Impurity profiling and structure elucidation of phosphoramidite raw materials used for oligonucleotide synthesis

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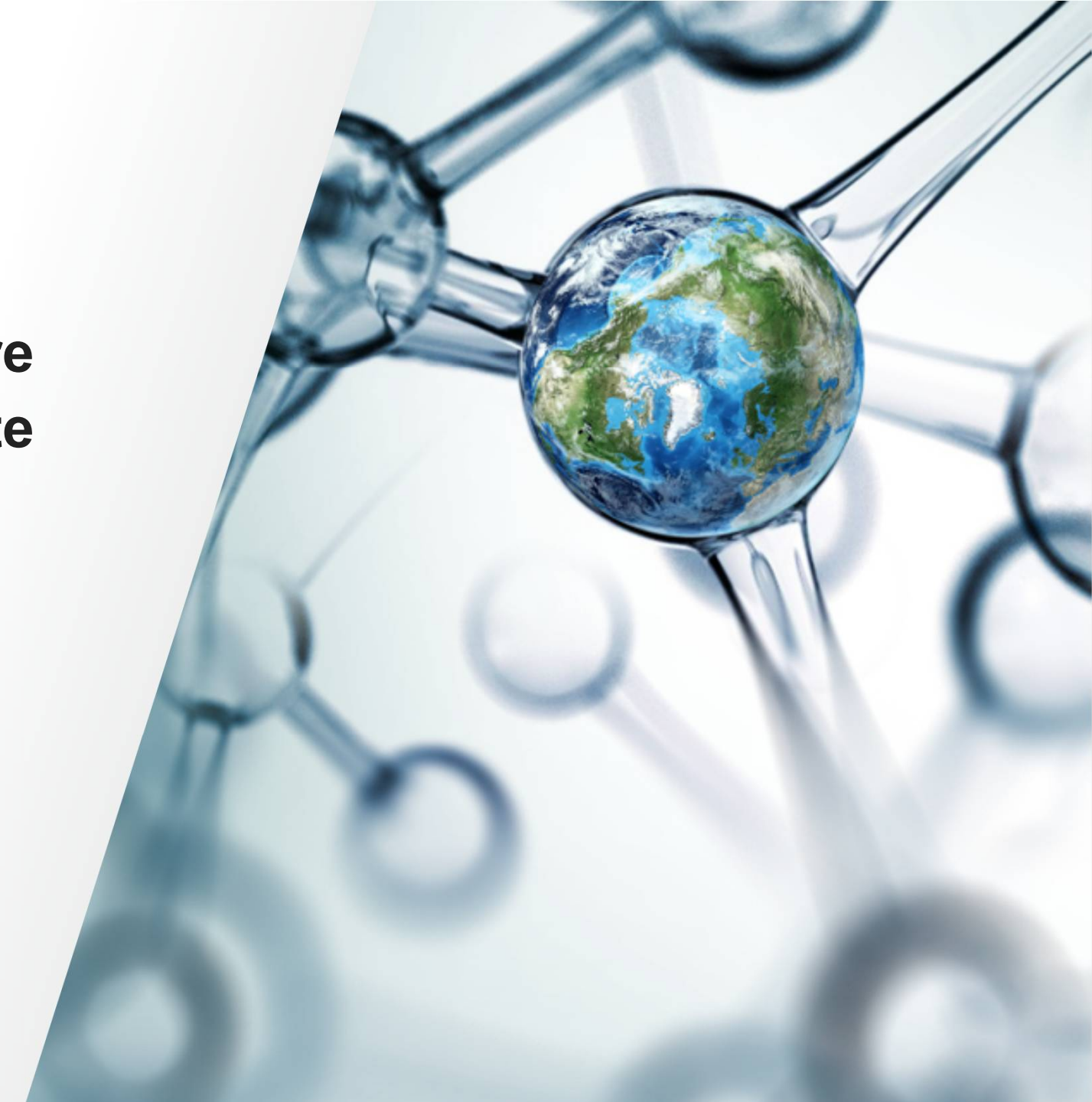
Senior Application Scientist, Structure Elucidation
13-April-2023



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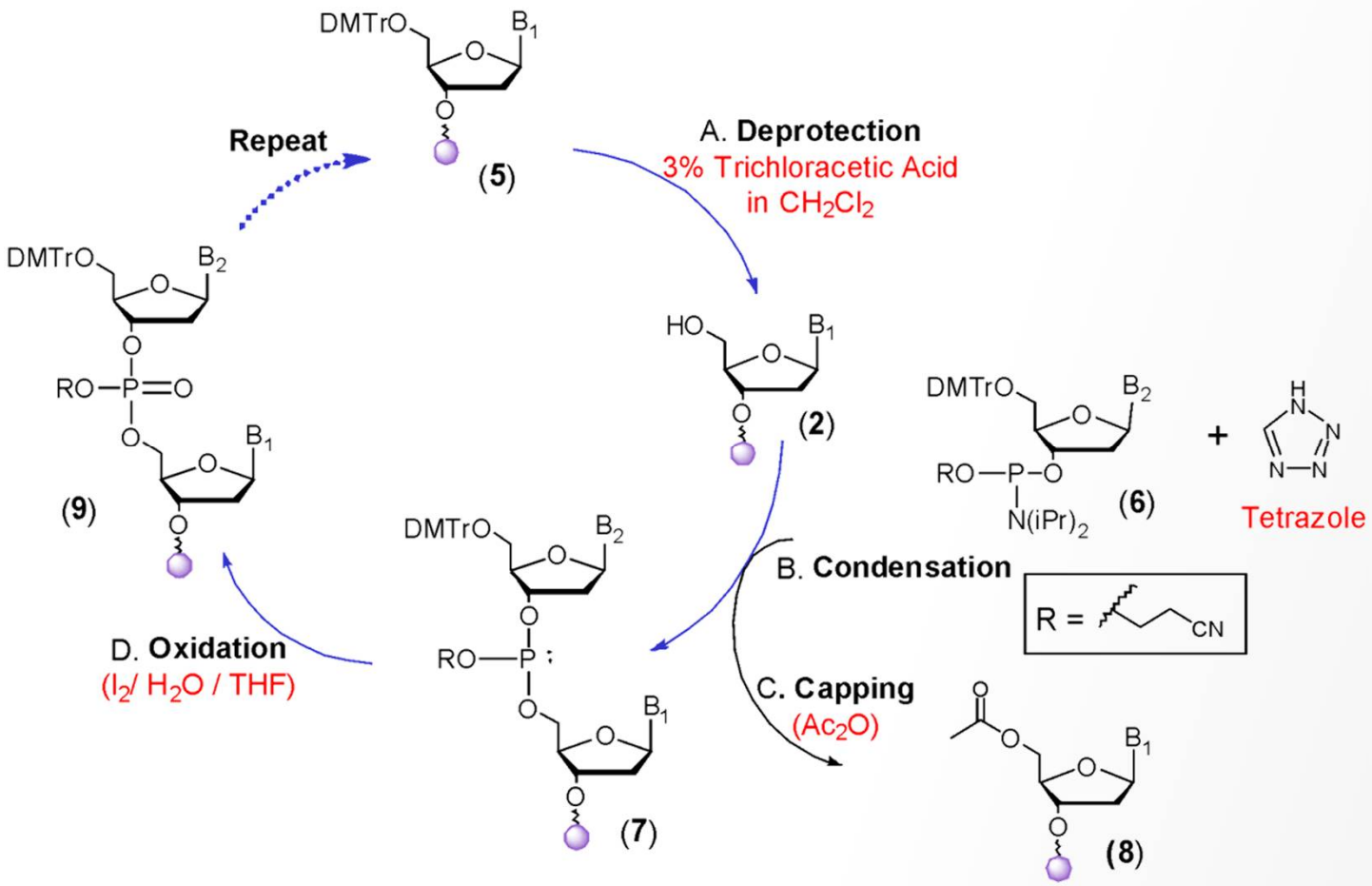
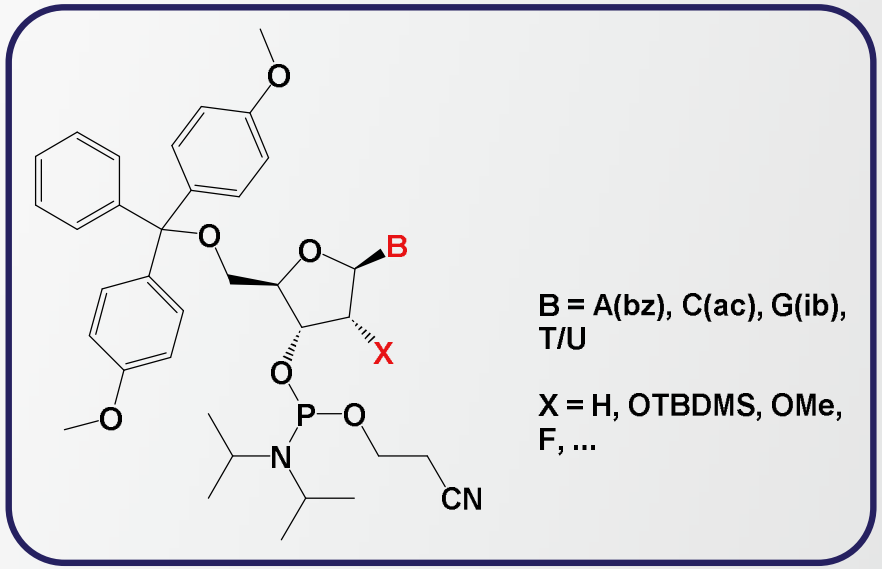
Solid phase oligonucleotide synthesis

Typical synthesis sequence (followed by purification)

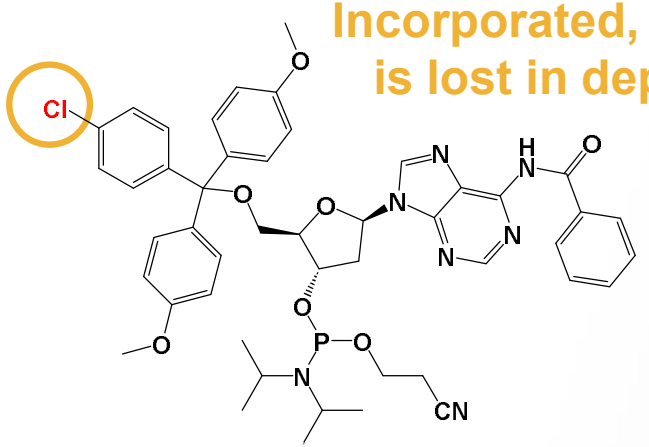




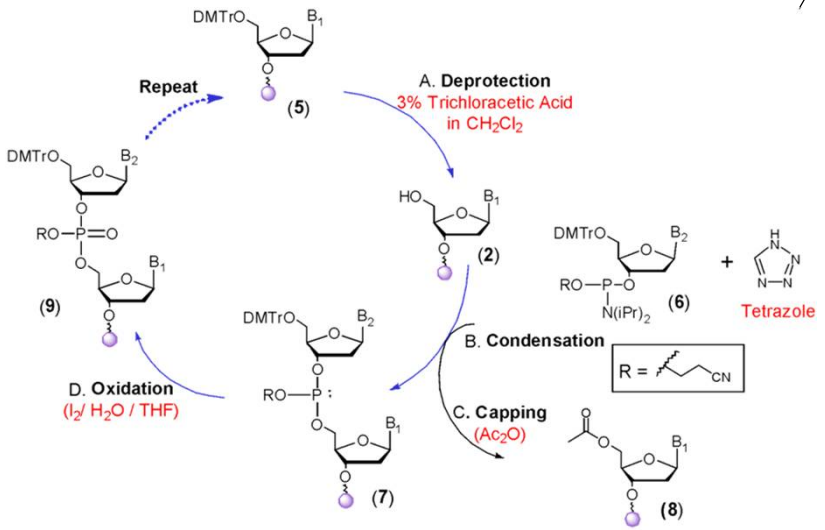
Phosphoramidite Building Blocks

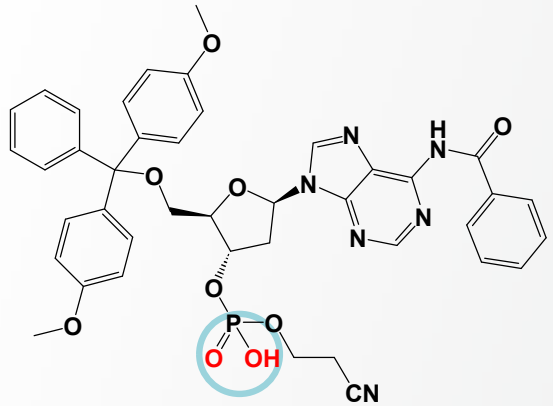
1. Roy, S.; Caruthers, M. "Synthesis of DNA/RNA and Their Analogs via Phosphoramidite and H-Phosphonate Chemistries" *Molecules*, **2013**, *18*, 14268-14284. (doi: 10.3390/molecules181114268)

Phosphoramidite impurities can impact the final product

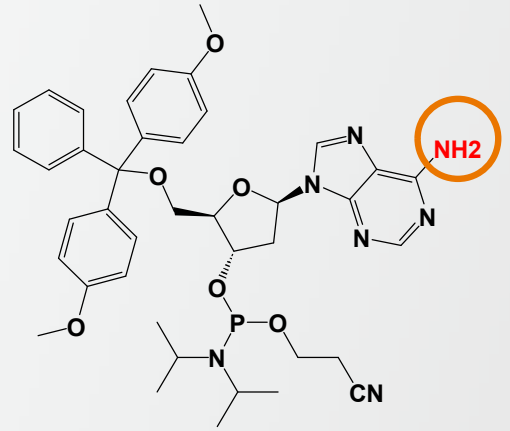


Incorporated, but modification is lost in deprotection step

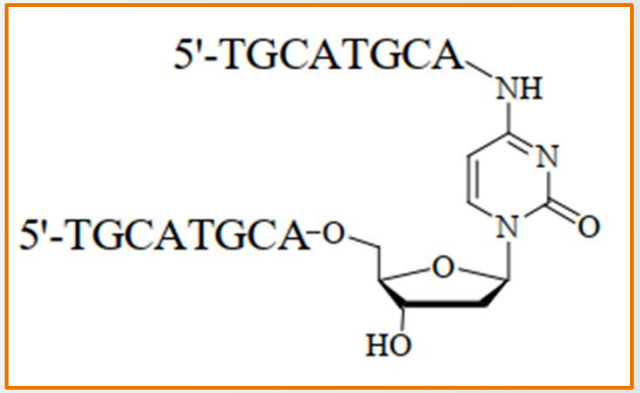




Not incorporated due to Oxidation to P(V)



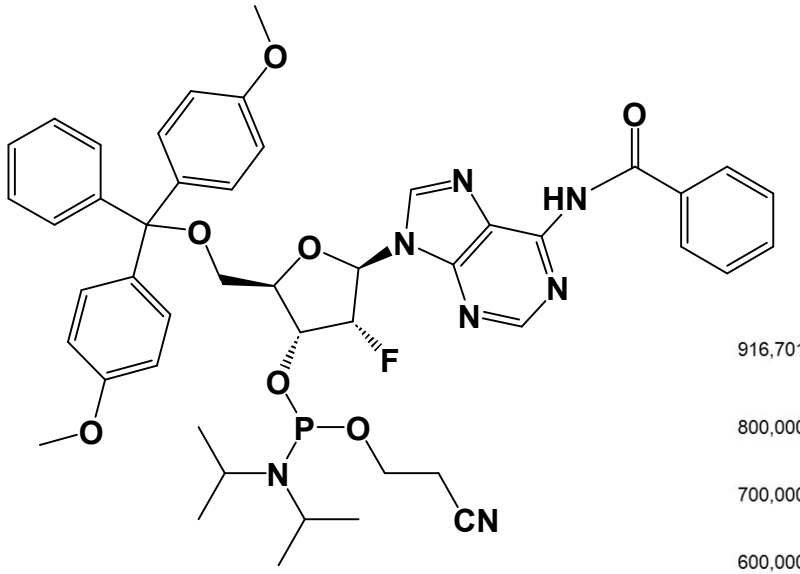
Incorporated, and may form branched oligomer



