-related impurities using a rapid method for synthetic peptide impurity profiling.

## Application benefits

- For a synthetic peptide impurity profiling, a rapid method with combined UV and single quadrupole MS detection was used.
- The extended mass range of the Thermo Scientific™ ISQ™ EM single quadrupole mass spectrometer of up to  $m/z$  2000 enables the detection of low charged peptide species and increases the confidence of identification.

## Introduction

To ensure high quality in the production of synthetic peptides, simple, rapid and reliable analytical methods to determine the purity of the peptide produced are needed. Analysis is often done with high-performance liquid chromatography (HPLC) with ultraviolet-detection (UV). However, sometimes it can be challenging to separate and detect process- and product-related impurities that were formed along with the main component during the production since they can be present in much lower concentrations and can often be structurally very similar. In addition, product-related impurities are often unknown and must be identified, which leads to a need for further advanced analytical techniques, such as mass spectrometry (MS). Synthetic peptides are mainly manufactured by either one of the two different technical approaches, solid phase peptide synthesis (SPPS) or solution phase peptide synthesis (SPS). The SPPS method is preferred today because this process can be fully automated. Simplified, in SPPS, one amino acid is coupled to the next, washed through between, and the process is repeated until the desired

peptide is completed.<sup>1</sup> Knowledge about the chemicals involved, including resins, amino acids, and reagents used in coupling and deprotection is helpful in assessing the resulting impurities during the production. Additionally, the success of the manufacturing process should be controlled.

In the current work, peptide impurity profiling and mass-based compound confirmation were demonstrated for the antimicrobial human LL-37 peptide, a compound of high medical importance due to its antibacterial, antimycotic, antiviral, wound healing, anticancer, and immunomodulatory activity.<sup>2</sup> The sequence consists of 37 amino acids with a peptide mass of about 4500 Da. The level of impurities was determined with UV detection, and the identity checked simultaneously by mass spectrometry. The extended mass range of the novel ISQ EM single quadrupole mass spectrometer enabled the detection of lower charge states of the selected peptides and increased the confidence of identification.

## Experimental

### Consumables

- Deionized water, 18.2 MΩ·cm resistivity or higher (P/N N/A)
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS Grade (P/N 10001334)
- Fisher Scientific™ Formic acid, Optima™ LC/MS Grade (P/N 10596814)
- Thermo Scientific™ Acclaim™ RSLC 120 C18 column, 50 × 2.1 mm, 2.2 μm (P/N 068981)

The synthetic peptides LL-37 (active pharmaceutical ingredient; API) and the two related fragments LL-37 RKS and LL-37 SKE were purchased from a reputable vendor.

### Preparation of standards

An overview of the peptide amino acid sequences used in the study is given in Table 1. A solution of 1 mg/mL of each peptide was prepared in mobile phase A (water + 0.1% formic acid). The fragment peptides LL-37 RKS (fragment 1) and LL-37 SKE (fragment 2) were spiked with a concentration of 150 μg/mL and 50 μg/mL, respectively, into a 500 μg/mL LL-37 (API) peptide solution to simulate a sample containing product related impurities.

### Instrumentation

A Thermo Scientific™ Vanquish™ Flex Binary UHPLC system equipped with an ISQ EM single quadrupole mass spectrometer was used for the analysis.

- Thermo Scientific™ Vanquish™ System Base Vanquish Horizon/Flex (P/N VH-S01-A-02)
- Thermo Scientific™ Vanquish™ Binary Pump F (P/N VF-P10-A-01)
- Thermo Scientific™ Vanquish™ Split Sampler FT (P/N VF-A10-A-02)
- Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A-02)
- Thermo Scientific™ Vanquish™ Variable Wavelength Detector F (P/N VF-D40-A)
- Flow Cell Semi-Micro, 2.5 μL, 7 mm light path (SST) (P/N 6077.0360)
- ISQ EM Mass Spectrometer (P/N ISQEM-ESI)

**Table 1. Peptide amino acid sequences and theoretical molecular mass**

Compound	Amino acid sequence	Theoretical average mass [Da]	Theoretical monoisotopic mass [Da]	Theoretical most abundant isotope mass [Da]
LL-37 (API)	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	4493.299	4490.565	4492.582
LL-37 RKS (fragment 1)	RKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	3800.488	3798.211	3799.225
LL-37 SKE (fragment 2)	SKEKIGKEFKRIVQRIKDFLR	2619.130	2617.544	2618.558

