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Importance of fragmentation data for the identification of phosphoramidite impurities

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ABSTRACT

Purpose: Demonstrate the confident characterization of phosphoramidite raw material using LC/UV/HRAM-MS and structure elucidation of trace level impurities with fragmentation data.

Methods: 2' modified RNA phosphoramidite material from multiple vendors were separated using RP-LC and analyzed with an Orbitrap high-resolution accurate mass spectrometer.

Results: Differences in the impurity profiles of 5'-DMT-2'-OMe-A(bz)-CEP from different vendors were readily observed from the UV data. Fragmentation spectra were essential in determining transformation sites for the impurities, providing information that can be used in controlling for their presence.

INTRODUCTION

Solid-phase chemical synthesis based on phosphoramidite chemistry is one of the most employed approaches to synthesize oligonucleotides, allowing for a variety of modifications to the core structure and protection groups highlighted in Figure 1. Because phosphoramidite raw material impurities can directly impact the quality of therapeutic oligonucleotides, it is essential to characterize the impurity profile of these oligonucleotide building blocks and control for them in manufacturing processes.[2]

Figure 1. Phosphoramidite building blocks used in oligonucleotide synthesis
Here we demonstrate the characterization of phosphoramidite raw materials from different vendors and structure elucidation of their impurities using a UHPLC system coupled with an Orbitrap high-resolution accurate mass spectrometer.

MATERIALS AND METHODS

5'-Dimethyloxytrityl-2'-O-methoxy-N-benzoyladenosine cyanoethyl phosphoramidite (5'-DMT-2'-OMe-A(bz)-CEP) was obtained from four different vendors, with specified purities of 98% or higher. Samples were dissolved at 1.0 mg/mL in anhydrous acetonitrile and analyzed using LC/MS.
LC separation was carried out using a Thermo Scientific™ Vanquish™ Horizon UHPLC system and a Thermo Scientific™ Accucore™ C18 column (2.1x100 mm, 2.6 µm). The mobile phases used were (A) 10 mM ammonium acetate in water and (B) acetonitrile, employing the gradient separation detailed in Table 1. Mass spectral data were acquired in both polarities within one run using a Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer. The MS method is detailed in Table 2.

Table 1. Gradient conditions

Time (min)	Mobile Phase B (%)
0.0	30
14.0	95
15.0	95
15.1	30
20.0	30

Table 2. MS Method parameters

Polarity Switching ddMS² Top2 experiment	
MS1 Mass Range	*m/z* 200-1200
Easy-IC	Scan-to-Scan
MS¹/MS² Resolution	60,000/15,000 @ *m/z* 200
HCD Collision Energy	10,20,40 %
MS² Max. IT	100 ms

RESULTS

Based on the UV chromatograms of the different raw materials of 5'-DMT-2'-OMe-A(bz)-CEP, differences in their impurity profiles could readily be observed, as depicted in Figure 2.

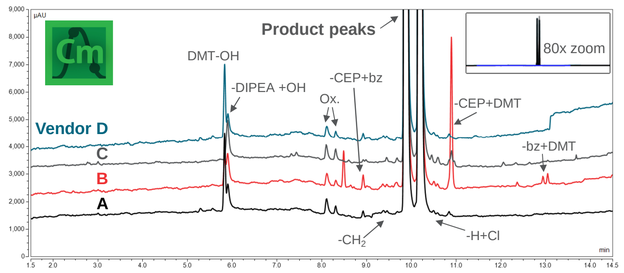

Figure 2. Overlay of the total UV spectra for 5'-DMT-2'OMe-A(bz)-CEP from all four vendors, zoomed in to show the different trace impurity peaks, using Chromeleon CDS.

Using Thermo Scientific™ Compound Discoverer™ software, the detected UV peaks could be manually correlated to compounds detected in the MS data and based on the accurate mass and predicted composition, the transformation relative to the parent structure could be determined.
Using the acquired MS² fragmentation spectra, it was possible to narrow down the possibilities and/or determine the site of transformation to allow confident annotation of the impurities, as detailed in Figures 5 and 6.

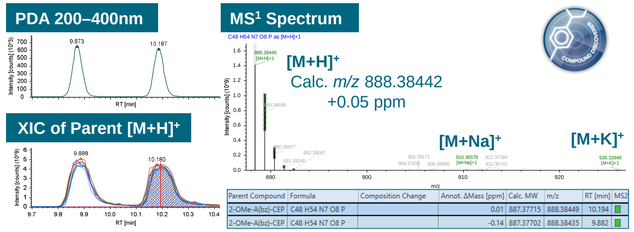

Figure 3. Correlation of the main UV peaks with the XIC of 5'-DMT-2'OMe-A(bz)-CEP in Compound Discoverer, with the MS1 spectrum at apex showing the isotopic peak pattern matching the expected elemental composition within tolerances (green bars).