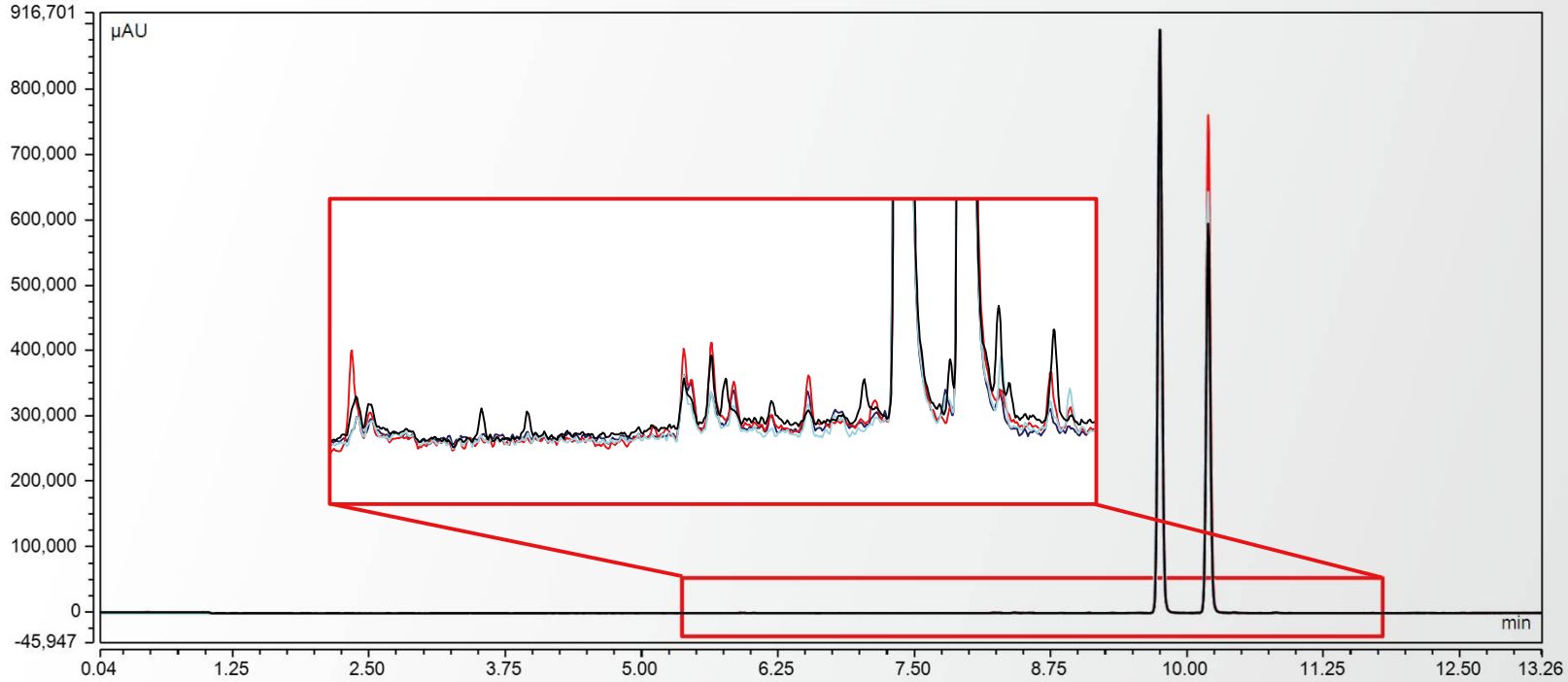
2. Kiesman, W.F. *et al.* "Perspectives on the Designation of Oligonucleotide Starting Materials" *Nucleic Acid Therapeutics,* **2021**, *31*, 93-113. (doi: 10.1089/nat.2020.0909)

Case study: 5’-DMT-2’-F-A(bz)-CEP

5’-Dimethoxytrityl-2’-fluoro-benzoyl-adenosine cyanoethyl phosphoramidite

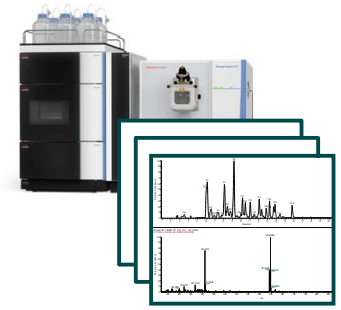


- Obtained material from four different vendors
- Analyze to detect and identify impurities above 0.01% relative abundance using LC/HRAM-MS

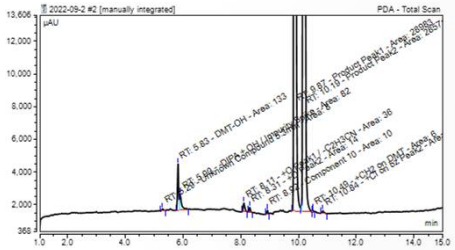


Overview of impurity profiling workflow

Raw Material Sample Analysis By LC/UV/MS

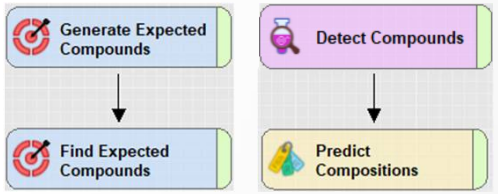


UV Component Detection and Quantitation



MS Compound Detection

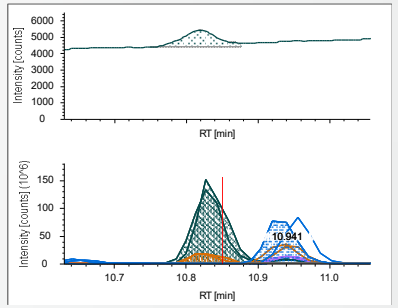
Expected + Unexpected Compounds

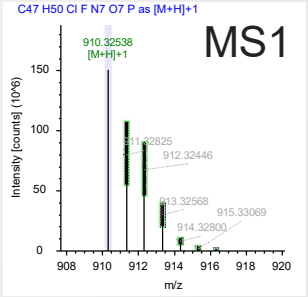


Correlation to MS Compounds + Structure Elucidation based on MS²

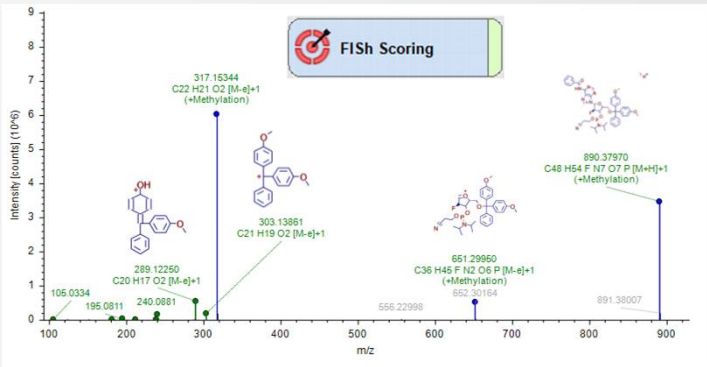
UV

MS





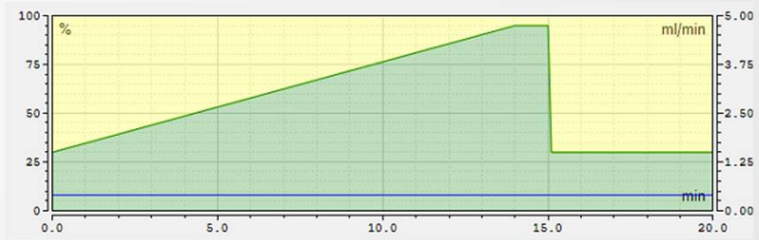
MS²



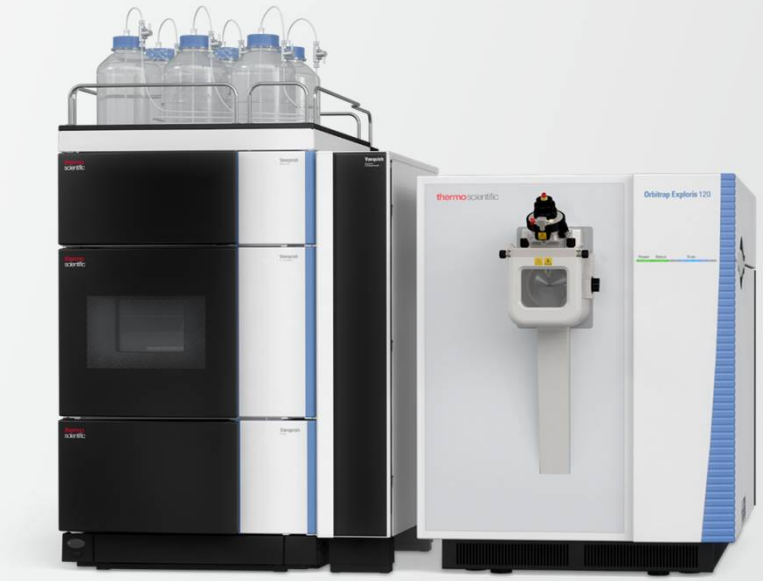
LC/MS Setup

Thermo Scientific™ Vanquish™ Horizon UHPLC system + Orbitrap Exploris™ 120 mass spectrometer

- Thermo Scientific™ Hypersil GOLD™ C18 column (2.1x100 mm, 1.9 µm)
- Mob. Phases: (A) 10 mM Ammonium acetate, (B) ACN
- **15 min gradient** + 5 min re-equilibration
- **UV 200-400 nm**



- **Full Scan** data acquired w/ **Polarity Switching**
- Resolution Setting 60,000 / 15,000 (MS^1 / MS^2)
- ***m/z* 200-1200 Da**
- **Top2 data-dependent MS^2** with HCD (stepped NCE 10,20,40)



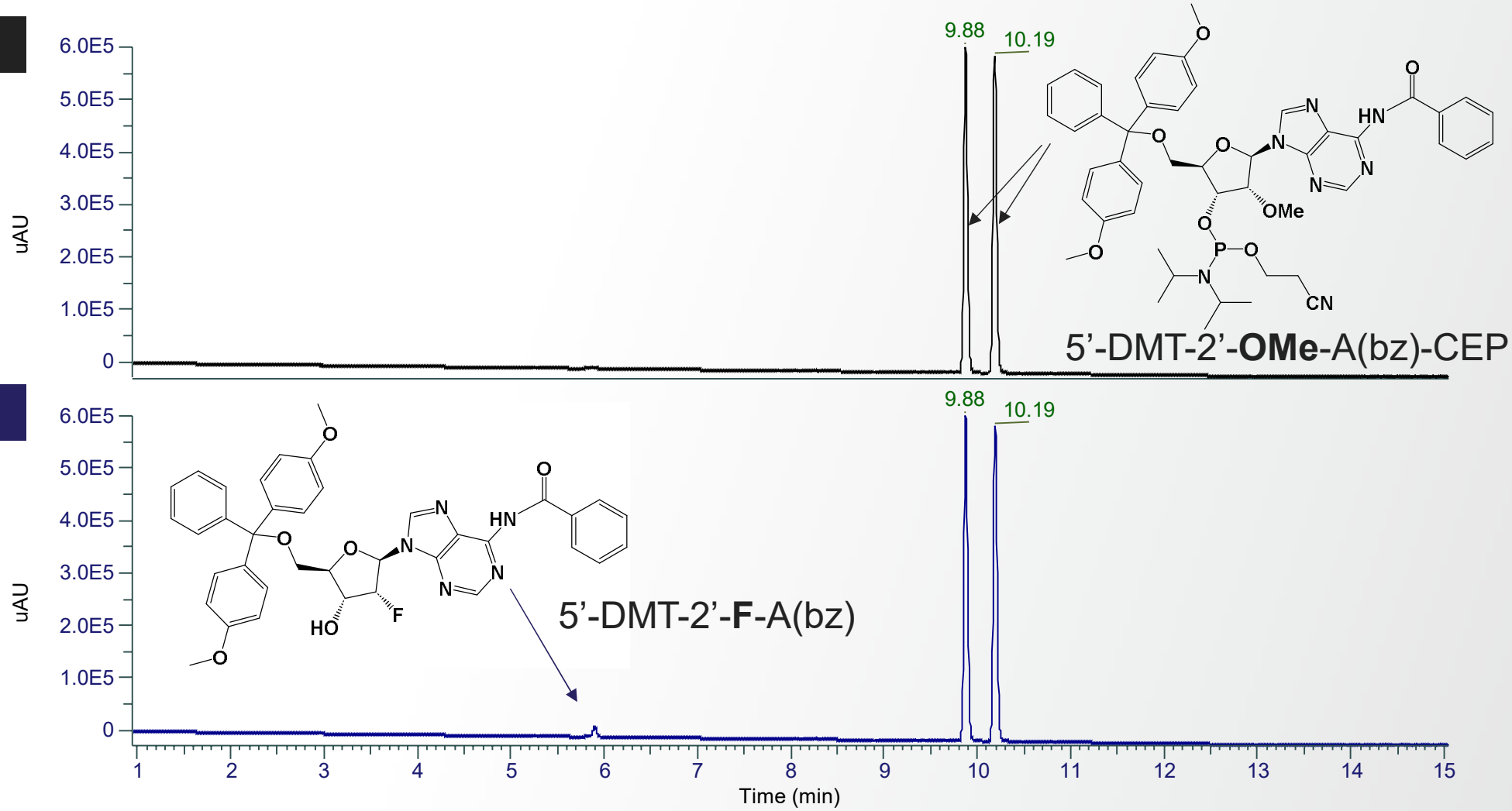
2'-F-A(bz)-CEP samples dissolved in anhydrous ACN at 1 mg/mL to prevent oxidation prior to analysis

Impurity spike-in experiments

Confirming sensitivity of analytical method to detect impurities at 0.1% relative to main component