## Liquid chromatographic conditions

Column	Acclaim RSLC 120 C18, 50 × 2.1 mm, 2.2 μm	
Mobile phase	A: Water + 0.1% formic acid B: Acetonitrile + 0.1% formic acid	
Flow rate	0.5 mL/min	
Gradient	Time (min)	%B
	0	20
	2	50
	2.1	20
5.5	20	
Mixer volume	10 + 25 μL	
Column temperature	50 °C (forced air mode, fan speed 5)	
Autosampler temperature	4 °C	
UV wavelength	214 nm	
UV data collection rate	10 Hz	
UV response time	0.5 s	
Injection volume	1 μL	

## Mass spectrometry settings

### MS source parameters

Sheath gas pressure	49.9 psig
Aux gas pressure	5.7 psig
Sweep gas pressure	0.5 psig
Vaporizer temperature	282 °C
Ion transfer tube temperature	300 °C
Source voltage	3000 V

### MS method parameters

Method type	Full Scan
Ion polarity	Positive
Mass range	<i>m/z</i> 500–2000
Dwell time	0.2 s
Source CID voltage	10 V

## Data processing

The Thermo Scientific™ Chromeleon™ 7.2.9 Chromatography Data System (CDS) was used for data acquisition and processing.

## Results and discussion

In this study the UV data served for the determination of impurity levels and the MS data primarily for the identification of product related impurities in a synthetic peptide sample. The relative peak areas (Rel. Area) of associated impurities were calculated and evaluated based on the guidelines published by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).<sup>3</sup> As a result, Rel. Area obtained from UV, total ion current (TIC), and extracted ion chromatograms (XIC) were compared. The method also allows a screening for unknown impurities and increases the confidence in the results.

Figure 1 shows the UV chromatogram of the LL-37 peptide and the corresponding TIC chromatogram for a scan range of *m/z* 500–2000. The LL-37 peptide could be detected at retention time (RT) 1.7 min. Just from the UV chromatogram no additional peaks can be observed, indicating that there are no major impurities present in the sample. Nevertheless, co-eluting impurities cannot be excluded based on the UV trace alone. A major advantage of using MS spectral data is that the peak purity can be determined by running in full scan mode. However, the spectrum obtained also shows no significant additional mass peaks that would indicate co-elution of the API with other impurities (Figure 4).

Chromeleon CDS offers a feature to use custom columns in the sequence list to calculate the theoretical mass of peptides and other chemical components via an entry of the peptide sequence or the chemical formula. It also allows the calculation of different charge states and various adduct masses (Figure 2). This is a helpful tool in identifying the multiple charge states in the mass spectra of peptides and other biomolecules.

