Impact of HFIP Vendor Variability on MS Sensitivity In IP-RP LC-MS Oligonucleotide Analysis

Toshi Ono,[1] Makoto Ozaki,[2] Tomomi Kuwayama,[2] Motoshi Shimotsuma,[2] Junji Kawakami,[3] Tsunehisa Hirose[2]

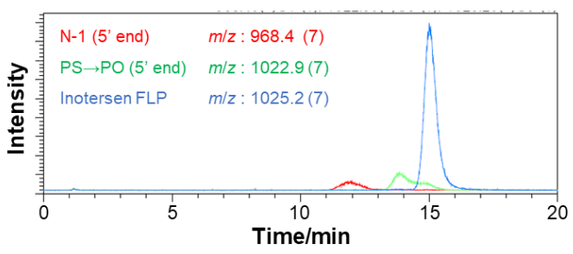
[1]Nacalai USA Inc., [2]Research and Development Department, Nacalai Tesque Inc., [3]FIRST, Konan University

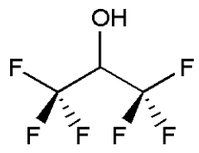
• Introduction

Ion-pair reversed-phase liquid chromatography coupled with mass spectrometry (IP-RP LC-MS) is the most widely used approach for oligonucleotide and nucleic acid impurity analysis. This method typically employs volatile mobile phases composed of alkyl amines and hexafluoroisopropanol (HFIP), which provide efficient chromatographic retention while maintaining electrospray ionization compatibility.

Despite the routine use of HPLC grade HFIP, variability in mass spectrometric sensitivity has been observed in practice, raising concerns regarding reproducibility and data interpretation. In this sturdy, we evaluated the impact of HFIP vendor variability on MS sensitivity using two representative analytes: oligo dT as a model synthetic oligonucleotide and Inotersen, a clinically relevant antisense oligonucleotide.

Chromatographic Separation of Inotersen and Related Impurities





HFIP
1,1,1,3,3,3-hexafluoro-2-propanol

LC conditions
Column: COSMOCORE 2.6C_{18}, 2.1 mm I.D. × 100 mm
Flow rate: 0.2 mL/min Temperature: 65ºC
Injection vol.: 1 µL
Solvent A: 100 mM HFIP - 10 mM triethylamine mixture
Solvent B: Solvent A / Methanol / Acetonitrile = 50 / 25 / 25 (v / v)
Gradient: Solvent B conc. 23%-28% (0-20 min)

MS conditions
Equipment: LCMS 2050 (Shimadzu)
Ionization: ESI/APCI (Negative) Mode: Scan and SIM
Mass range: 550-2000
Nebulizing gas flow: 2.0 L/min Drying gas flow: 5.0 L/min
Heating gas flow: 7.0 L/min DL temperature: 200ºC
Desolvation temperature: 450ºC Interface voltage: -2.0 kV

Table. Purity and metal content of each HFIP

Vendor	In-house	Vendor A	Vendor B	Vendor C*	Vendor D*
Grade	HPLC	HPLC	HPLC	LC-MS	LC-MS
Purity	> 99.9%	> 99.9%	> 99.9%	> 99.8%	> 99.8%
Al	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm
Ba	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	**
Bi	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	**
Ca	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm
Cd	< 0.05 ppm	< 0.05 ppm	< 0.05 ppm	< 0.05 ppm	**
Co	< 0.02 ppm	< 0.02 ppm	< 0.02 ppm	< 0.02 ppm	**
Cr	< 0.02 ppm	< 0.02 ppm	< 0.02 ppm	< 0.02 ppm	**
Cu	< 0.02 ppm	< 0.02 ppm	< 0.02 ppm	< 0.02 ppm	< 0.02 ppm
Fe	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm
K	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 2 ppm
Li	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	**
Mg	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm
Mn	< 0.02 ppm	< 0.02 ppm	< 0.02 ppm	< 0.02 ppm	**
Mo	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	**
Na	< 1 ppm	< 1 ppm	**3.7 ppm**	< 1 ppm	< 10 ppm
Ni	< 0.02 ppm	< 0.02 ppm	< 0.02 ppm	< 0.02 ppm	**
Pb	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	**
Sr	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	**
Zn	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	**

* Vendor C and D are specified values.

All metal contents in the HFIP were found to be within specifications except for Na of vendor B.

An optimized analysis protocol was established to eliminate residual effects from different HFIP vendors.