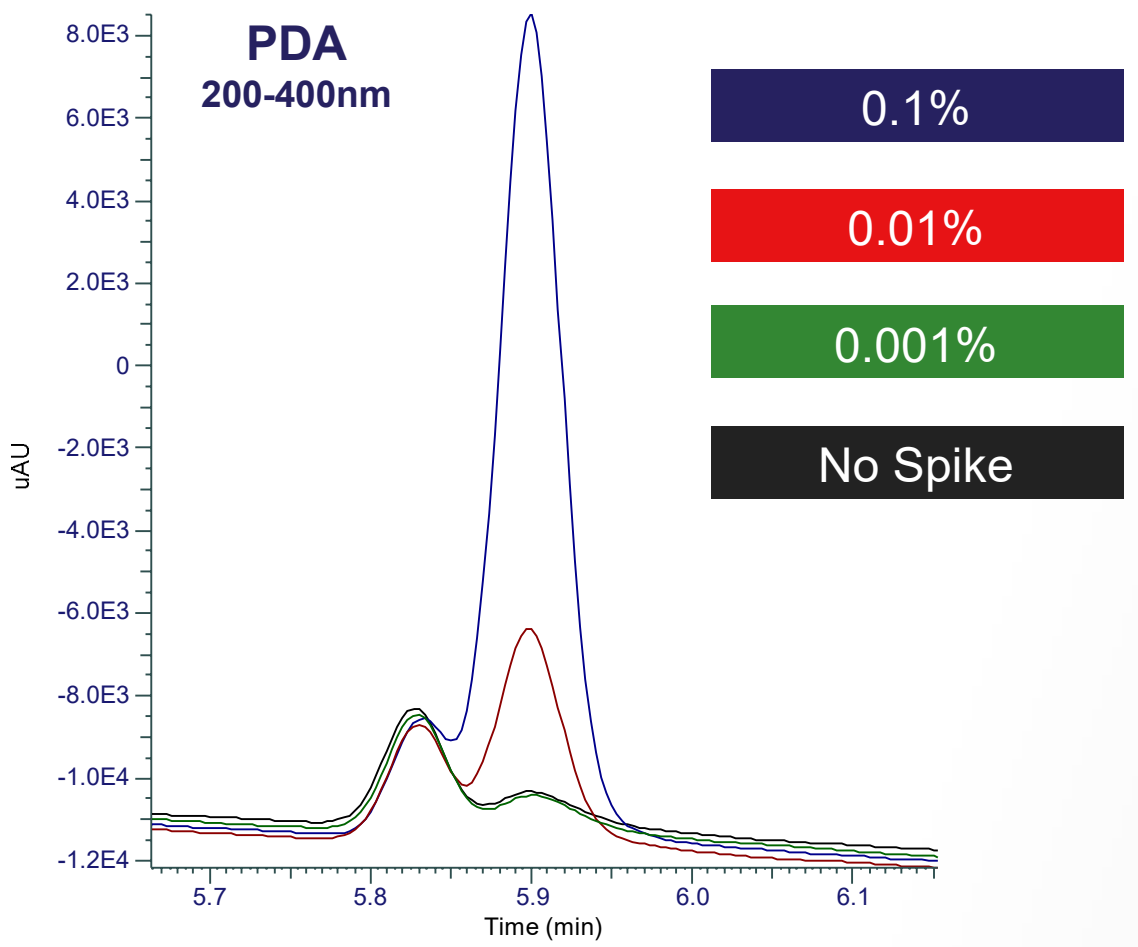
No Spike

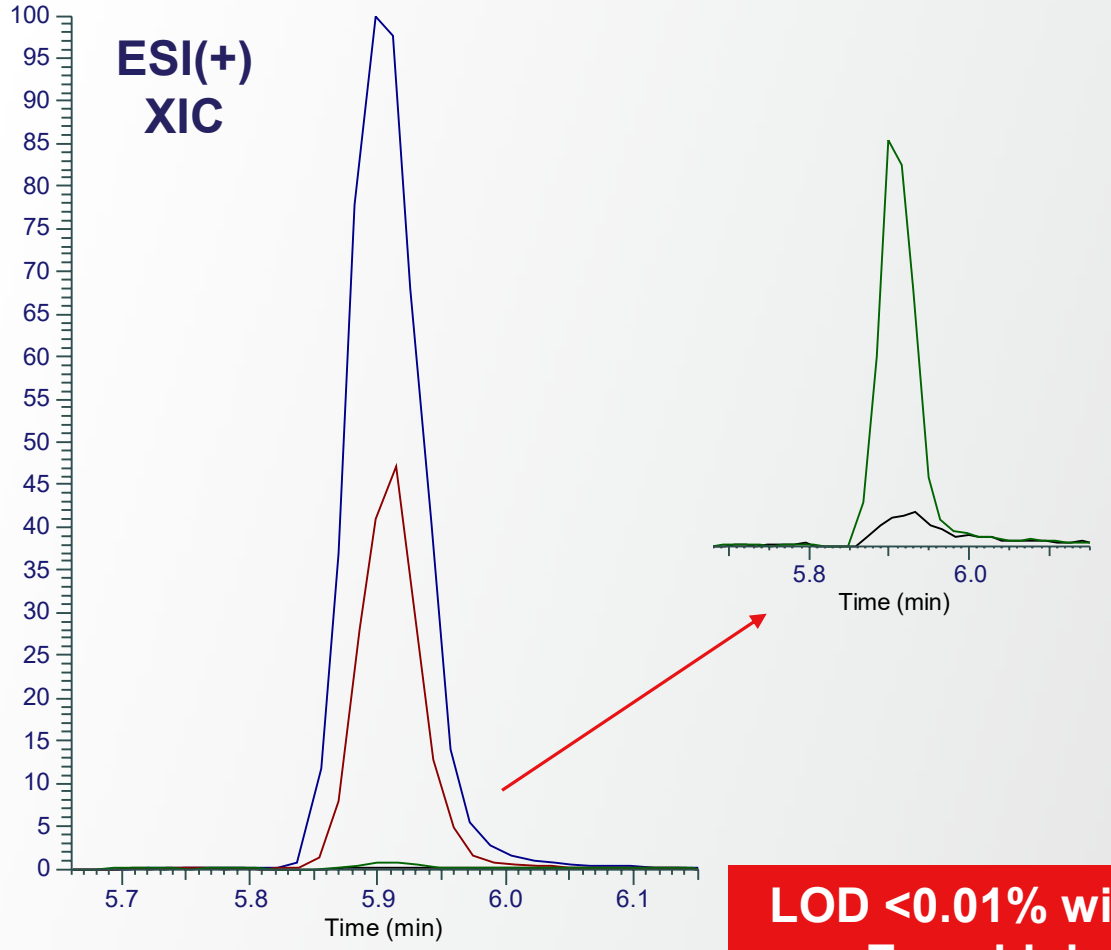
0.1%



Impurity spike-in experiments

Different levels 5'-DMT-2'-**F**-A(bz) spiked into 5'-DMT-2'-**OMe**-A(bz)-CEP

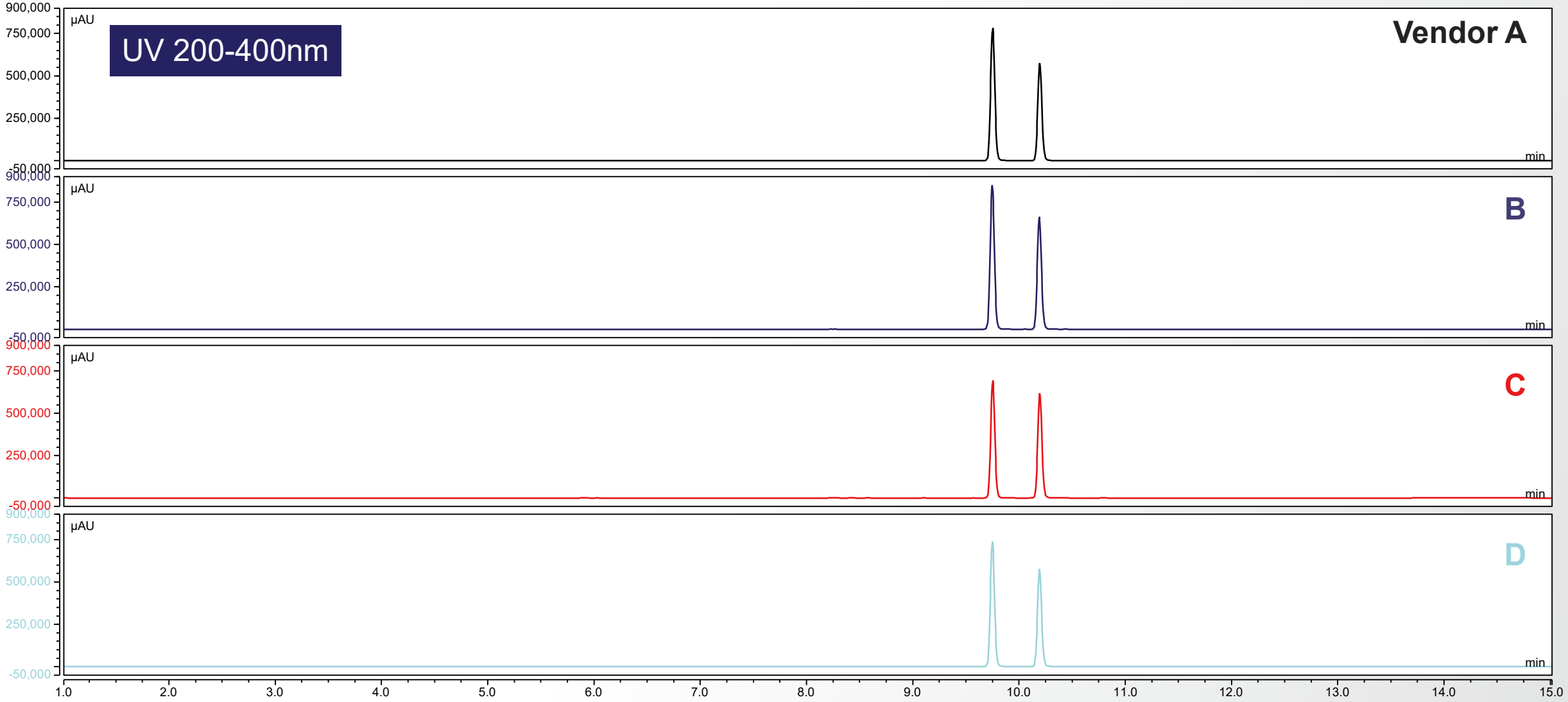




LOD <0.01% with UV
Even higher Sensitivity with MS

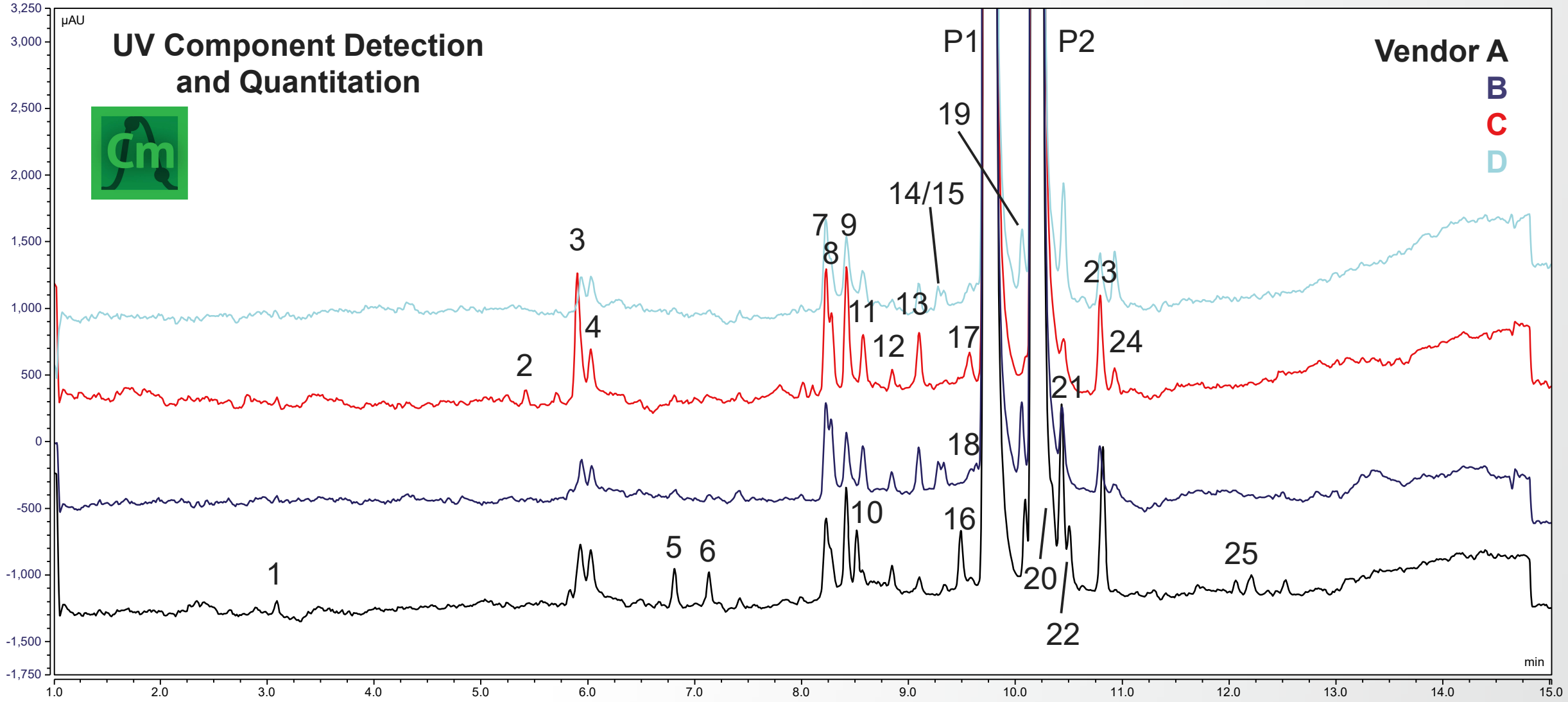
Analysis of several vendor's materials

5'-DMT-2'-F-A(bz)-CEP – Purity > 99% for all



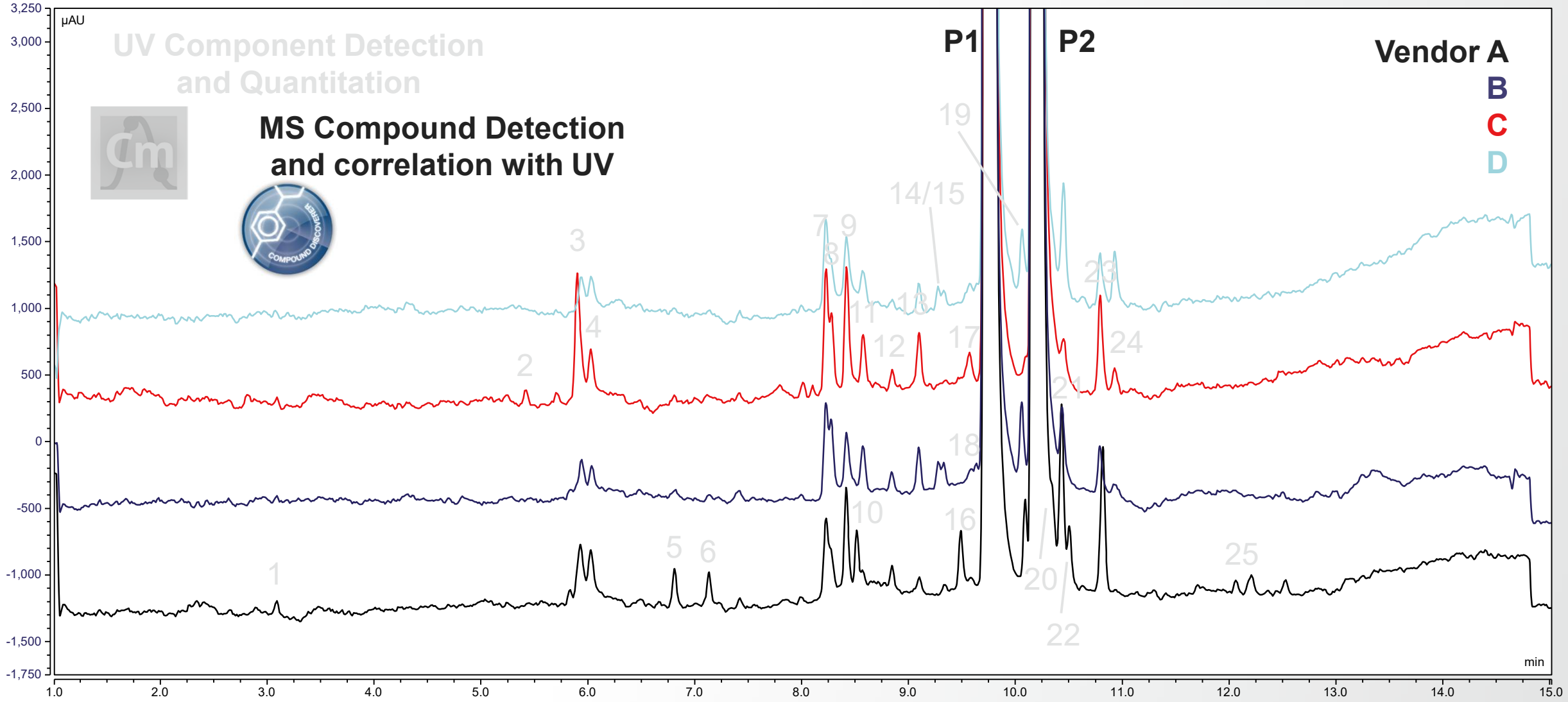
Analysis of several vendor's materials – detail view

5'-DMT-2'-F-A(bz)-CEP **UV 200-400nm** 200X zoom



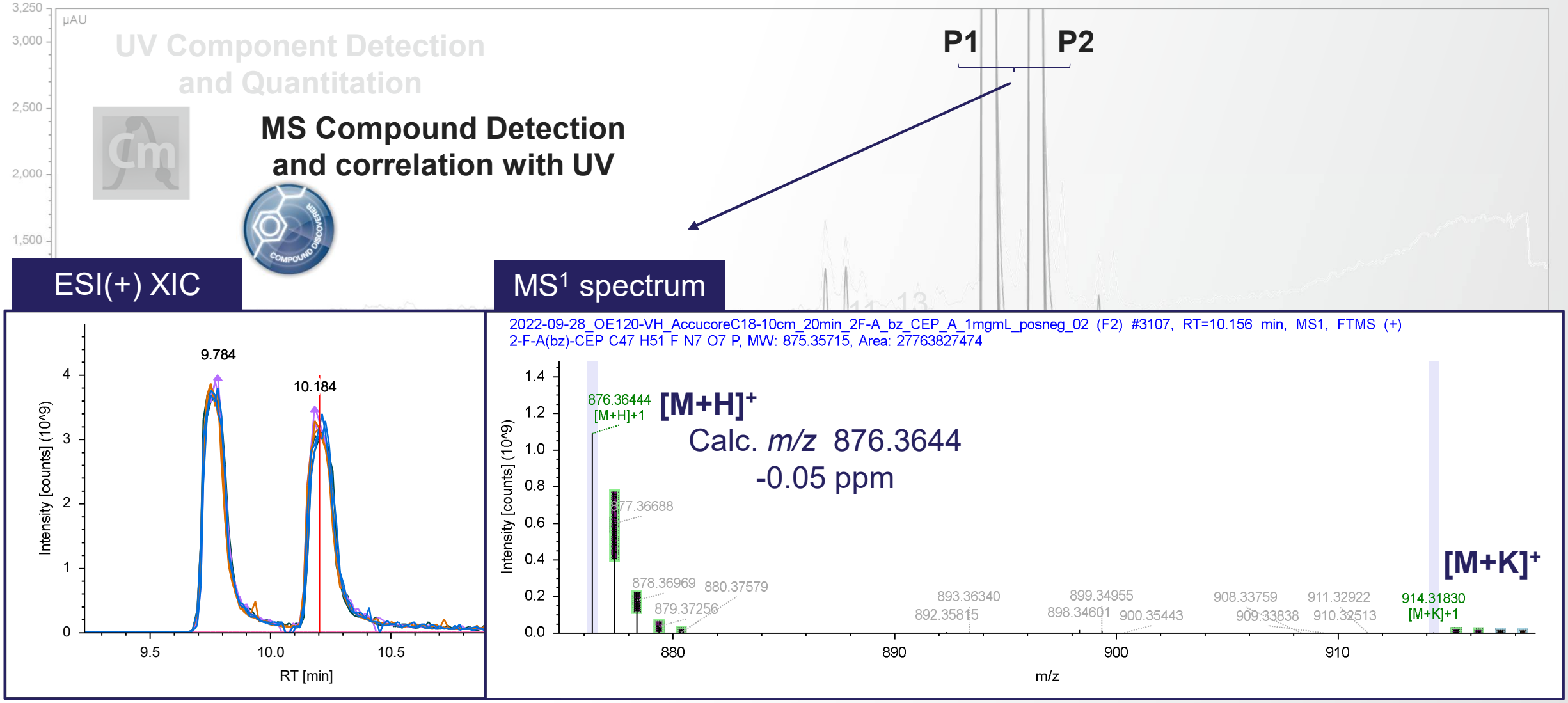
Analysis of several vendor's materials – detail view

