Biopharma

LC/UV/HRMS-based impurity profiling and structure elucidation of phosphoramidite raw materials used for oligonucleotide synthesis

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Abstract

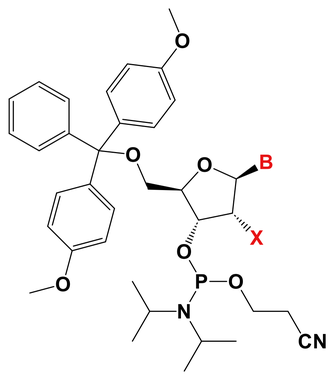
Purpose: Demonstrate the confident characterization of phosphoramidite raw material using LC/UV/HRAM-MS and structure elucidation of trace level impurities with fragmentation data.

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'-F-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.[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]



B = A(bz), C(ac), G(ib), T/U

X = H, OTBDMS, OMe, F, …

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'-fluoro-N-benzoyladenosine cyanoethyl phosphoramidite (5'-DMT-2'-F-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

To establish the sensitivity of the LC/MS method for the detection of impurities at or below the level typically required, spike-in experiments were carried out using 5'-DMT-2'-F-A(bz), a potential impurity of 5'-DMT-2'-F-A(bz)-CEP resulting from the loss of the cyanoethyl phosphoramidite group.

Since this impurity was already present in the phosphoramidite at detectable levels, it was spiked into a different amidite modality, 5'-DMT-2'-OMe-A(bz)-CEP, at relative concentrations ranging from 0.001% to 0.1%. This was done to model the case of an impurity that was not present in the unspiked sample. The resulting UV traces and ESI(+) XICs from the different injections are shown in Figure 2. As can be seen,

The spiked impurity eluting at 5.9 min was readily detected down to a relative concentration of 0.01% using the total UV trace, while the 0.001% spike was only detectable in the high-resolution accurate mass (HRAM) mass spectral data.

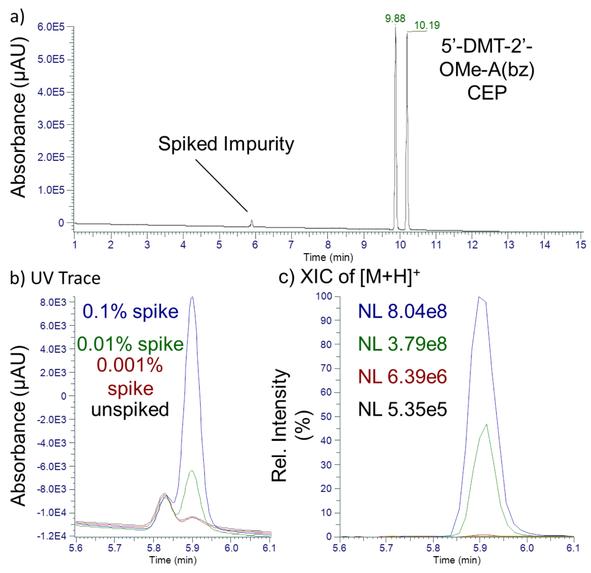


Figure 2. (a) UV chromatogram showing both the spiked impurity 5'-DMT-2'-F-A(bz) and 5'-DMT-2'-OMe-A(bz)-CEP; (b) and (c) UV and ESI(+) XIC traces of the spiked impurity, highlighting the sensitive detection of impurities at spiked levels down to 0.01% and 0.001% by UV and MS.

After establishing the necessary sensitivity of the method, supplies of 5'-DMT-2'-F-A(bz)-CEP obtained from four different vendors were analyzed using the established method. Based on their UV chromatograms, differences in their impurity profiles could readily be observed, as depicted in Figure 3.

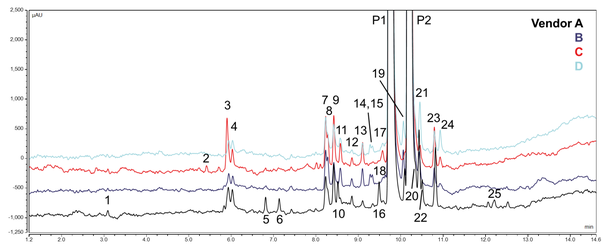


Figure 3. Zoomed-in overlay of the UV chromatograms of 5'-DMT-2'-F-A(bz)-CEP from vendors A (black), B (blue), C (red), and D (light blue) with impurity peaks labeled.

As highlighted by Kiesman *et al.*,[2] the structure and reactivity of amidite impurities plays a critical role for the oligonucleotide synthesis process beyond the overall purity level of the building blocks. To that end, the data were first processed using the Qualitative workflow in Thermo Scientific™ Chromeleon™ 7.2.10 CDS to automatically detect all peaks in the Total UV spectra present at or above 0.01% relative intensity, after automatic background subtraction of a solvent blank injection. Then, both expected and unexpected (i.e., "untargeted") peak detection of the MS data were carried out using the Thermo Scientific™ Compound Discoverer™ 3.3 SP2 software, allowing the detected UV peaks to be manually correlated to compounds detected in the MS data as shown in Figures 4.