• Analysis protocol

Equilibration with HFIP mobile phase for 1 hour
↓
Analysis (n=2)
↓
Washing with 50% methanol for 2 hour
↓
Equilibration with HFIP mobile phase for 1 hour
↓
Analysis (n=2)

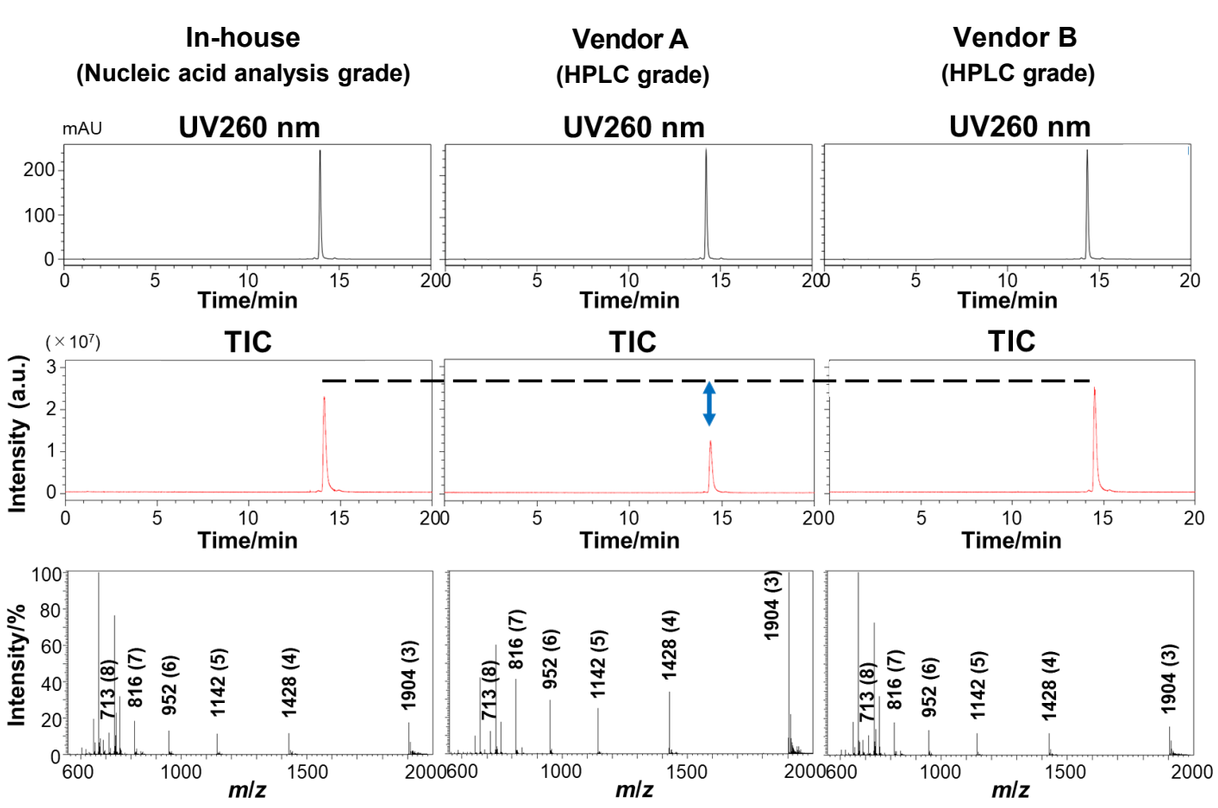
Mobile phase is prepared immediately before analysis.

