Quality Control of Synthetic Biomolecules Using Rapid Methods with Serial Coupling of UV and MS Detectors

Sylvia Grosse, Katherine Lovejoy, Martin Samonig, Mauro De Pra, Frank Steiner, Martin Ruehl, Thermo Fisher Scientific, Dornierstrasse 4, 82110 Germering, Germany

ABSTRACT

Quality control is an important step during the production of synthetic biomolecules, both in peptide and oligonucleotide synthesis. HPLC-UV is commonly used to determine the purity of the API product. This technique is simple and efficient when the API is known. However, impurity analysis of the synthetic biomolecules may be quite challenging due to a wide variety of possible related impurities, quite often present at low concentration.

INTRODUCTION

Peptide synthesis distinguishes between process and product related impurities. In particular, the determination of the product related impurities is difficult as they are mostly not known. Therefore, mass detection is needed for reliable identification and confirmation. Oligonucleotide synthesis requires confident confirmation of oligonucleotide mass, as well as rough quantification of yield and impurity levels. Quantification of yield can easily be performed by UV. Rough estimation of impurities requires a mass detector, as aborted sequences (N-1) are not usually chromatographically separated from complete sequences (N) during a quick QC method. Mass confirmation of oligo identity also requires a mass detector.

Mass spectrometry is often considered to be too complex and too difficult to use in routine quality control applications. The Thermo Scientific™ ISQ™ EM Single Quadrupole Mass Spectrometer is developed for operation by chromatographers. Its full integration into the Thermo Scientific™ Chromeleon™ 7.2 chromatography data system (CDS) software and the Autospray smart method set-up make LC-MS operation and data analysis straightforward and intuitive. The ISQ EM mass spectrometer has a mass range from *m/z* 50 to 2000, allowing chromatographers to collect data over many charge states. The orthogonal source design provides high levels of instrument robustness, even for the challenging conditions posed by the ion pairing eluents used for oligonucleotides. 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 [1]. The sequence consists of 37 amino acids with a peptide mass of about 4500 Da. Also in this work, a quality control method for DNA oligomers of 29, 31, 37, and 40 base pairs in length was augmented with MS to provide mass-based compound confirmation and impurity profiling.

SAMPLE PREPARATION

Samples Peptide Application

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.

Table 1. Peptide samples.

Compound	Sequence	Average Mass (Da)
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	4492.3
LL-37 RKS	RKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	3800.5
LL-37 SKE	SKEKIGKEFKRIVQRIKDFLR	2619.1

Samples DNA Application

Lyophilized desalted, detritylated samples were reconstituted in Dnase-free water to a concentration of 100 µM and stored at -20° C. Samples for injections were diluted to the concentrations described in the text using micropipettes and water from the purification system.

Table 2. Oligomer samples (provided by Thermo Fisher Scientific, Pleasanton, CA).

Length	Sequence	Average Mass (Da)
40	CTCTCTGACACAATTAAGGGATAAAATCTCTGACGGAATG	12312.0
37	CAGGAAACAGCTATGACCCGCGCTCACCTCGCCTCTG	11240.3
31	ATGATATTATGATTAGGAGCCGCGCAGGGAG	9664.3
29	TGAAGGAITGCACTGAAAGGCAGGCTAAT	9024.9

INSTRUMENTATION AND METHODS

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

- System Base Vanquish Horizon/Flex (P/N VH-S01-A-02)
- Binary Pump F (P/N VF-P10-A-01)
- Split Sampler FT (P/N VF-A10-A-02)
- Column Compartment H (P/N VH-C10-A-02)
- 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)

Data Analysis

Chromeleon 7.2.9 chromatography data system (CDS) software was used for data acquisition and processing.