5'-DMT-2'-F-A(bz)-CEP **UV 200-400nm** 200X zoom



Identity of raw material from MS data

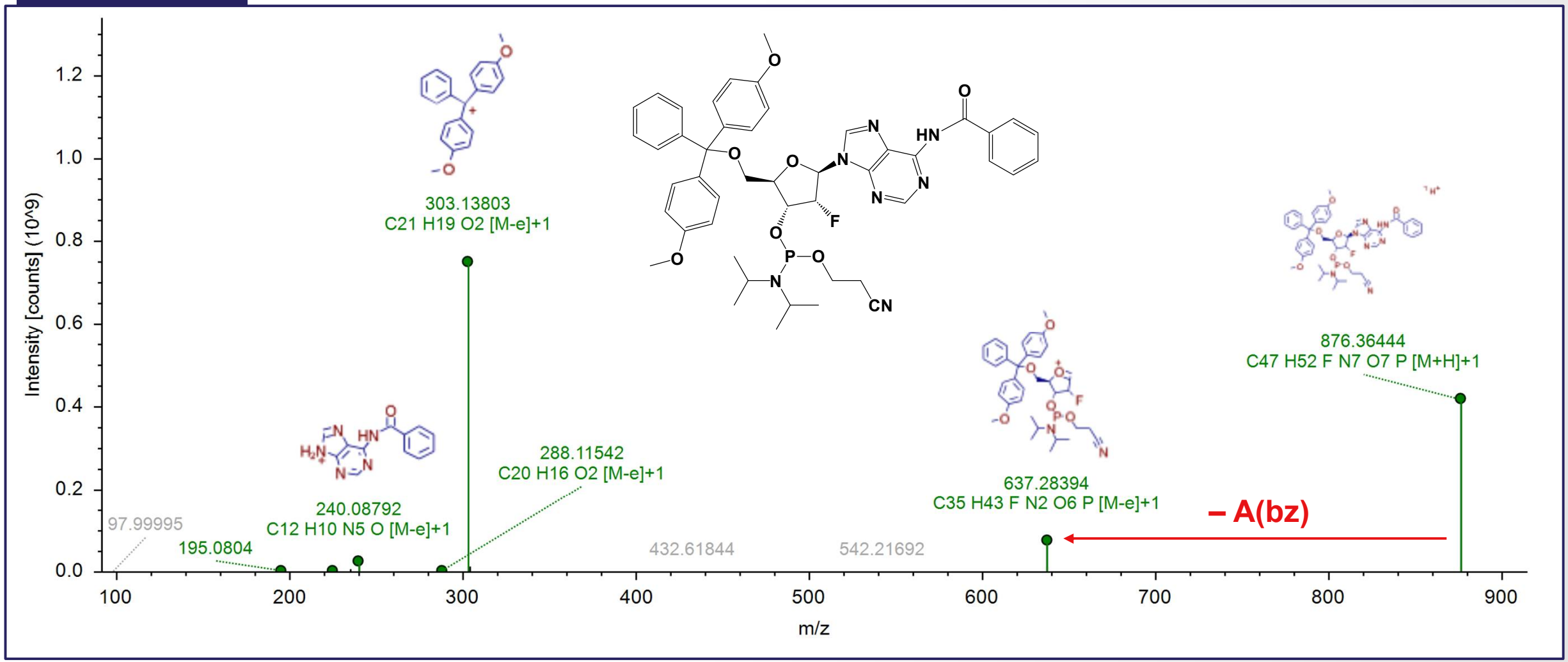
5’-DMT-2’-F-A(bz)-CEP



Structure confirmation of raw material identity by MS^2

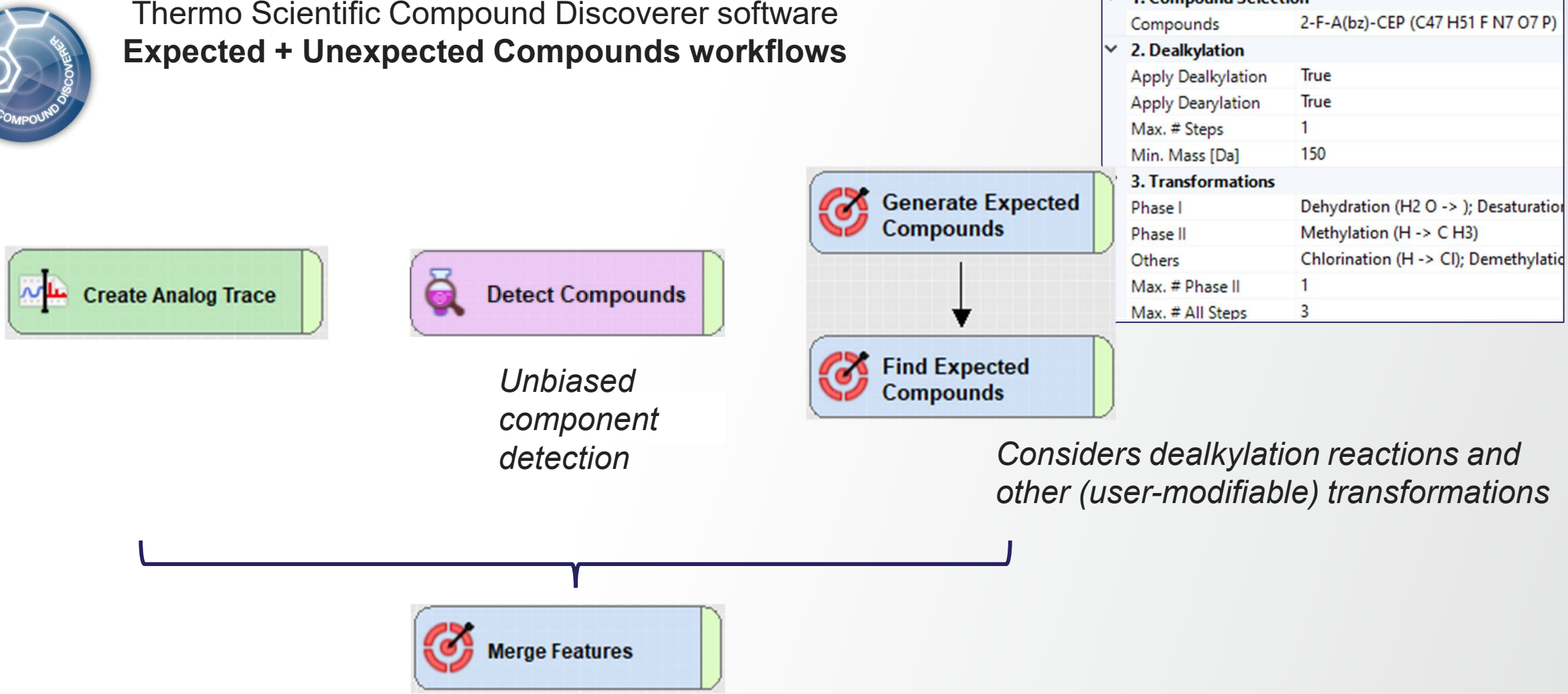
5’-DMT-2’-F-A(bz)-CEP – automatic fragment structure assignment from *in-silico* prediction

MS^2 spectrum



Identification of impurities in Compound Discoverer

Thermo Scientific Compound Discoverer software
Expected + Unexpected Compounds workflows



Unbiased component detection

Considers dealkylation reactions and other (user-modifiable) transformations

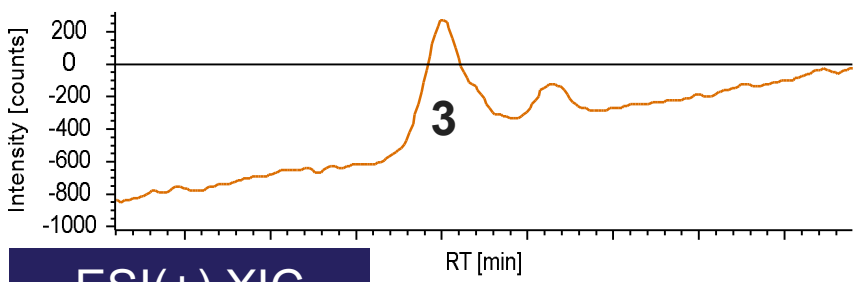
Impurity identification from predicted transformation

Compound **3** - ΔMW = 200 Da (– $C_9H_{17}N_2OP$)

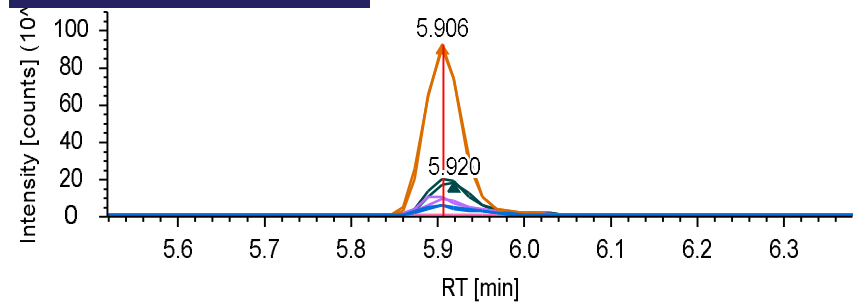
Expected Compounds

Formula	Parent Compound	Dealkylated	Transformations	Composition Change	Calc. MW	m/z	Reference Ion	Annot. ΔMass [ppm]	RT [min]	MS2
C38 H34 F N5 O6	2-F-A(bz)-CEP	X	Reduction	-(C9 H17 N2 O P)	675.24910	676.25637	[M+H]+1	-0.32	5.907	

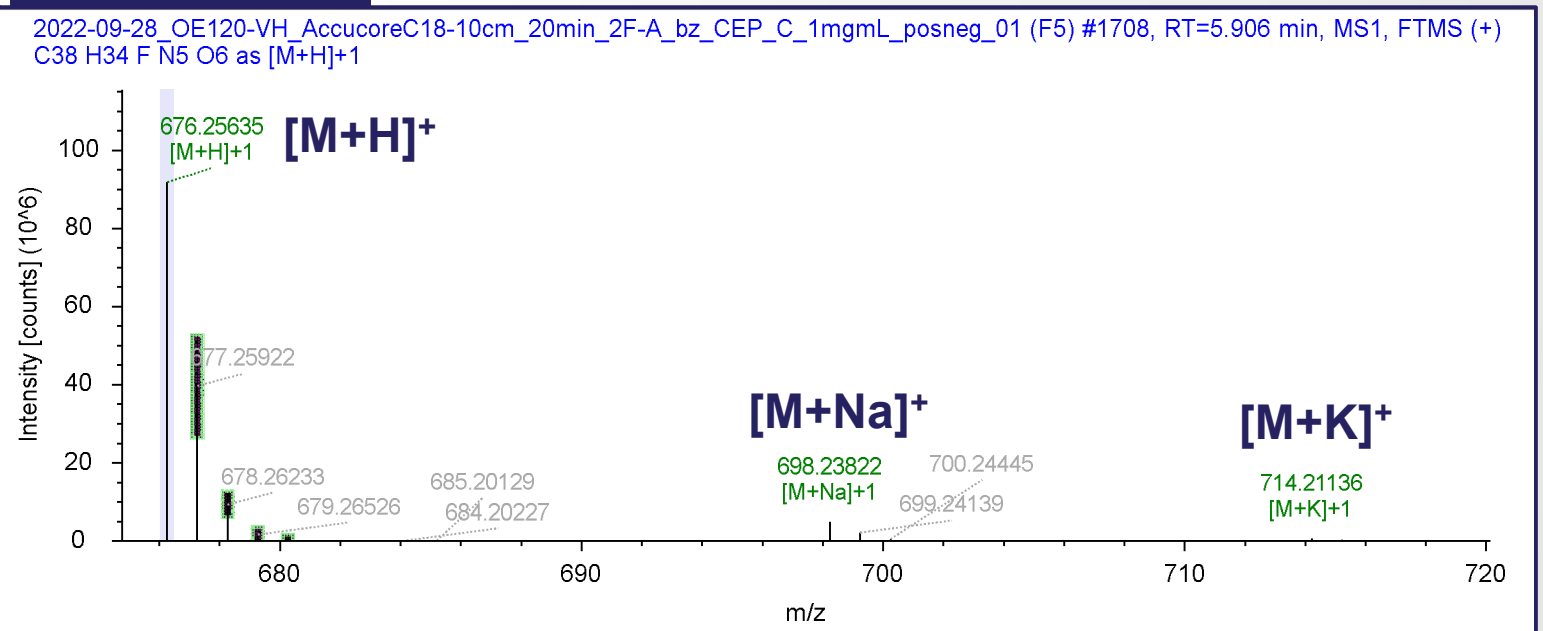
UV 200-400nm



ESI(+) XIC



MS^1 spectrum



Only one peak → loss of stereocenter

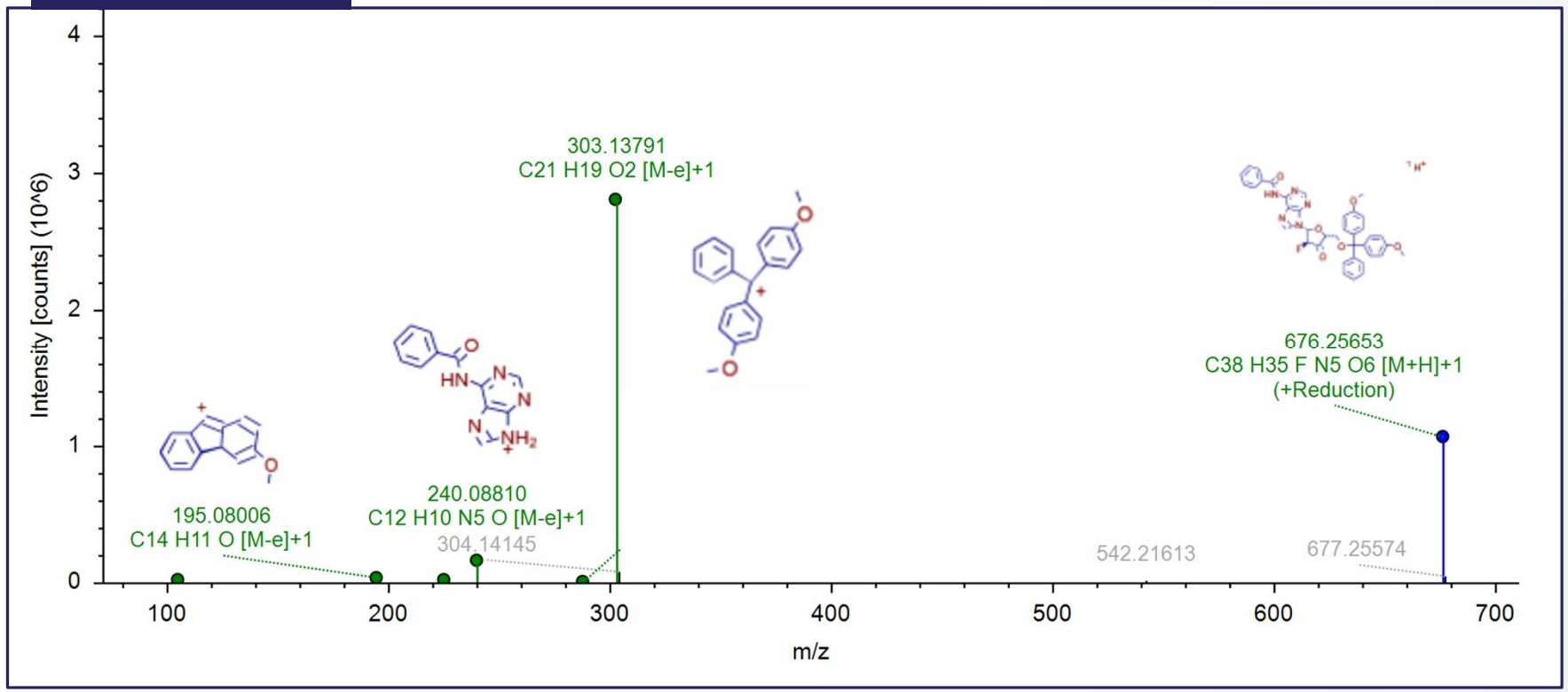
Confirmation of impurity 3 structure with MS^2