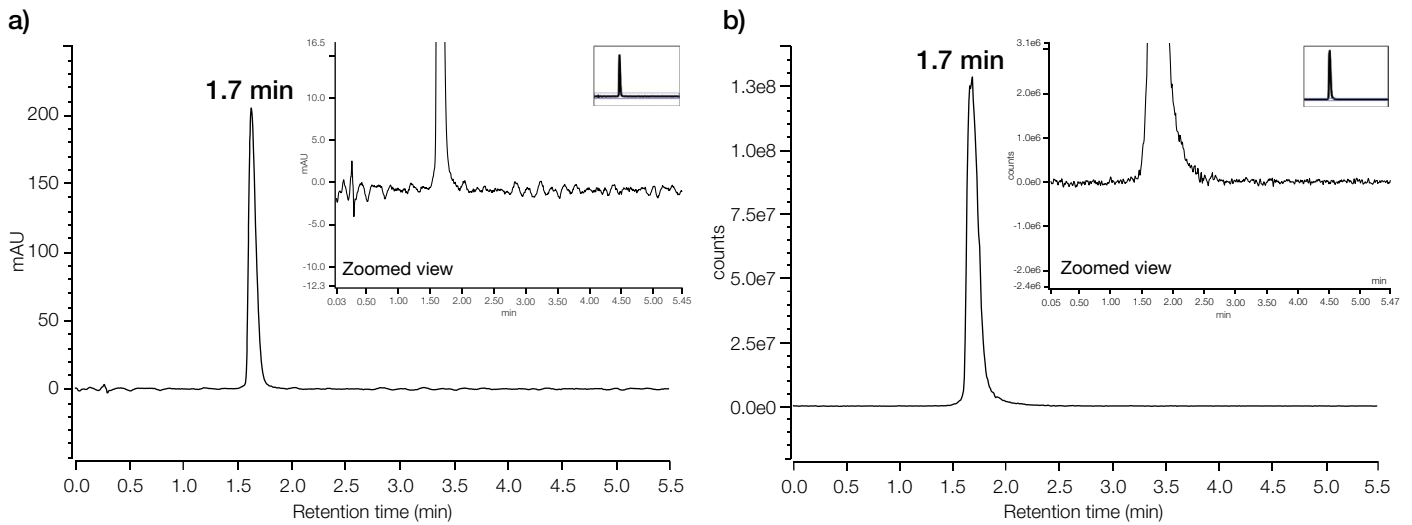


Figure 1. a) UV chromatogram at 214 nm and b) TIC chromatogram of the LL-37 peptide with zoom into the baseline

#	TIC	Name	*API_peptide_sequence	#API_Calculated most abundant Mass [M] [Da]	#API_Calculated most abundant Mass [M+4H] [m/z]	*Fragment_1_peptide_sequence	#Fragment_1_Calculated most abundant Mass [M] [Da]	#Fragment_1_Calculated most abundant Mass [M+4H] [m/z]	*Fragment_2_peptide_sequence	#Fragment_2_Calculated most abundant Mass [M] [Da]	#Fragment_2_Calculated most abundant Mass [M+4H] [m/z]
1	None	Blank		n.a.	n.a.		n.a.	n.a.		n.a.	n.a.
2	None	API + fragments	LLGDFFRKSKEKIGKEFKRIV...	4492.58213	1124.15281	RKSKEKIGKEFKRIVQRIKDF...	3799.22542	950.81363	SKEKIGKEFKRIVQRIKDFLR	2618.55776	655.64672
3	None	API + fragments	LLGDFFRKSKEKIGKEFKRIV...	4492.58213	1124.15281	RKSKEKIGKEFKRIVQRIKDF...	3799.22542	950.81363	SKEKIGKEFKRIVQRIKDFLR	2618.55776	655.64672
4	None	API + fragments	LLGDFFRKSKEKIGKEFKRIV...	4492.58213	1124.15281	RKSKEKIGKEFKRIVQRIKDF...	3799.22542	950.81363	SKEKIGKEFKRIVQRIKDFLR	2618.55776	655.64672
5	None	Blank		n.a.	n.a.		n.a.	n.a.		n.a.	n.a.

Figure 2. Screenshot of the Chromeleon injection list with added custom columns for theoretical mass calculations of the target components (light blue: API; light green: fragment 1; light red: fragment 2)

To simulate a product impurity profiling, two separate peptide fragments were spiked into the LL-37 peptide solution and measured under the same condition. Baseline separation could be achieved with a short gradient method within 2 min, as it is shown in Figure 3.

Due to the coupling to the ISQ EM mass spectrometer, the peaks can be assigned to the respective peptide by comparing the theoretical and the observed mass. The peak assignment would not have been possible based on the UV signal alone and without additional experiments.