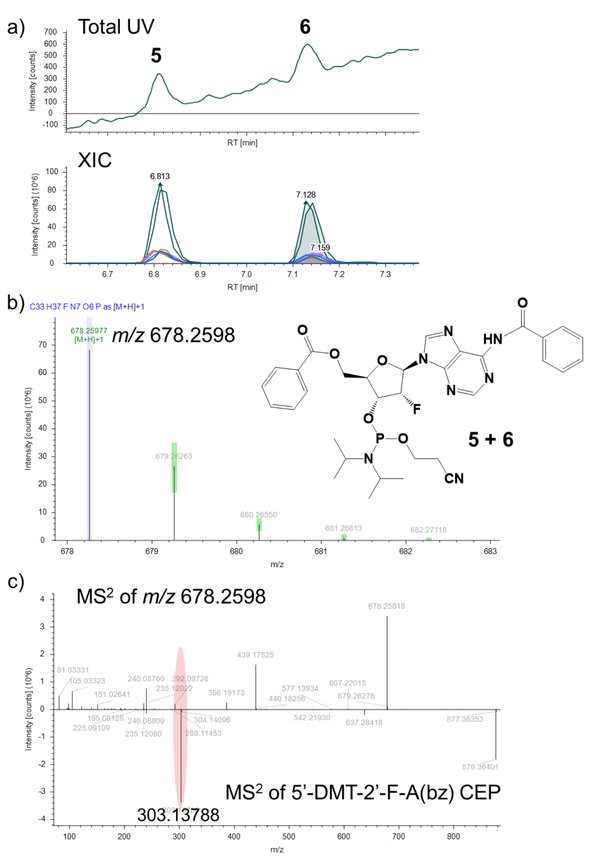


Figure 4. (a) Correlation of peaks 5 and 6 in the total UV chromatogram of material A with the XIC of *m/z* 678.2598 (compound V) in Compound Discoverer software; (b) isotopic peak pattern of the detected compound matching to the elemental composition of $C_{33}H_{37}FN_7O_6P$ within tolerances (green bars); and (c) mirror plot of the fragmentation spectrum for impurity V and its parent compound, showing the absence of the trityl fragment at *m/z* 303.13788 in the former.

Impurity structure elucidation with Compound Discoverer software

The expected compounds workflow used the default transformation list for the "Impurity ID Related and Unknown with Molecular Networks" workflow template including dealkylation and dearylation, as well as (de)hydration, (de)saturation and oxidation and reductions, with methylation added as a custom transformation. The added methylation transformation was detected in Impurity XVII, with an isotope pattern match for the assigned elemental formula, as shown in Figure 5.

Additionally, the software automatically generated fragment ion predictions using the FISh algorithm to match the MS² fragmentation spectra, with fragment ions matching the parent compound highlighted in green and mass-shifted fragments in blue. Based on this, the methylation could be localized to one of the methoxy groups on the DMT protection group and the impurity classified as *reactive and noncritical,* as the DMT group is removed in the oligonucleotide synthesis cycle and not incorporated into the final product.

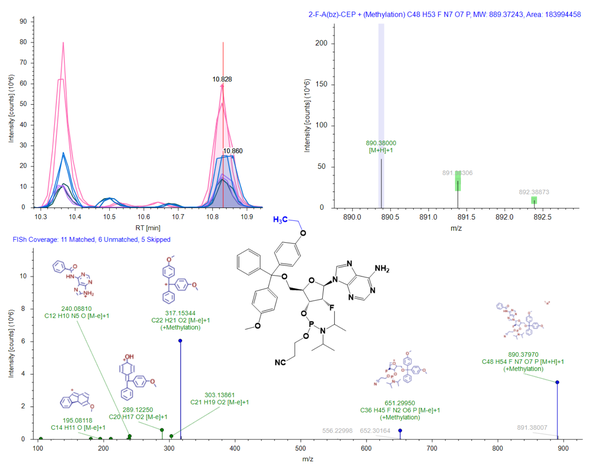


Figure 5. XIC and zoomed MS spectrum of the M+H⁺ ion of the methylated impurity XVII (peaks 21 + 23). The site of methylation could be localized to the DMT group based on the observed shift in the trityl fragment ion to *m/z* 317, and the unshifted ion detected at *m/z* 289 indicates the likely substitution of the methoxy group with ethoxy, resulting in the proposed structure of the impurity in the center.