$\Delta MW = 200$ Da ($- C_9H_{17}N_2OP$) ≙ **CEP**

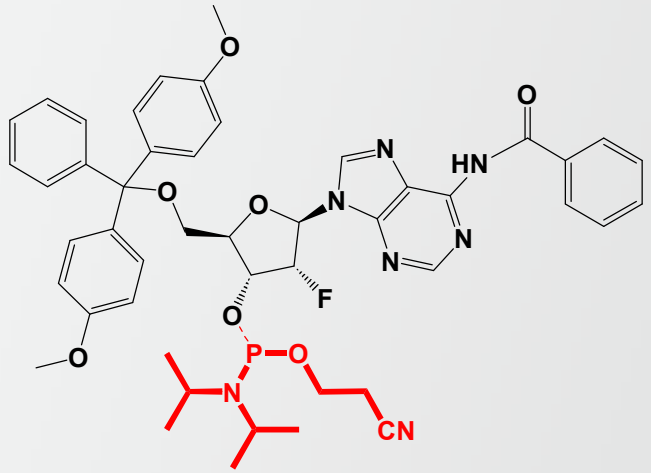
Expected Compounds

Formula	Parent Compound	Dealkylated	Transformations	Composition Change	Calc. MW	m/z	Reference Ion	Annot. ΔMass [ppm]	RT [min]	MS2
C38 H34 F N5 O6	2-F-A(bz)-CEP	X	Reduction	-(C9 H17 N2 O P)	675.24910	676.25637	[M+H]+1	-0.32	5.907	

MS^2 spectrum



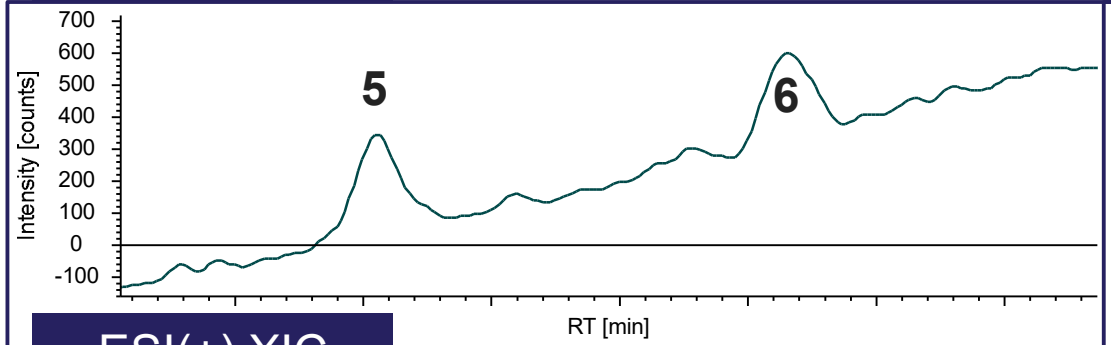
Fragmentation indicates absence of CEP



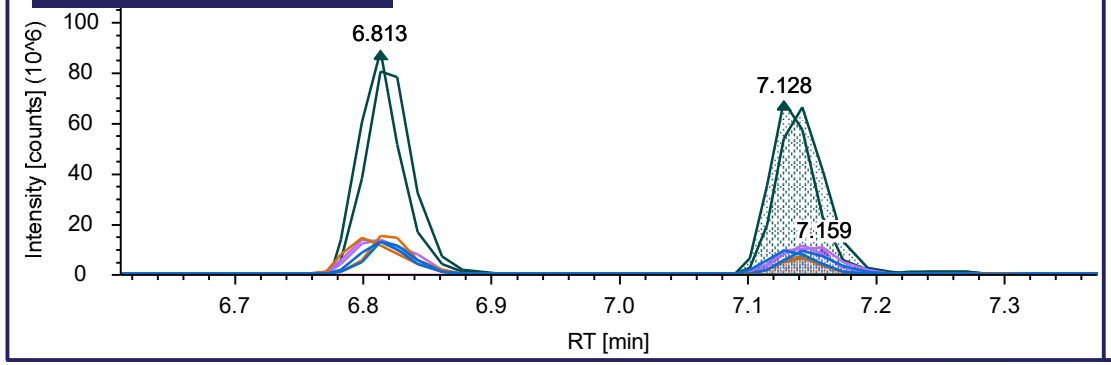
Structure elucidation for unexpected impurities

Compounds **5** + **6** – pair of diastereomers

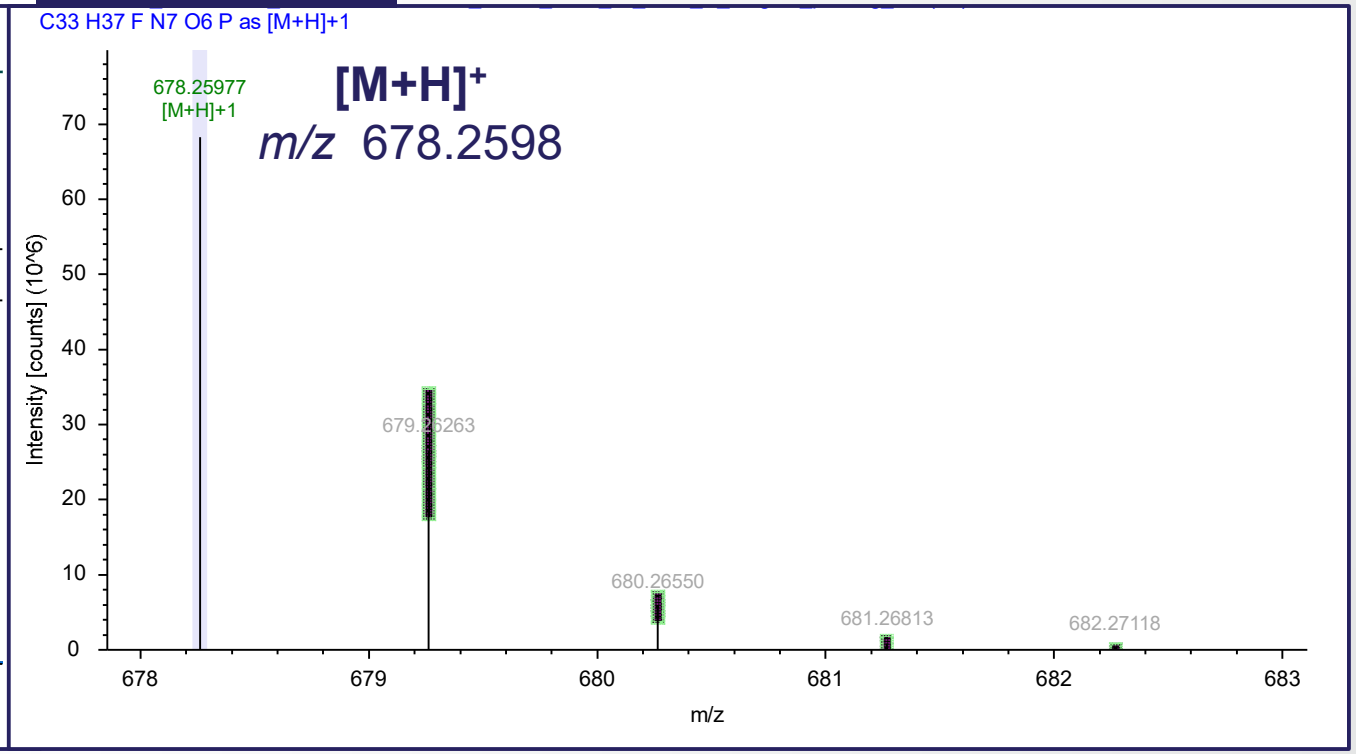
UV 200-400nm



ESI(+) XIC



MS^1 spectrum



Compounds	Compounds per File	Expected Compounds	Expected Compounds per File	Featu

	Formula	Calc. MW	Annot. ΔMass [ppm]	Reference Ion	m/z	RT [min]	MS2
1	C33 H37 F N7 O6 P	677.25252	-0.26	[M+H]+1	678.25981	6.815	

ΔFormula = – $C_{14}H_{14}O$

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Structure elucidation for unexpected impurities

Compounds **5** + **6** – pair of diastereomers

Compounds | Compounds per File | Expected Compounds | Expected Compounds per File | Featu

	Formula	Calc. MW	Annot. ΔMass [ppm]	Reference Ion	m/z	RT [min]	MS2
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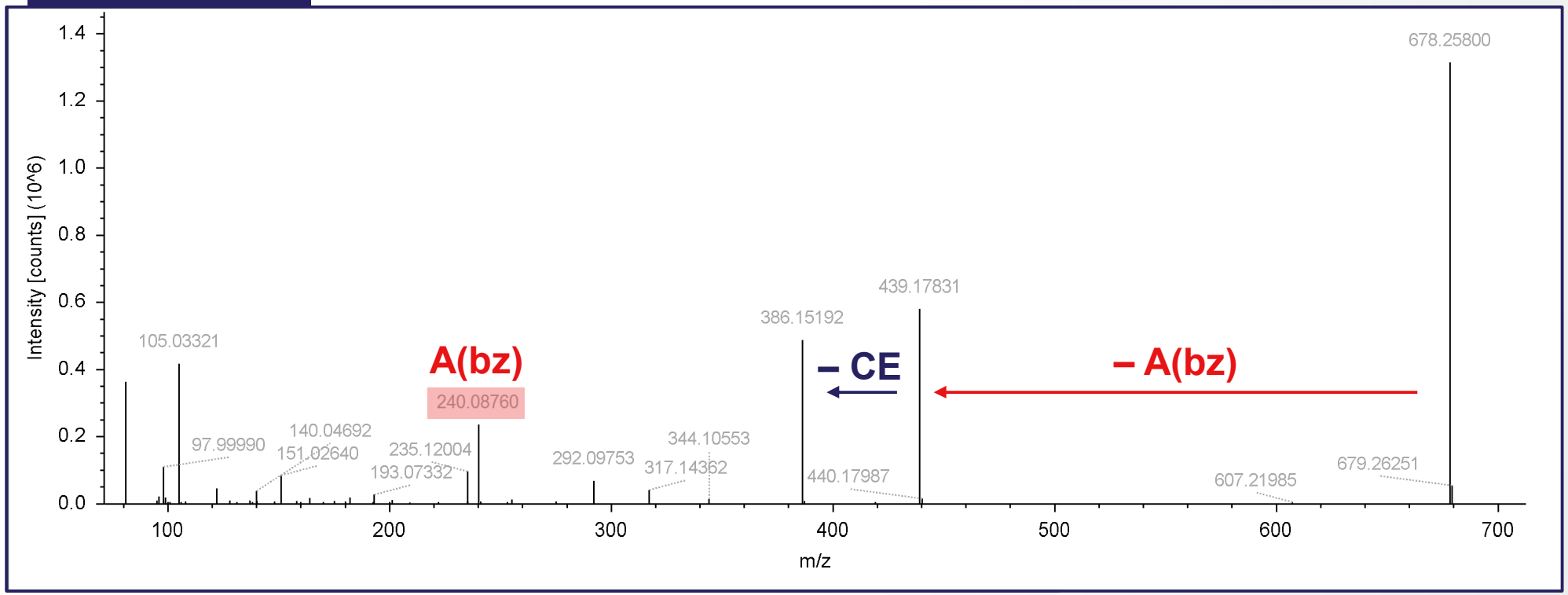
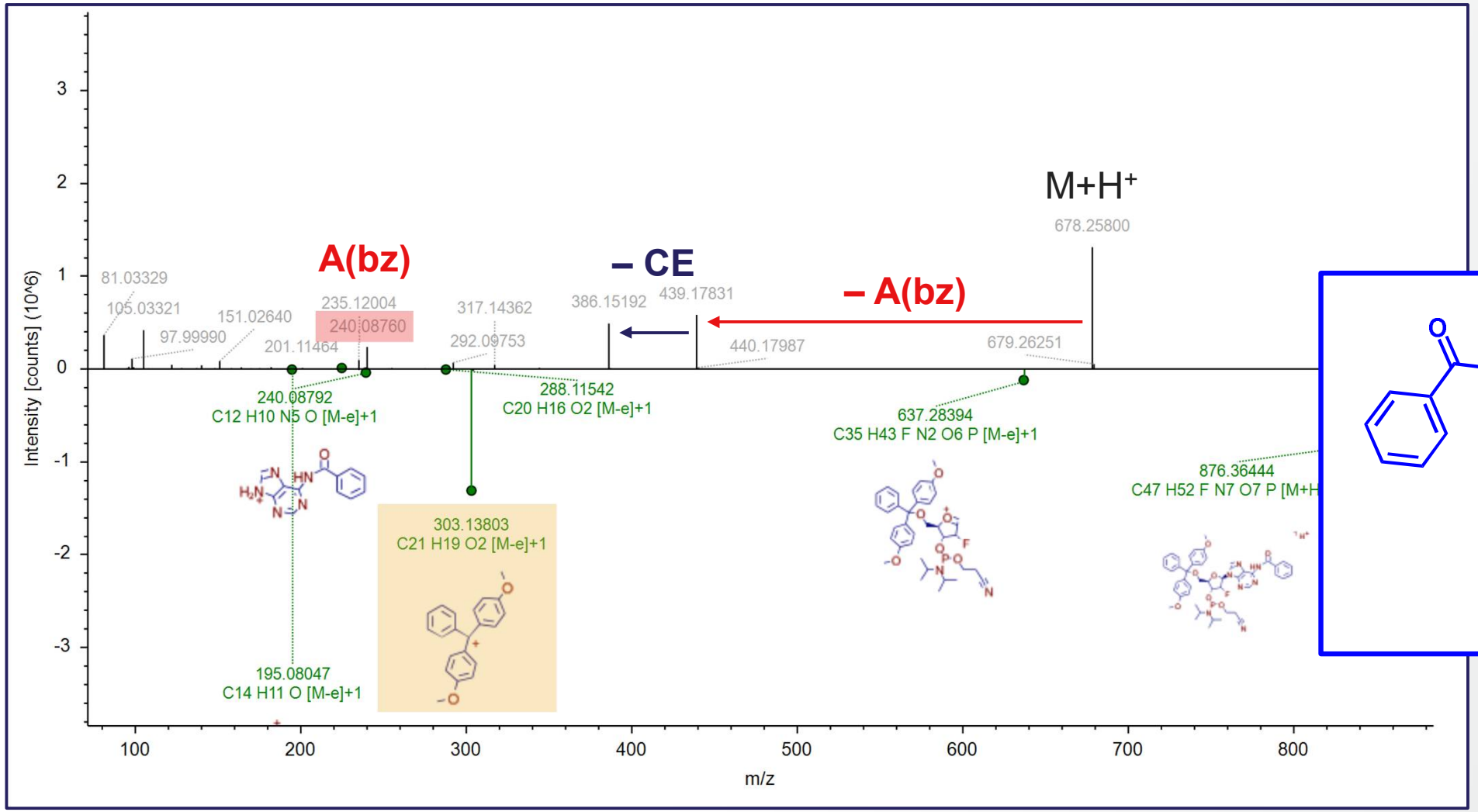
MS^2 spectrum



Structure elucidation for unexpected impurities

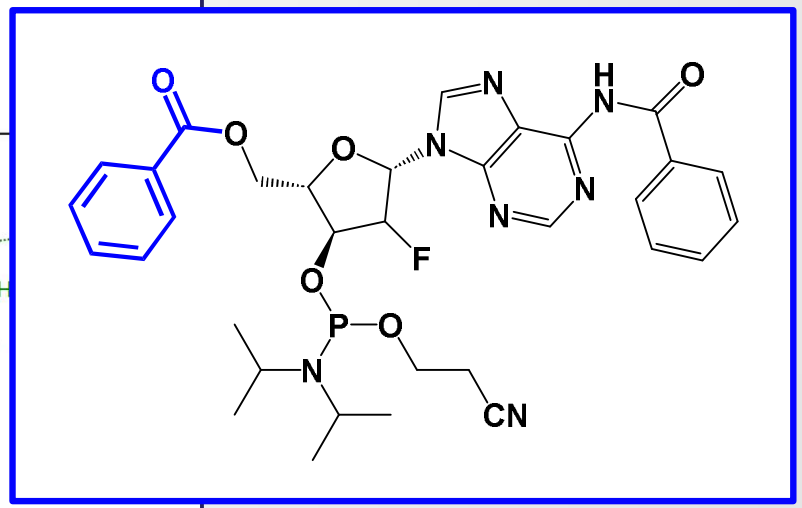
Compounds **5** + **6** – pair of diastereomers – Comparison to 5'-DMT-2'-F-A(bz)-CEP



ΔFormula = – $C_{14}H_{14}O$

– DMT = – $C_{21}H_{19}O_2$

+ R = + C_7H_5O



Structure elucidation for unexpected impurities

Compounds **5** + **6** – pair of diastereomers – 5'-**bz**-2'-F-A(bz)-CEP

Compounds	Compounds per File	Expected Compounds	Expected Compounds per File	Featu