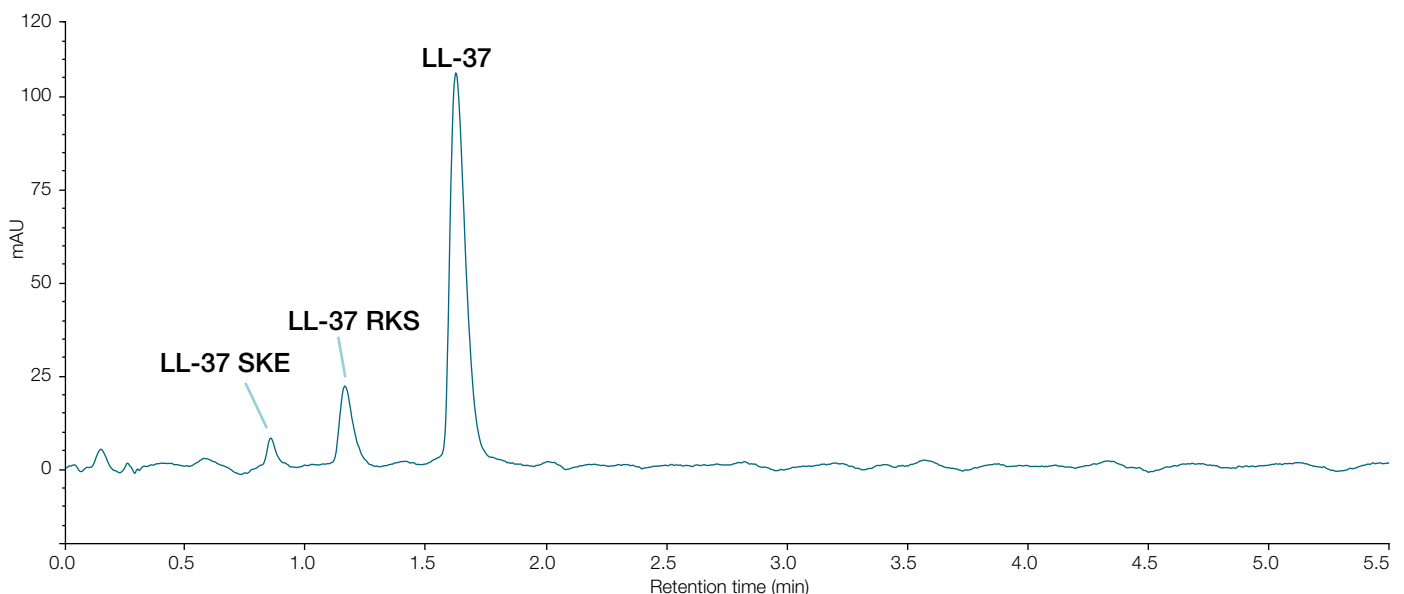


Figure 3. UV chromatogram at 214 nm of simulated peptide impurity profiling

The extended mass range of the ISQ EM mass spectrometer allows the detection of multiple charge states between 2 and 8 for the peptides used in the study (Figure 4). Low resolution mass spectrometers such as quadrupole spectrometers do not sufficiently resolve isotope masses at high charge states. Therefore, the average mass is used instead of the monoisotopic mass, as is common for high resolution mass spectra.

Table 2 summarizes the measured and theoretical  $m/z$  values (averaged and most abundant isotope) of all charge states obtained for the LL-37 peptide and the two fragments. The mass deviation between the averaged theoretical and the measured mass ( $\Delta m/z$ ) was found to be  $\leq 0.4$  for all charge states. The uncharged averaged mass was calculated for each charge state using Equation 1 and averaged, resulting in the intact deconvoluted average mass. The mass deviation for the intact molecule was  $\leq 0.5$  Da for all peptides.

$$M = z(m/z - H^+)$$

Equation 1. Calculation of the uncharged mass from the  $m/z$  value and the corresponding charge state