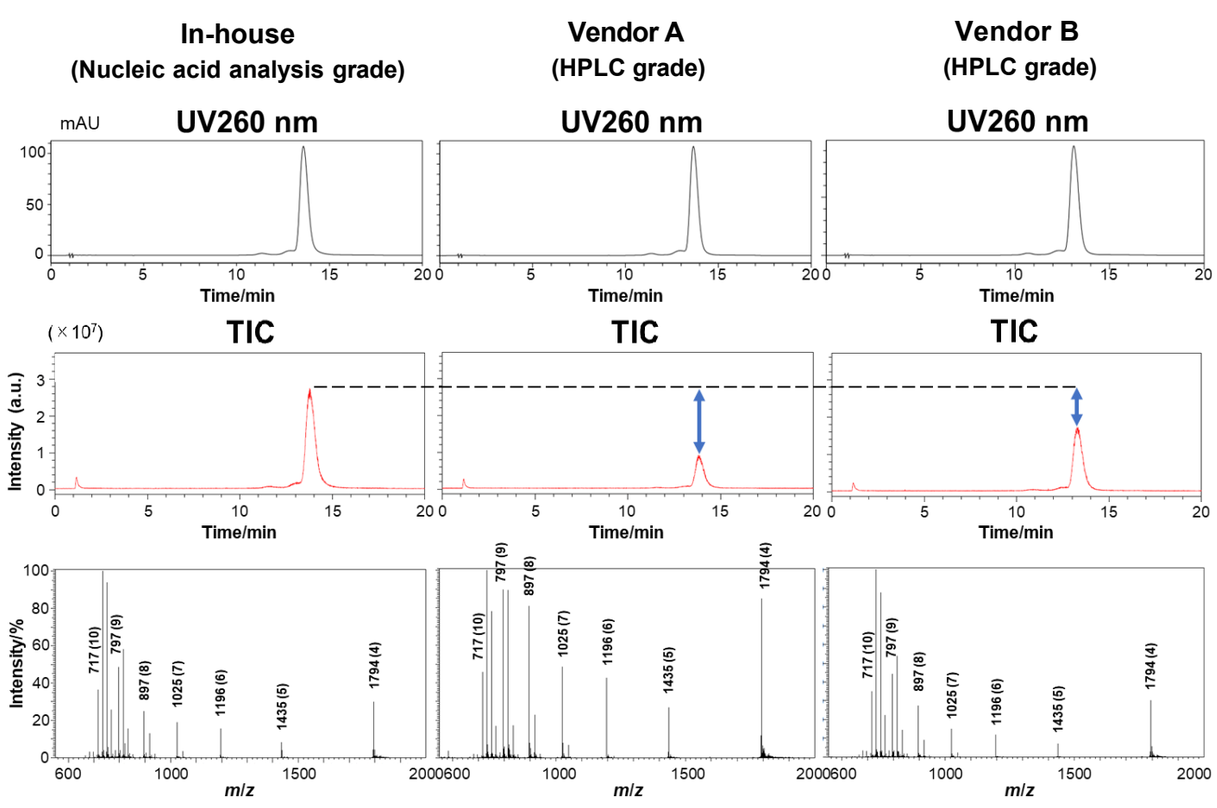
• Comparison of MS sensitivity obtained using mobile phases prepared with HFIP from different sources.

Poly(dT_{19}) 5'-TTTTTTTTTTTTTTTTTTT-3'

Inotersen 5'-**TCTTG**GTTA**C**ATGAA**A**TCCC-3'

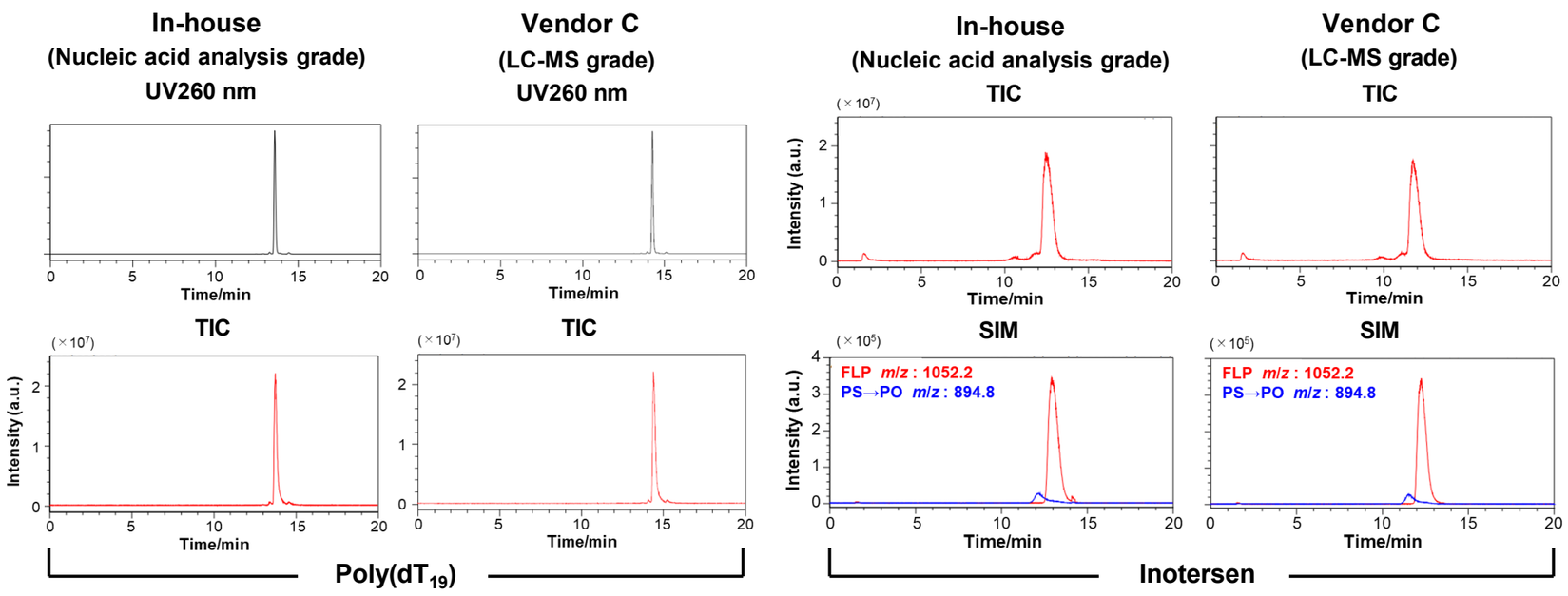
Red font: 5-Methyl pyrimidine
Blue font: 2'-*O*-methoxyethyl