Table 3. Chromatographic Conditions

Parameter	Peptide Application [2]		DNA Application [3]	
Column	Thermo Scientific™ Acclaim™ 120 C18, 50 x 2.1 mm, 2.2 µm (P/N 068981)		Thermo Scientific™ DNAPac™ RP 2.1 x 10 mm, 4 µm (P/N 088925)	
Mobile Phase	A: water + 0.1% formic acid B: acetonitrile + 0.1% formic acid		A: 200 mM HFIP, 8.0 mM TEA, pH 8.0 B: methanol	
Flow rate	0.5 mL/min		0.5 mL/min	
Gradient	Time [min]	%B	Time [min]	%B
	0	20	0	15
	2	50	0.5	15
	2.1	20	1	60
	5.5	20	1.3	60
			1.4	15
			6	15
Mixer vol.	10 + 25 µL		10 + 25 µL	
Column Temp	50 °C (forced air mode, fan speed 5, active preheater)		50 °C (forced air mode, fan speed 5, active preheater)	
Sampler Temp	4 °C		4 °C	
UV	λ = 214 nm, data collection rate = 10 Hz, response time = 0.5 s		λ = 260 nm, data collection rate = 10 Hz, response time = 0.5 s	
Injection vol.	1 µL		1 µL	

Table 4. Mass Detector Settings.

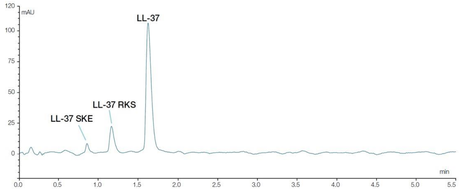
Source Parameters	Both Applications Autospray HESI source settings for 0.5 mL/min	
Sheath gas pressure	49.9 psig	
Aux gas pressure	5.7 psig	
Sweep gas pressure	0.5 psig	
Vaporizer temperature	282 °C	
Ion transfer tube temperature	300 °C	
Source voltage	3000 V for peptides, -2000 V for DNA	
Method Parameters	**Peptide Application Setting**	**DNA Application Setting**
Method type	Full Scan	Full Scan
Ion polarity	positive	Negative
Mass range	*m/z* 500-2000	*m/z* 600-2000
Dwell Time	0.2 s	0.2 s
Source CID voltage	10 V	15 V

RESULTS

Peptide Analysis

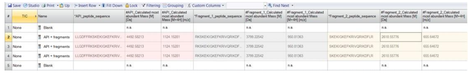
To simulate a product impurity profiling, two separate peptide fragments were spiked into the LL-37 peptide solution and measured under the same condition. Baseline separation could be achieved with a short gradient method within 2 min, as it is shown in Figure 1. Due to the coupling to the mass detector, 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 1. UV chromatogram at 214 nm of simulated peptide impurity profiling



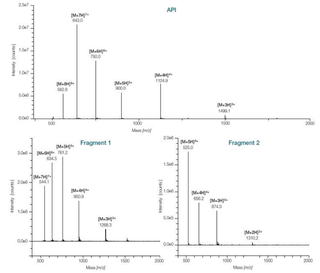
Chromeleon CDS offers a feature to use custom columns in the sequence list to calculate the theoretical mass of peptides and other chemical components via an entry of the peptide sequence or the chemical formula. It also allows calculation of different charge states and various adduct masses (Figure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

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



The mass range *m/z* 50-2000 of the ISQ EM single quadrupole mass spectrometer allows the detection of multiple charge states between 2 and 8 for the peptides (Figure 3).

Figure 3. Obtained mass spectra for the API and the two fragments

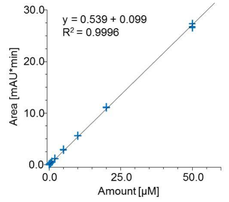


DNA Oligomer Analysis

Oligonucleotide with sequences shown in Table 2 were analyzed by ion pair reversed phase chromatography using a short quality control method.

Quantification of the 37mer oligonucleotide by UV shows that the concentration has a linear relationship with the absorbance at 260 nm over the entire examined concentration range of 0.05 to 50 µM. This curve is shown in Figure 4.

Figure 4. Calibration curve over the values 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 and 50 µM



Injections of 5 pmol of 29mer, 31mer, 37mer and 40mer oligomers were made in triplicate for the purpose of identification by mass confirmation.

Table 5 shows the mass confirmation experiments of each of the four oligomers. Mass accuracy is excellent over various oligonucleotide lengths at a relatively low mass on column. The observed masses are all within the mass accuracy specification of < ± 0.1 Da of the mass detector. The repeatability of the observations is excellent, with an RSD of 0.02% or less.

Table 5. Mass confirmation for 29mer, 31mer, 37mer and 40mer using the relatively high-abundance [M-9H]9- charge state. The mass on column was 5 pmol.