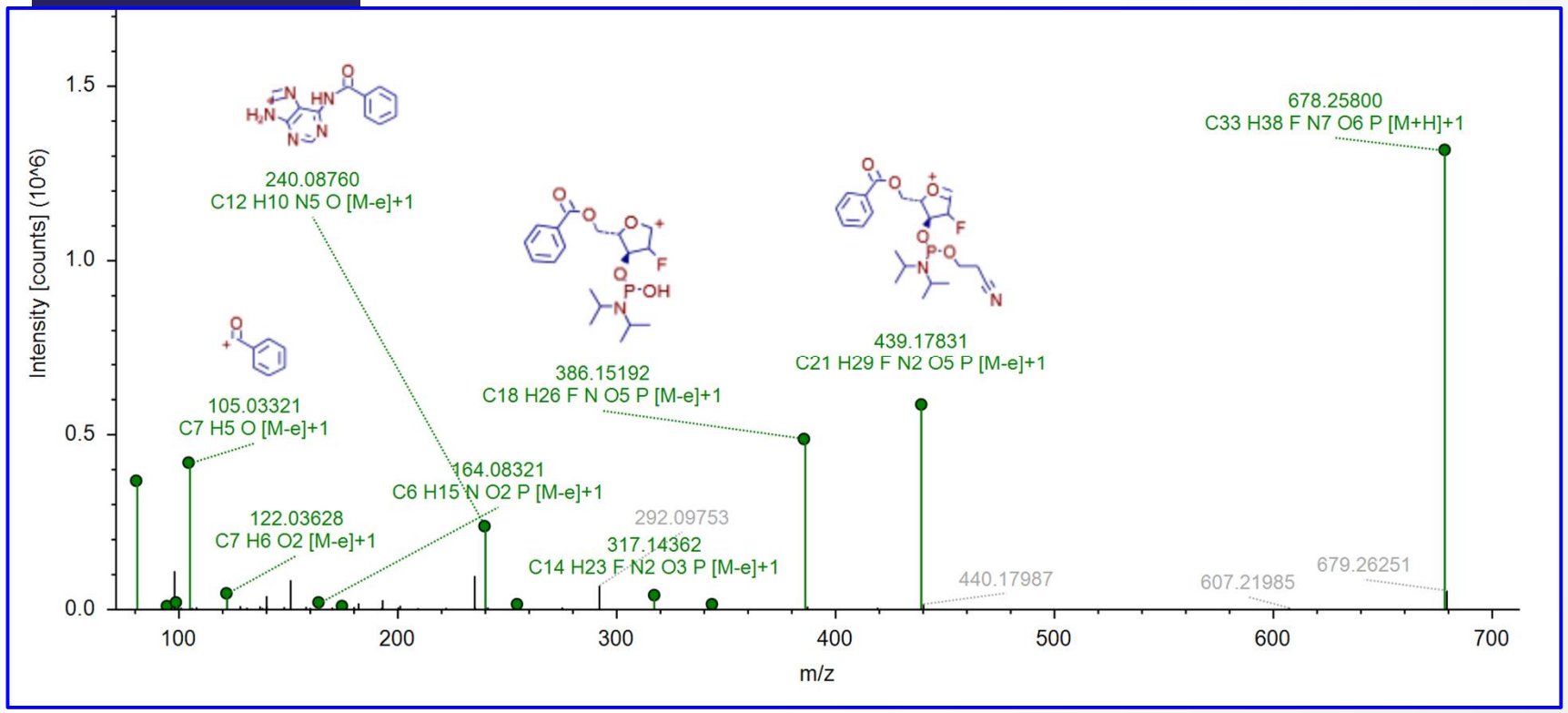
	Formula	Calc. MW	Annot. ΔMass [ppm]	Reference Ion	m/z	RT [min]	MS2
1	C33 H37 F N7 O6 P	677.25252	-0.26	[M+H]+1	678.25981	6.815	

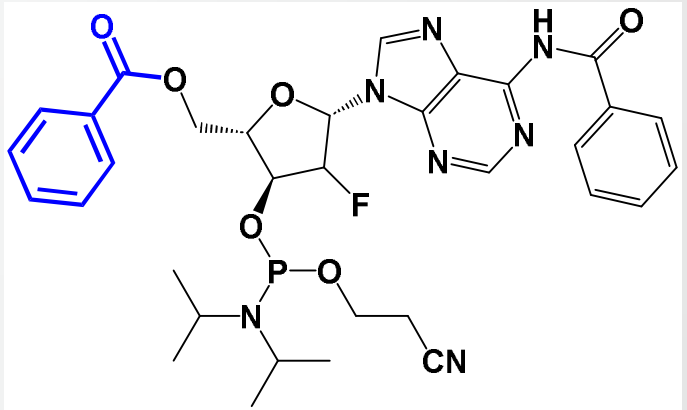
ΔFormula = – $C_{14}H_{14}O$

– DMT = – $C_{21}H_{19}O_2$

+ R = + C_7H_5O

MS^2 spectrum

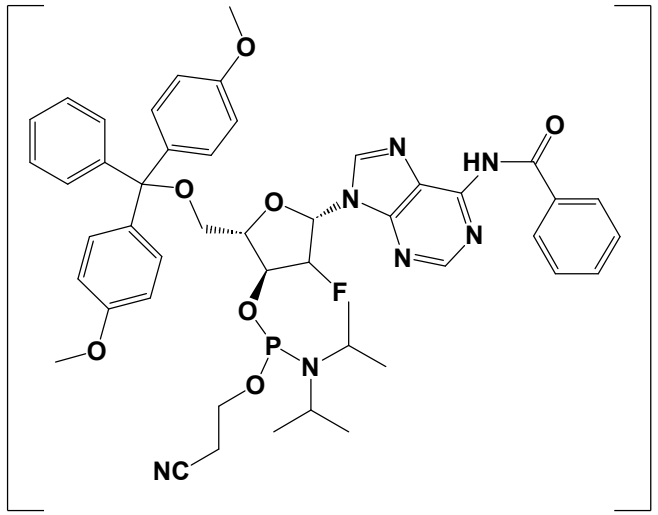


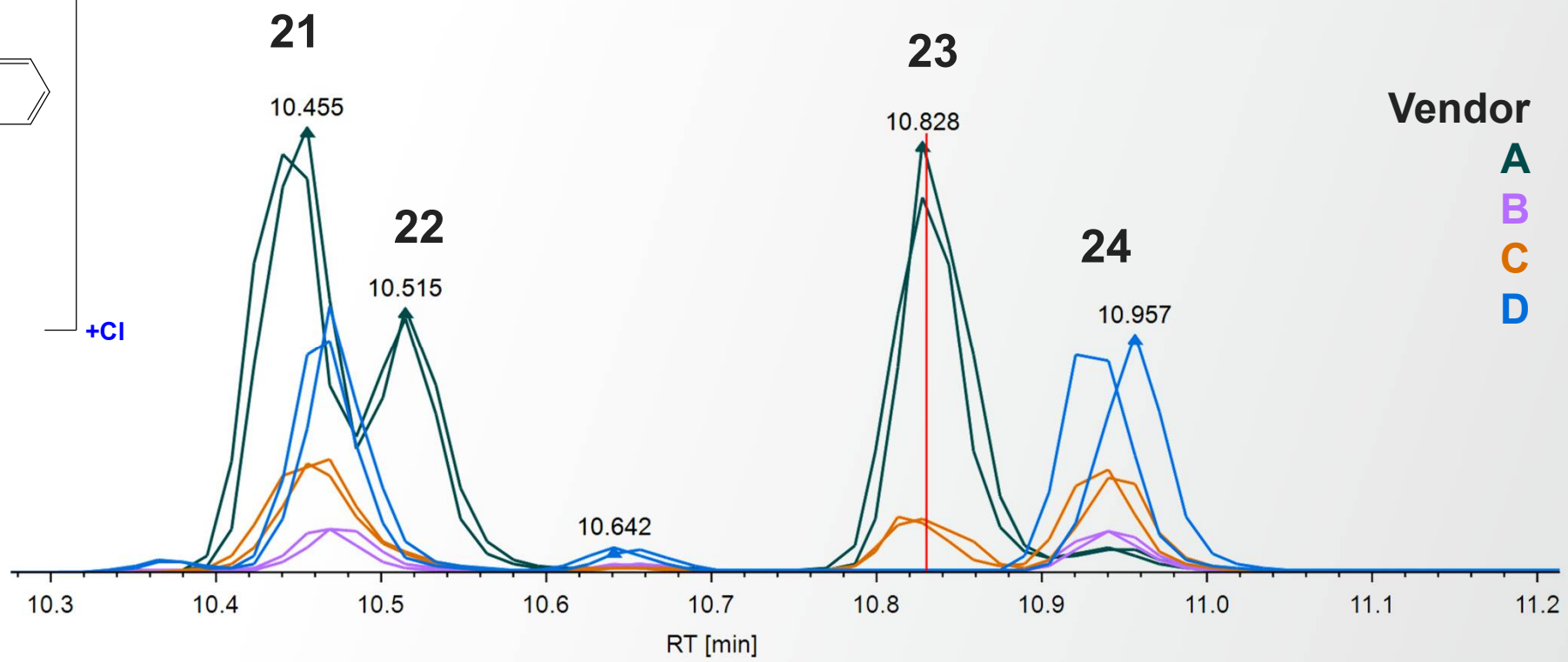


Structure proposal fits observed fragments

MS^2 distinguishes isomeric impurities 21-24

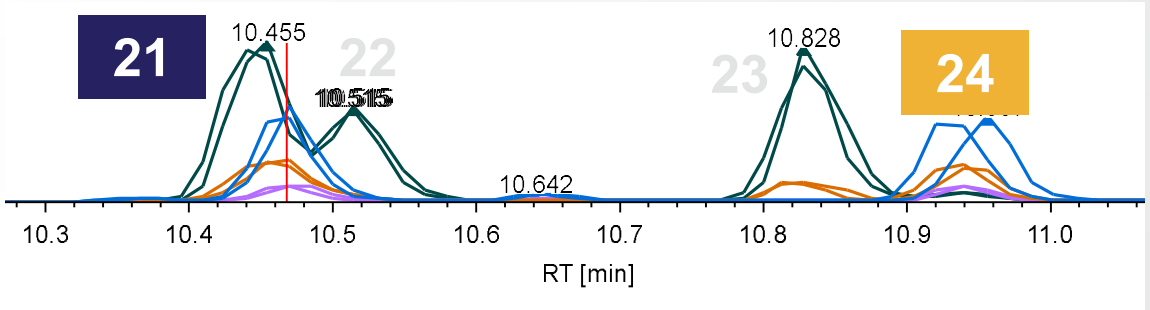
[5'-DMT-2'-F-A(bz)-CEP]**–H+Cl** *m/z* 910.3254