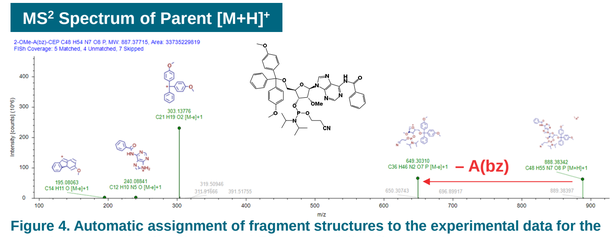

Figure 4. Automatic assignment of fragment structures to the experimental data for the parent compound in the Compound Discoverer 3.3 software.

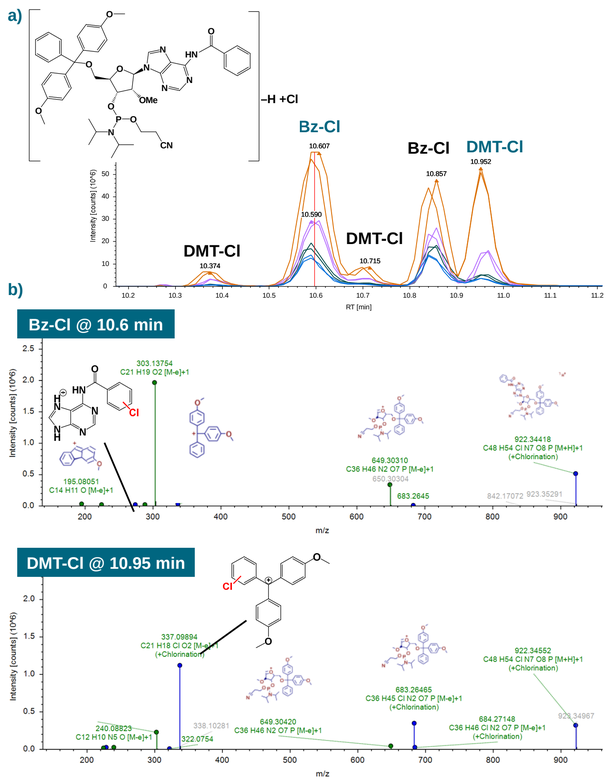

Figure 5. a) XIC of impurities with chlorine substitution (MW 921.3378 Da), highlighting the difference in the impurity profiles for the different supplier materials. b) Comparison of the MS² spectra for the impurity peaks at 10.6 min and 10.95 min, revealing differences in the fragmentation pattern due to different locations of the chlorine substitution on the Bz and DMT protection groups, respectively (transformation shifted peaks colored blue in the Compound Discoverer 3.3 software).

CONCLUSIONS

- Reactive phosphoramidite impurities can directly impact the quality of synthetic oligos.
- Fragmentation data allows the confident determination of the structure of trace impurities, enabling the determination of critical impurities.
- Compound Discoverer software automates the mass spectral annotation process from peak detection to elemental composition and transformation prediction and facilitates the localization of transformation sites using FISh fragment ion predictions and labeling of transformation-shifted fragment ions.

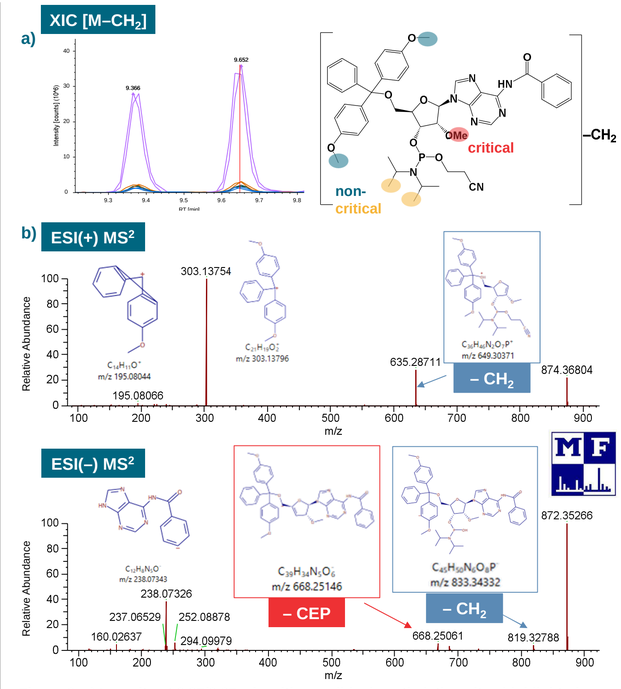

Figure 6. a) Impurity at 9.37/9.65 min with possible demethylation sites highlighted. b) Fragmentation spectra of [M+H]⁺ and [M-H]⁻ ions allowing the localization of the demethylation to one of the isopropyl groups on the phosphoramidite from the negative mode data, which was annotated using the improved negative mode fragment ion prediction in Thermo Scientific Mass Frontier 8.1 software.

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4. Hackbusch, S.; et al. "LC/UV/HRAM MS-based impurity profiling and structure elucidation of phosphoramidite raw materials used for oligonucleotide synthesis" Thermo Fisher Scientific Application Note 001949, **2023**.

TRADEMARKS/LICENSING

PO2023-50EN

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