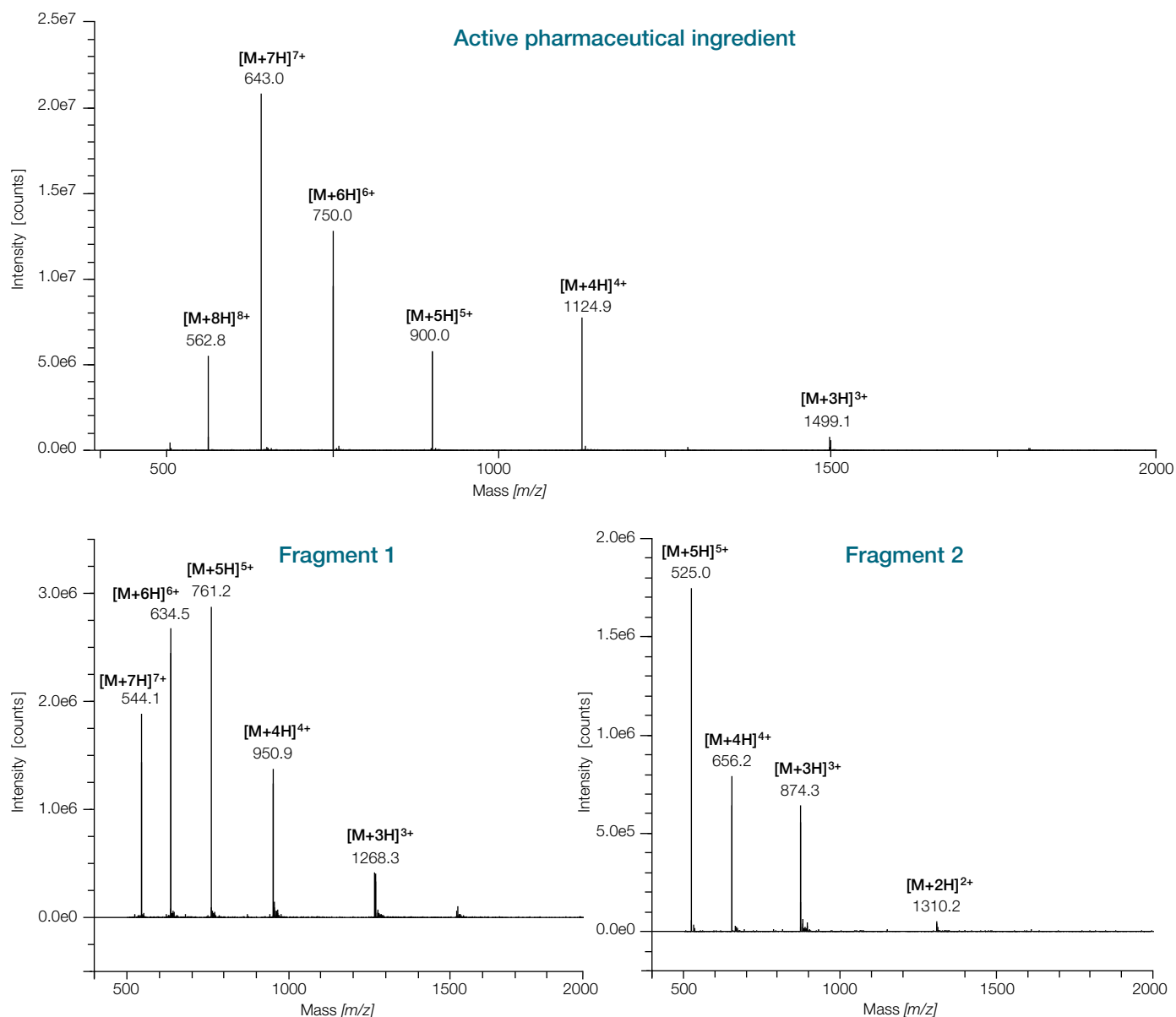


Figure 4. Obtained mass spectra for the API and the two fragments

Table 2. Comparison of theoretical and measured masses with corresponding absolute mass deviation of all assigned charge states in each peptide spectrum for Figure 4