Length	Average Mass [Da]	Average Mass [M-9H]$^{9-}$	Observed Mass Trial 1 [M-9H]$^{9-}$	Observed Mass Trial 2 [M-9H]$^{9-}$	Observed Mass Trial 3 [M-9H]$^{9-}$	Mean (n=3)	Std. Dev. (n=3)	RSD	Mass Accuracy
40	12312.0	1367.0	1367.1	1366.9	1366.9	1367.0	0.12	0.01	0.0
37	11240.3	1247.9	1247.9	1247.8	1247.8	1247.8	0.06	0.00	-0.1
31	9664.3	1072.8	1072.9	1073.0	1072.8	1072.9	0.10	0.01	0.1
29	9024.9	1001.8	1001.6	1001.7	1002.0	1001.8	0.21	0.02	0.0

Multiple charge states were observed for all oligomers examined. For mass confirmation of all charge states between *m/z* 600 – 2000, injections of 50 pmol of the 37mer oligonucleotide were made in quadruplicate.

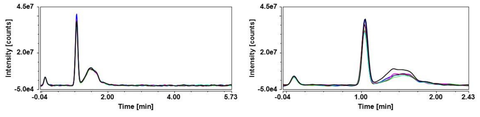
The charge states for the 37mer are shown in Table 6. The observed masses, except for the -13 charge state, are within the mass accuracy specification. The repeatability of the observations is excellent, with a %RSD of 0.02% or less except for the -14 charge state, which had a %RSD of 0.07%.

Table 6. Charge states calculated and found for 37mer (TIC, scan *m/z* 600 – 2000, 50 pmol on-column). Found values and standard deviations (Std. Dev.) are from the average of four injections.

	Expected and Found Masses for Charge States [M-xH]$^{x-}$									
	6	7	8	9	10	11	12	13	14	15
Expected [m/z]	1872.4	1604.8	1404.0	1247.9	1123.0	1020.9	935.7	863.6	801.9	748.4
Observed [m/z]	1872.4	1604.8	1403.8	1247.9	1122.9	1020.7	935.7	864.0	802.1	748.6
Mass Accuracy	-0.0	-0.0	-0.2	-0.0	-0.1	-0.2	-0.0	+0.4	+0.2	+0.2
Std. Dev. (n=4)	0.16	0.20	0.15	0.10	0.15	0.10	0.10	0.21	0.56	0.17
RSD	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.02%	0.07%	0.02%

When using ion pairing reagents in MS, longer equilibration time improves peak area reproducibility. As shown in Figure 5, the RSD for the peak area of the full scan of six 1 µL injections (5 µM 37mer sample) improved from 15.4% to 8.1% when the equilibration time was increased from 1 to 4.5 minutes.

Figure 5. Overlay of six injections with long (left side) and short (right side) equilibration times. The RSD for peak area is 8.1% for the long equilibration time and 15.4% for the short equilibration time. The trace shown is the full scan (*m/z* 600-2000).



CONCLUSIONS

- A rapid gradient method allowed the separation of the LL-37 peptide impurity profiling within 2 min.
- Adding mass detection to existing UV workflows for quality control provides peak purity, mass-based identity confirmation and impurity identification.
- The extended mass range up to *m/z* 2000 enables the detection of low-charged peptide species and allows the detection of the complete charge state profile of mid-sized biomolecules. Ten charge states were detected for a 37mer oligomer.
- The orthogonal source design of the mass detector did not require cleaning over 100 injections of "sticky" HFIP and TEA reagents. A long equilibration time improved peak area reproducibility when using these ion pairing reagents

REFERENCES

1. Yufei Chen, Sidi Yang, and Emmanuel A. Ho, Development of an Analytical Method for the Rapid Quantitation of Peptides Used in Microbicide Formulations (**2014**), *Chromatographia*, 77(23-24), 1713–1720
2. Thermo Fisher Application note 72818: Impurity profiling of the synthetic peptide LL-37 using high-performance liquid chromatography with combined UV and single quadrupole mass spectrometric detection
3. Thermo Fisher Application note 72820: Oligonucleotide characterization for quality control and increased productivity by single quadrupole mass spectrometer with extended mass range

TRADEMARKS/LICENSING

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