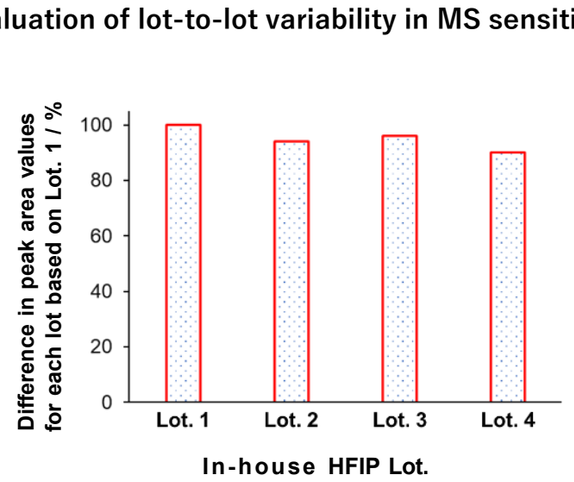


UV chromatographic intensities were comparable across mobile phases prepared with HFIP from different sources; however, MS sensitivity differed among HFIP sources and varied depending on the nucleic acid analyzed.

• Comparison of MS sensitivity between in-house and commercial LC-MS grade HFIP



• Evaluation of lot-to-lot variability in MS sensitivity



No significant differences in MS sensitivity were observed among different lots of the in-house HFIP.

HFIP prepared from the in-house source showed comparable MS sensitivity to one of the evaluated commercial LC-MS grade.

LC condition (Poly(dT_{19}))
Column: COSMOCORE 2.6C_{18}, 2.1 mm I.D. × 100 mm
Flow rate: 0.2 mL/min Temperature: 40ºC
Injection vol.: 1 µL (H_2O 0.5 µL + FLP 0.5 µL) Detection: UV260 nm
Solvent A: 100 mM HFIP - 15 mM triethylamine mixture
Solvent B: Solvent A / Methanol = 1 / 1 (v / v)
Gradient: Solvent B conc. 20%-50%-50%-20%-20% (0-20-25-25.1-40 min)

MS condition
Equipment: LCMS 2050 (Shimadzu)
Ionization: ESI/APCI (Negative) Mode: Scan or SIM
Mass range: 550-2000
Nebulizing gas flow: 2.0 L/min Drying gas flow: 5.0 L/min
Heating gas flow: 7.0 L/min DL temperature: 200ºC
Desolvation temperature: 450ºC Interface voltage: -2.0 kV

LC condition (Inotersen)
Column: COSMOCORE 2.6C_{18}, 2.1 mm I.D. × 100 mm
Flow rate: 0.2 mL/min Temperature: 65ºC
Injection vol.: 1 µL (H_2O 0.5 µL + FLP 0.5 µL) Detection: UV260 nm
Solvent A: 100 mM HFIP - 10 mM triethylamine mixture
Solvent B: Solvent A / Methanol / Acetonitrile = 50 / 25 / 25 (v / v)
Gradient: Solvent B conc. 23%-28%-28%-23%-23% (0-20-25-25.1-40 min)

MS condition
Equipment: LCMS 2050 (Shimadzu)
Ionization: ESI/APCI (Negative) Mode: Scan or SIM
Mass range: 550-2000
Nebulizing gas flow: 2.0 L/min Drying gas flow: 5.0 L/min
Heating gas flow: 7.0 L/min DL temperature: 200ºC
Desolvation temperature: 450ºC Interface voltage: -2.0 kV

Conclusions

Our result demonstrate that the source of HFIP significantly impacts MS signal response for both synthetic and antisense oligonucleotides. These variations in sensitivity suggest that trace impurities or solvent quality differences between vendors can influence ionization efficiency in IP-RP LC-MS workflows.

Acknowledgements

This research was supported by AMED under Grant Number JP21ae0121022, JP21ae0121023, JP21ae0121024.