LL-37 (API)					
Charge state	Measured average mass [m/z]	Measured most abundant mass [m/z]	Theoretical average mass [m/z]	Delta [m/z]	Calculated average mass [Da]
<b>[M]</b>			<b>4493.3</b>		
[M+1H] <sup>1+</sup>	—	—	4494.3	—	—
[M+2H] <sup>2+</sup>	—	—	2247.7	—	—
[M+3H] <sup>3+</sup>	1498.4	1499.1	1498.8	0.4	4492.2
[M+4H] <sup>4+</sup>	1124.2	1124.9	1124.3	0.1	4492.8
[M+5H] <sup>5+</sup>	899.5	900.0	899.7	0.2	4492.5
[M+6H] <sup>6+</sup>	750.0	750.0	749.9	-0.1	4494.0
[M+7H] <sup>7+</sup>	643.0	643.0	642.9	-0.1	4493.9
[M+8H] <sup>8+</sup>	562.8	562.8	562.7	-0.1	4494.3
<b>Deconvoluted average mass (n=6) [Da]</b>					<b>4493.3</b>
<b>Mass deviation to theoretical mass [Da]</b>					<b>-0.02</b>
LL-37 RKS (Fragment 1)					
<b>[M]</b>			<b>3800.5</b>		
[M+1H] <sup>1+</sup>	—	—	3801.5	—	—
[M+2H] <sup>2+</sup>	—	—	1901.3	—	—
[M+3H] <sup>3+</sup>	1267.5	1268.3	1267.8	0.3	3799.5
[M+4H] <sup>4+</sup>	950.9	950.9	951.1	0.2	3799.6
[M+5H] <sup>5+</sup>	761.2	761.2	761.1	-0.1	3801.0
[M+6H] <sup>6+</sup>	634.5	634.5	634.4	-0.1	3801.0
[M+7H] <sup>7+</sup>	544.1	544.1	543.9	-0.2	3801.6
[M+8H] <sup>8+</sup>	—	—	476.1	—	—
<b>Deconvoluted average mass (n=5) [Da]</b>					<b>3800.5</b>
<b>Mass deviation to theoretical mass [Da]</b>					<b>0.04</b>
LL-37 SKE (Fragment 2)					
<b>[M]</b>			<b>2619.1</b>		
[M+1H] <sup>1+</sup>	—	—	2620.1	—	—
[M+2H] <sup>2+</sup>	1310.2	1310.2	1310.6	0.4	2618.4
[M+3H] <sup>3+</sup>	873.7	874.3	874.1	0.4	2618.1
[M+4H] <sup>4+</sup>	655.5	656.2	655.8	0.3	2618.0
[M+5H] <sup>5+</sup>	525.0	525.0	524.8	-0.2	2620.0
[M+6H] <sup>6+</sup>	—	—	437.5	—	—
[M+7H] <sup>7+</sup>	—	—	375.2	—	—
[M+8H] <sup>8+</sup>	—	—	328.4	—	—
<b>Deconvoluted average mass (n=4) [Da]</b>					<b>2618.6</b>
<b>Mass deviation to theoretical mass [Da]</b>					<b>-0.5</b>

Typically, the UV signal response is used for relative quantitation in impurity analysis since the MS signal is highly dependent on the ionization efficiency for the individual analytes and ion suppression must be considered as well, which may lead to over- or underestimation of the analyte concentrations. Nevertheless, based on the higher sensitivity, MS data is often used to quantify the lower concentration levels, where UV detection is already reaching its limits. In Table 3, a comparison of relative peak areas obtained by the UV-, TIC- and XIC-traces is shown. The variation of the results clearly indicates how challenging the quantitation of product related impurities can be. The UV signal response is based on the strong peptide bond absorption at 214 nm. However, due to the quite different sequences the UV response is not uniform for the three peptides. Thus, the result obtained from relative peak areas differs from the theoretically calculated values, which should be 30% for fragment 1 and 10% for fragment 2, and can only serve as a rough estimation. For accurate quantification with UV, external calibration of each individual analyte should be performed or response factors need to be determined. In the case of MS quantification, isotope labeled standards are needed to accurately quantify, as MS responses are not uniform as well and ion suppression may occur.

## Conclusions

- The combination of UV and MS detection is beneficial for peptide impurity profiling due to the complementary information obtained about the analytes, e.g., peak purity and mass-based compound confirmation.
- The extended mass range up to  $m/z$  2000 enables the detection of low-charged species and allows the detection of the complete charge state profiles of mid-sized biomolecules.
- A rapid gradient method allowed the separation of the LL-37 peptide impurity profiling within 2 min.

## References

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**Table 3. Data for RT, Resolution, and Rel. Area of simulated impurities comparing UV, TIC and XIC trace with  $m/z$  525.0 for LL-37 SKE (fragment 2),  $m/z$  634.5 for LL-37 RKS (fragment 1) and  $m/z$  643.0 for LL-37 (API)**

Compound	RT [min]	Resolution	UV trace Rel. Area [%]	TIC trace Rel. Area [%]	XIC trace Rel. Area [%]
LL-37 SKE (fragment 2)	0.9	3.54	4.4	4.3	5.9
LL-37 RKS (fragment 1)	1.2	4.16	16.5	21.9	25.1
LL-37 (API)	1.7	—	100	100	100

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