The importance of fragmentation data for confident structure elucidation is further illustrated by the impurities XVIII and XX, representing multiple isomeric compounds with molecular mass of 909.3177 Da, corresponding to a chlorine substitution.

As highlighted in Figure 6, the chlorine substitution could be localized to either the DMT or benzoyl protecting groups from the shifted fragments present in the MS² spectra.

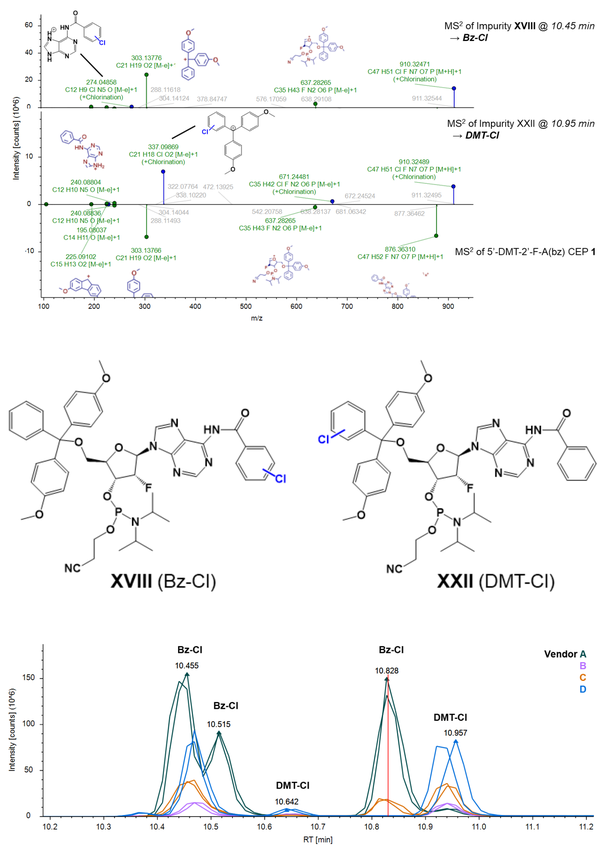


Figure 6. Comparison of the MS² spectra of isomeric impurities XVIII and XX to the expected product 5'-DMT-2'F-A(bz) CEP, revealing differences in the fragments due to different locations of the chlorine substitution on Bz and DMT, respectively (top) and XIC of the chlorine substitution (MW 909.3177 Da), highlighting the difference in the impurity profiles for the different supplier materials (bottom).

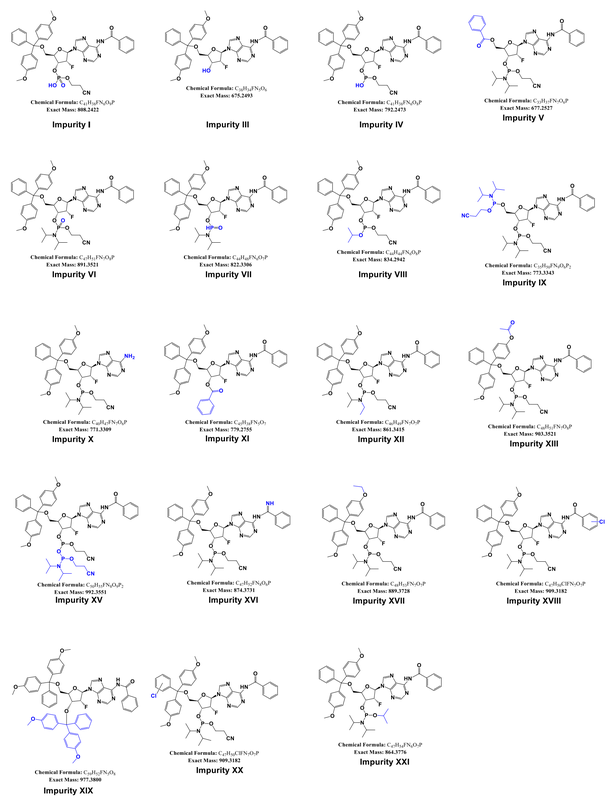


Figure 7. Proposed structures for the detected trace impurities in 5'-DMT-2'-F-A(bz)-CEP from the four vendors.

Conclusions

Here, we highlight that the Vanquish Horizon UHPLC system combined with the Orbitrap Exploris 120 mass spectrometer provide excellent sensitivity and high-quality MS data to facilitate the confident identification of phosphoramidite impurities and their profiling across different supplier's materials:

- Sensitive detection of trace impurities at levels of 0.01% and lower was demonstrated in a spike-in experiment.
- Differences in the impurity profiles of 5'-DMT-2'-F-A(bz)-CEP from different vendors were readily determined.
- Fragmentation data allowed the confident determination of the structure of trace impurities, enabling the determination of critical and non-critical impurities.
- The Compound Discoverer 3.3 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.

References

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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**.

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