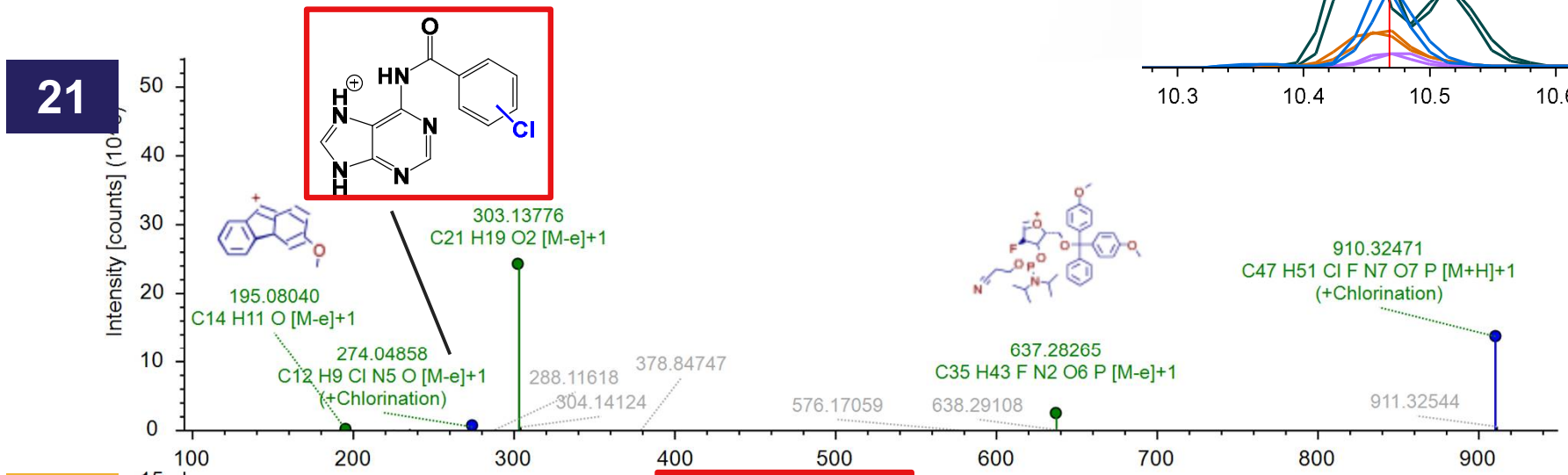


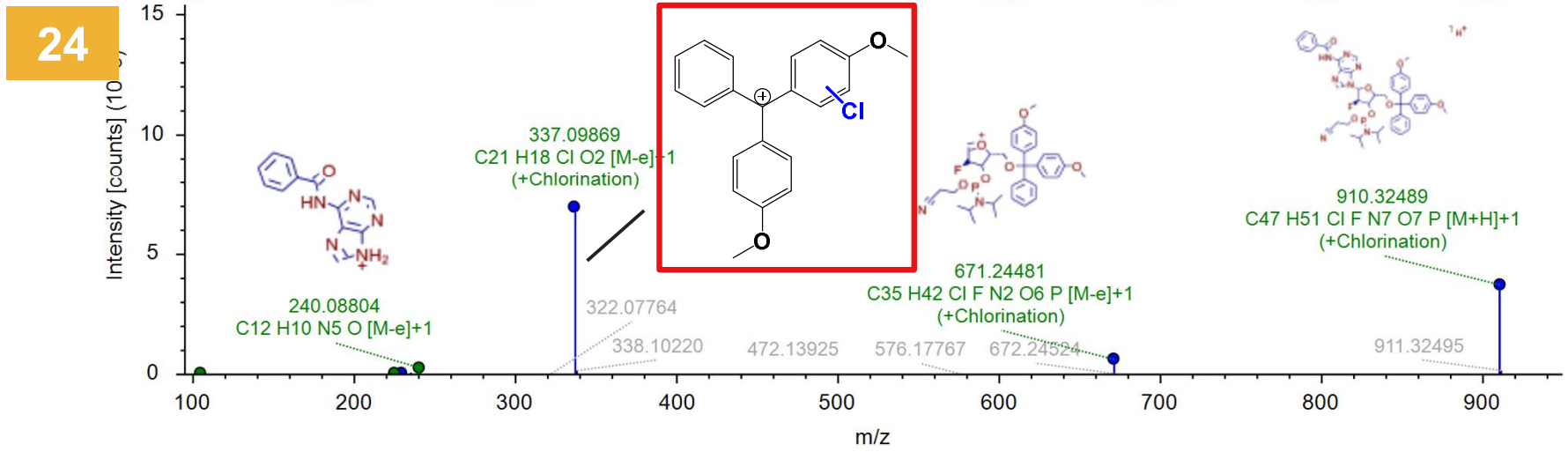


MS^2 distinguishes isomeric impurities 21-24

[5'-DMT-2'-F-A(bz)-CEP]–**H+Cl** *m/z* 910.3254

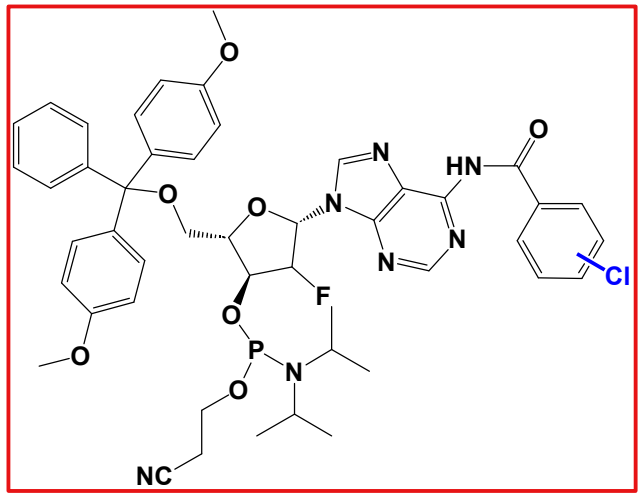


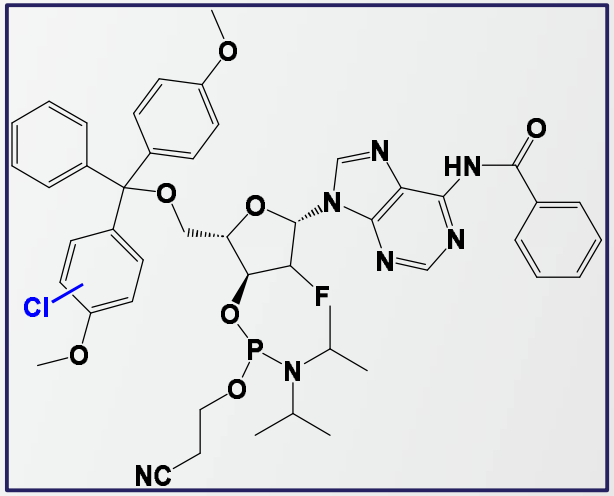


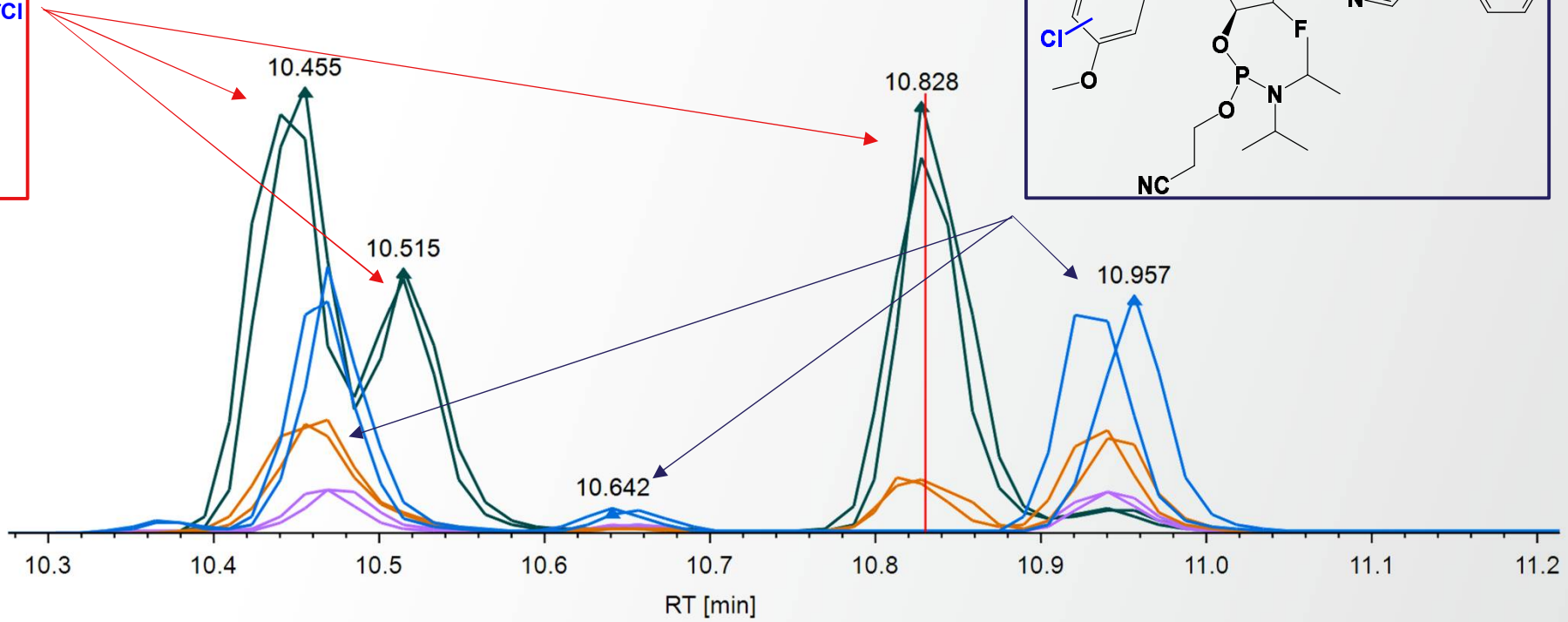


MS^2 distinguishes isomeric impurities 21-24

5’-DMT-2’-F-A(bz–**Cl**)-CEP *and* 5’-DMT–**Cl**-2’-F-A(bz)-CEP



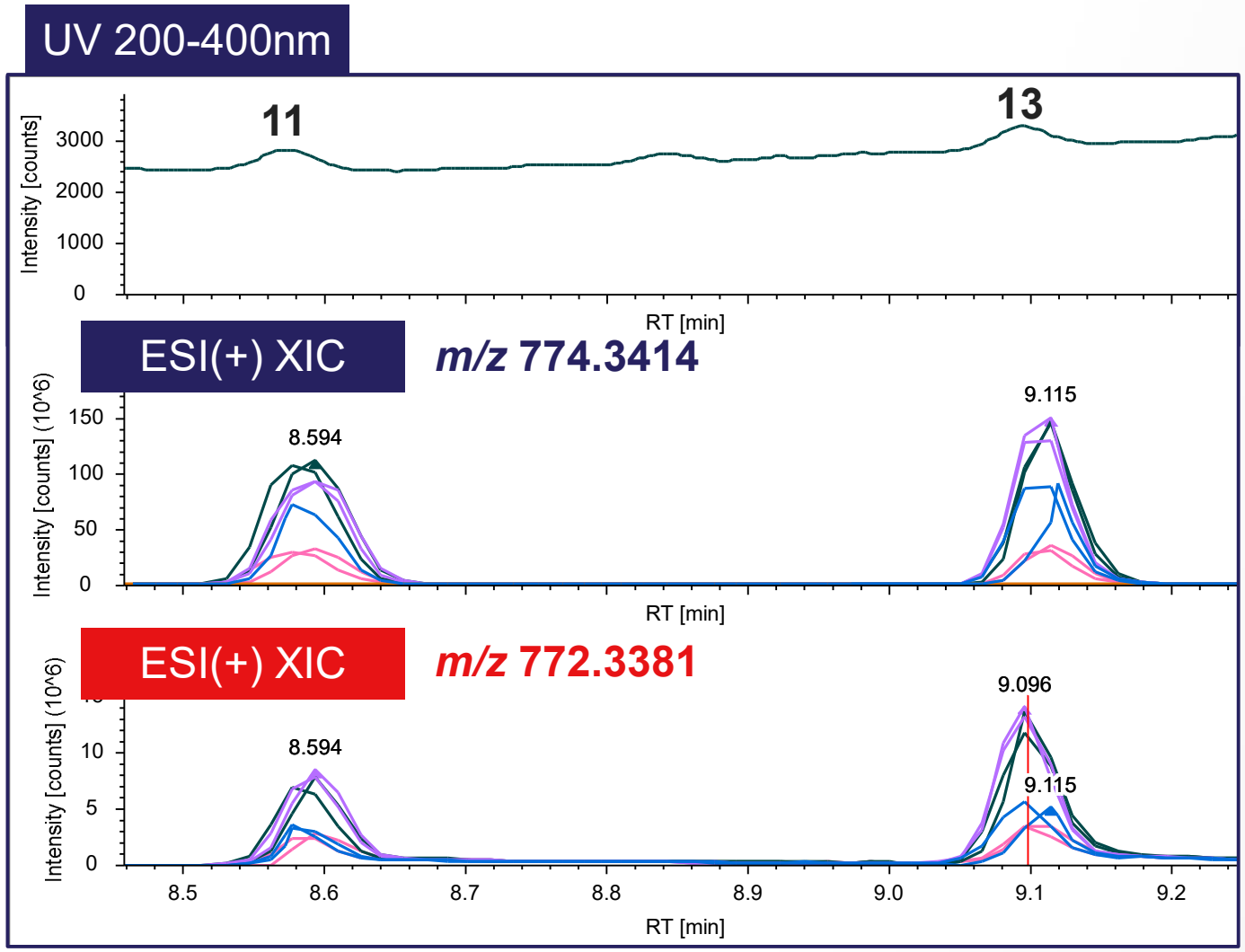


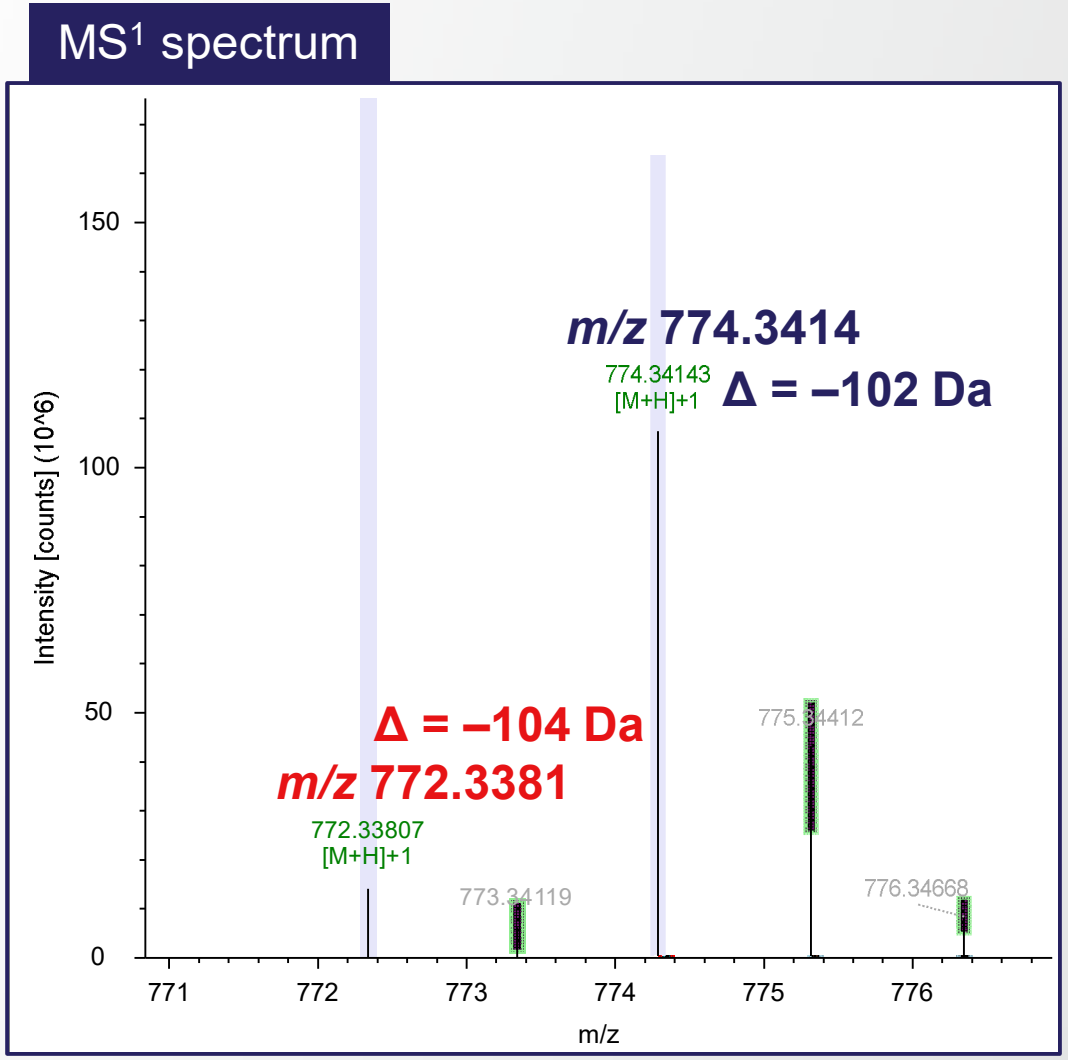


Determination of transformation site allows to trace back to origin of Chlorine substitution

Distinguishing co-eluting impurities with HRAM MS

Peaks **11** and **13** – overlapping MS components

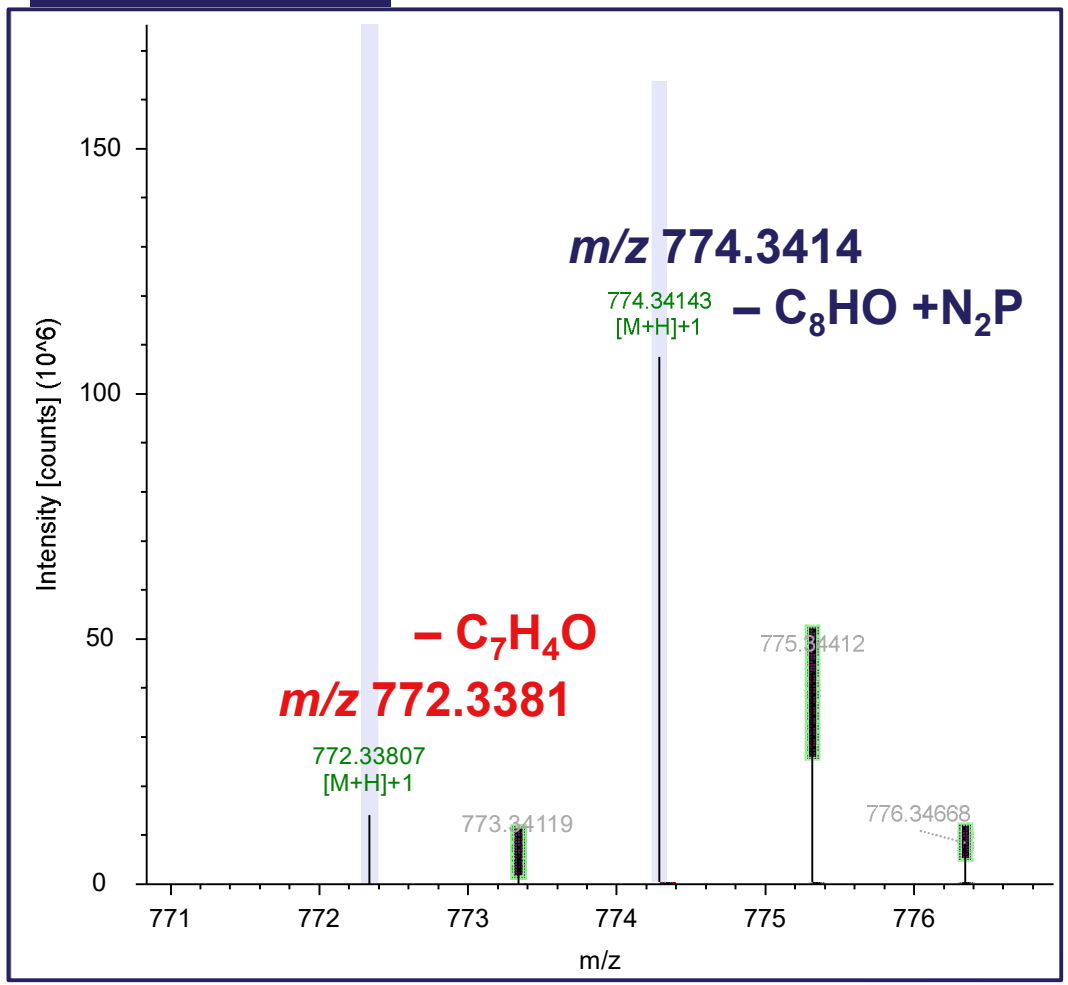




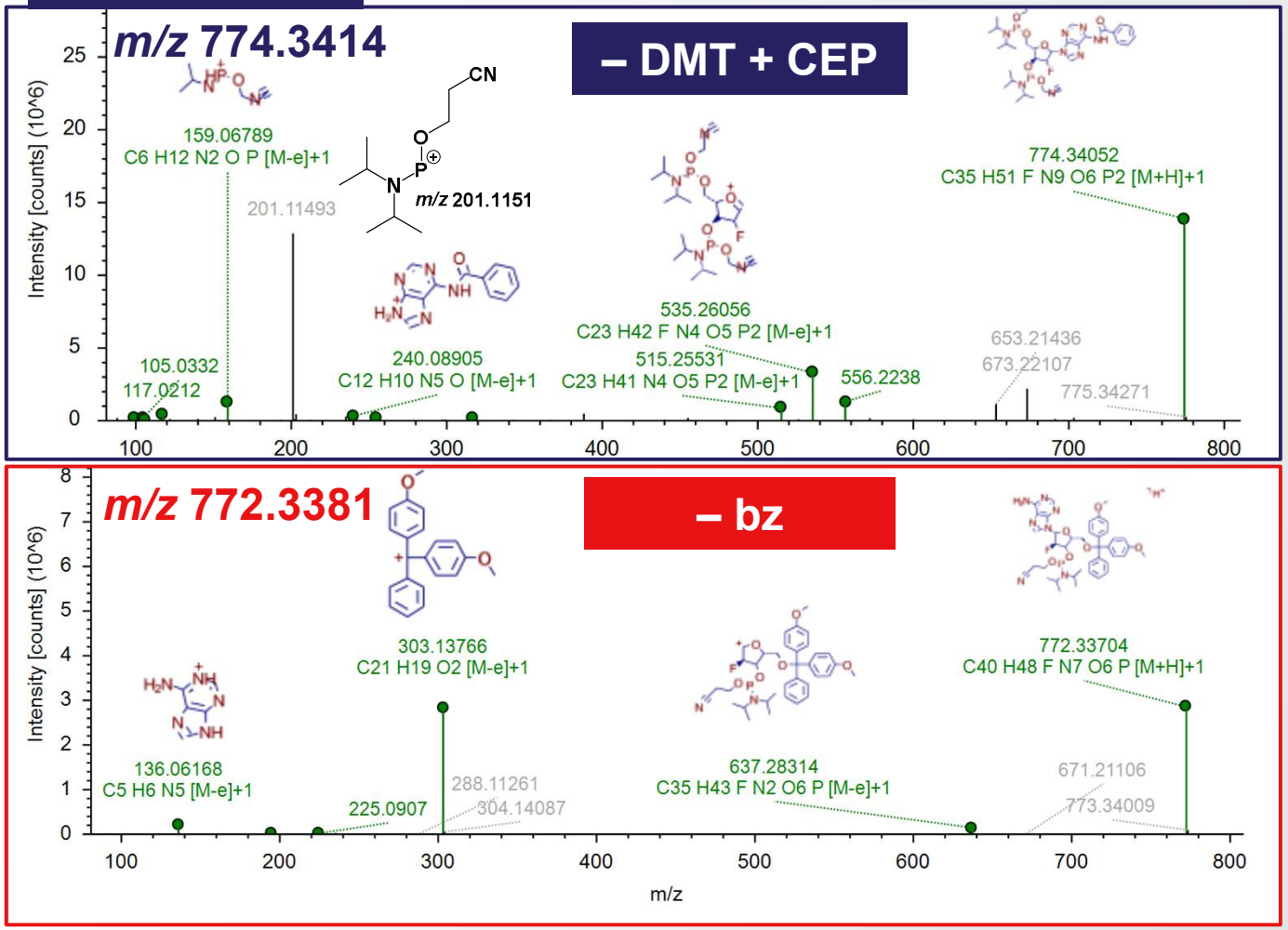
Distinguishing co-eluting impurities with HRAM MS

Peaks **11** and **13** – overlapping MS components – identification based on accurate mass and MS^2

MS^1 spectrum

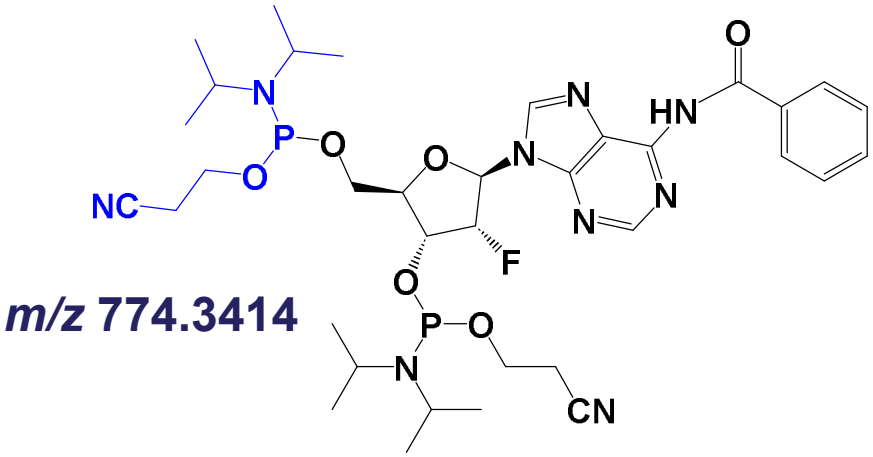


MS^2 spectrum

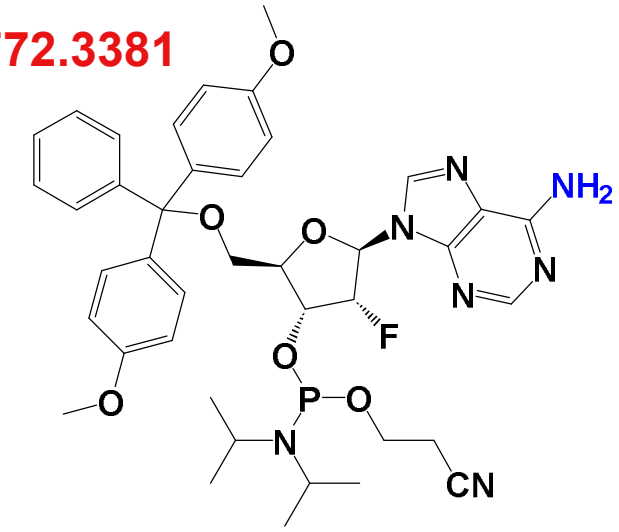


Distinguishing co-eluting impurities with HRAM MS

Peaks **11** and **13** – 5'-**CEP**-2'-F-A(bz)-CEP and 5'-DMT-2'-F-**A**-CEP

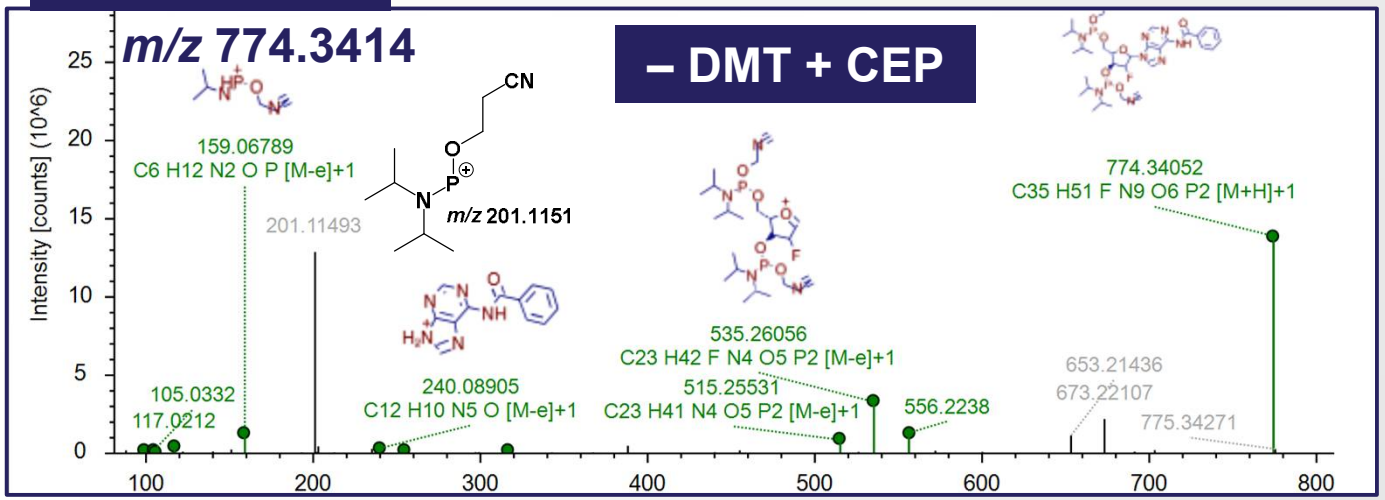


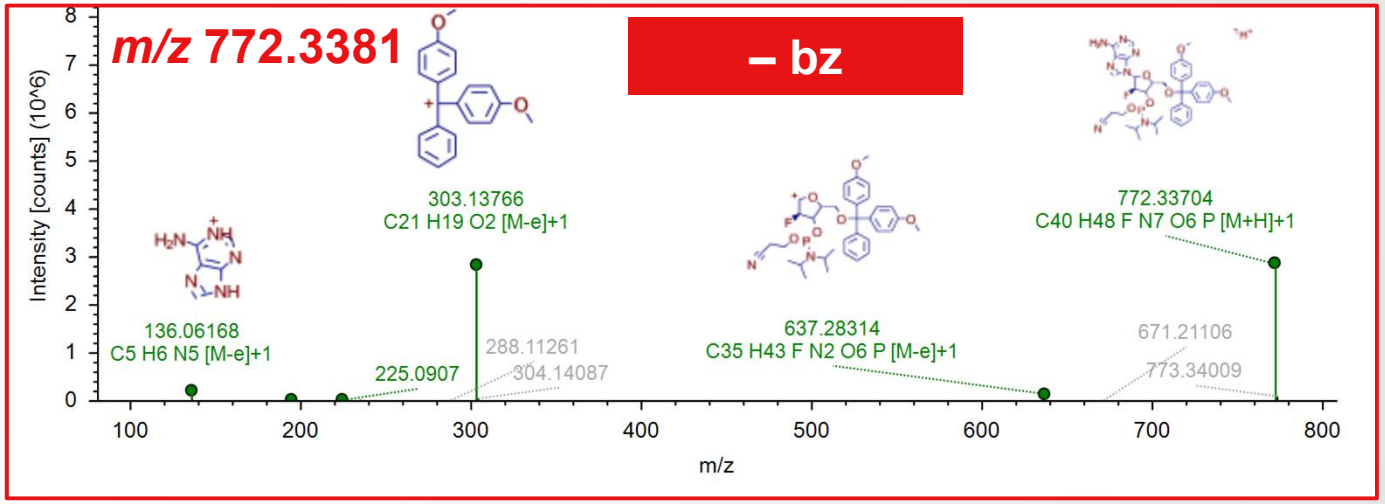
***m/z* 774.3414**



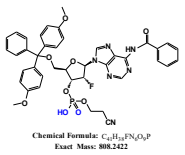
***m/z* 772.3381**

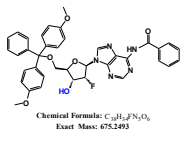
MS² spectrum

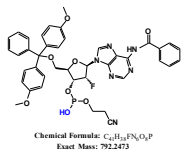


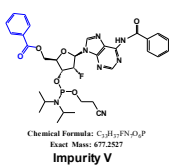


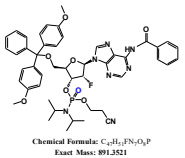
Summary of impurity analysis of 5'-DMT-2'-F-A(bz)-CEP

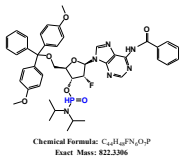

Impurity I

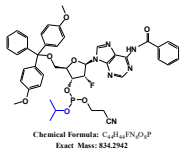

Impurity III

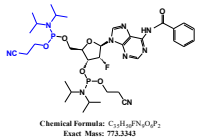

Impurity IV

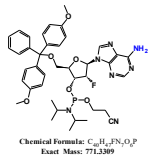

Impurity V

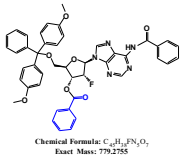

Impurity VI

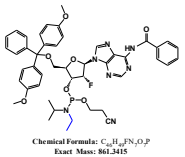

Impurity VII

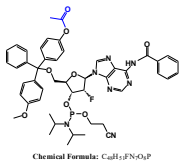

Impurity VIII

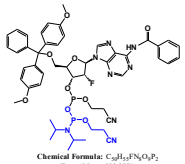

Impurity IX

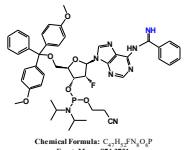

Impurity X

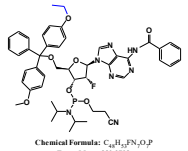

Impurity XI

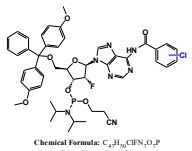

Impurity XII

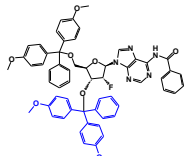

Impurity XIII

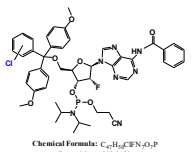

Impurity XV

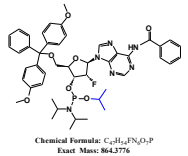

Impurity XVI


Impurity XVII


Impurity XVIII


Impurity XIX

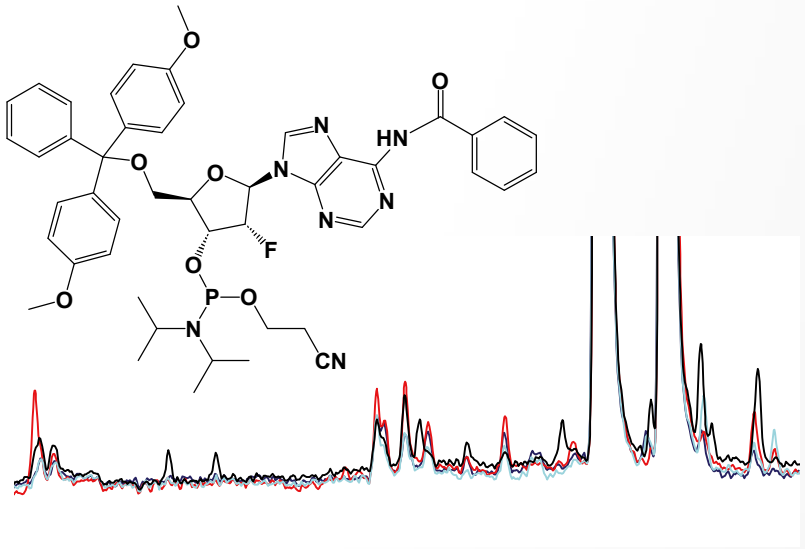

Impurity XX

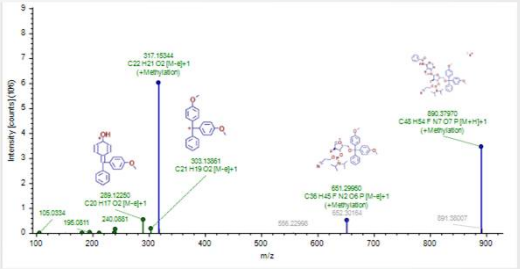

Impurity XXI

Impurity	MS peak (Da)	Formula	Putative identification	Example impurity classification	% of total UV peak area by vendor			
					A	B	C	D
I	808.2422	$C_{41}H_{38}FN_6O_9P$	-DIPA +O_2 to P(V) species	Noncritical	0.01%	0.00%	0.00%	0.00%
III	675.2491	$C_{38}H_{34}FN_5O_6$	-CEP	Noncritical	0.00%	0.00%	0.09%	0.00%
IV	792.2472	$C_{41}H_{38}FN_6O_8P$	-DIPA + OH	Noncritical	0.07%	0.05%	0.07%	0.05%
V	677.2525	$C_{33}H_{37}FN_7O_6P$	-DMT +bz	Noncritical	0.05%	0.00%	0.00%	0.00%
VI	891.3518	$C_{47}H_{51}FN_7O_8P$	Oxidation to P(V) species	Noncritical	0.06%	0.10%	0.13%	0.13%
VII	822.3306	$C_{44}H_{48}FN_6O_7P$	HP=O phosphite + loss of cyanoethyl group	Noncritical	0.01%	0.04%	0.04%	0.00%
VIII	834.2944	$C_{44}H_{44}FN_6O_8P$	Substitution of DIPA with iPrOH	Noncritical	0.06%	0.00%	0.00%	0.00%
IX	773.3341	$C_{35}H_{50}FN_9O_6P_2$	-DMT +CEP	Critical	**0.01%**	**0.05%**	**0.07%**	**0.04%**
X	771.3307	$C_{40}H_{47}FN_7O_6P$	-bz	Critical				
XI	779.2753	$C_{45}H_{38}FN_5O_7$	-CEP + bz	Noncritical	0.01%	0.01%	0.01%	0.00%
XII	861.3414	$C_{46}H_{49}FN_7O_7P$	Demethylation *on CEP*	Noncritical	0.00%	0.03%	0.00%	0.01%
XIII	903.3518	$C_{47}H_{55}FN_3O_{12}P$	Acetyl-methyl substitution on DMT	Noncritical	0.00%	0.01%	0.00%	0.01%
XV	992.3551	$C_{50}H_{55}FN_8O_9P_2$	-DIPA +CEP	Critical	0.00%	0.00%	0.03%	0.00%
XVI	874.3732	$C_{47}H_{52}FN_8O_6P$	-O +NH on bz	Noncritical	0.08%	0.00%	0.04%	0.00%
XVII	889.3721	$C_{48}H_{53}FN_7O_7P$	Methylation on DMT	Noncritical	0.09%	0.00%	0.00%	0.00%
XVIII	909.3177	$C_{47}H_{50}ClFN_7O_7P$	Chlorination on bz	Noncritical	0.12%	0.07%	0.05%	0.09%
XIX	977.3799	$C_{59}H_{52}FN_5O_8$	-CEP +DMT	Noncritical	0.02%	0.01%	0.03%	0.02%
XX	909.3177	$C_{47}H_{50}ClFN7O_7P$	Chlorination on DMT	Noncritical	0.00%	0.01%	0.01%	0.04%
XXI	864.3779	$C_{47}H_{54}FN_6O_7P$	-CN +CH_3 "M-11"	Critical	**0.02%**	**0.00%**	**0.00%**	**0.00%**
P	**875.3571**	**$C_{47}H_{51}FN_7O_7P$**	**5'-DMT-2'-F-A(bz)CEP**	**–**	**99.35%**	**99.61%**	**99.44%**	**99.61%**
				Critical impurity level	0.03%	0.05%	0.10%	0.04%

For more detail: [Application Note AN001949](https://view.highspot.com/viewer/644058d5d73ddb9595e641e5)

Summary





- **Phosphoramidites** are **raw materials** for oligonucleotide-derived drug substances, and their impurities need to be controlled in the **manufacturing of therapeutic oligonucleotides**

- **Vanquish UHPLC** system coupled with **Orbitrap Exploris 120 MS** facilitates **confident profiling and identification of phosphoramidite impurities**
- **Compound Discoverer** software **automates mass spectral annotation** process, allowing users to determine transformation sites
- **Fragmentation data** allows the distinction of isomeric impurities and to **determine the origin of transformations**

Thank you

Questions?