# The Identification of Methyl-Substituted Fentanyl Analogs Using Optimized Weighting Within the Inverted Library Search Algorithm (ILSA)

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## ABSTRACT

The National Institute of Standards and Technology/National Institute of Justice Data Interpretation Tool (NIST/NIJ DIT) was developed to help analysts interpret complex direct analysis in real time-mass spectrometry (DART-MS) spectra. However, the underpinning algorithm of the NIST/NIJ DIT, the ILSA, struggles to reliably differentiate isomers. This study explores the use of optimized weighting of the scoring metrics within the ILSA to improve the differentiation of methyl-substituted fentanyl analogs.

## INTRODUCTION

Given the continuously evolving drug landscape, the rapid identification of seized drug isomers is essential to address growing casework backlogs. The NIST/NIJ DIT was developed to address this challenge based on the comparison of in-source collision-induced dissociation (IS-CID) mass spectra at low, medium, and higher activation energies [1,2]. The NIST/NIJ DIT utilizes a novel algorithm known as the inverse library search algorithm (ILSA) to search for partial patterns within a mass spectrum. The ILSA generates two similarity scores, the fraction of peak intensity explained (FPIE) and the reverse match factor (RevMF) for spectral comparison [1,2]. Unfortunately, the ILSA struggles with the correct identification of isomers using the current equal weighting system [2]. This research demonstrates how optimizing the weighting of the low, medium, and high activation energy mass spectra leads to improved differentiation of methyl-substituted fentanyl analogs [3].

## MATERIALS & METHODS

*Chemicals and Sample Preparation*

Eight methyl-substituted fentanyl analogs representative of different R-group substitutions were analyzed in this study (Figure 1). All standards were prepared at a concentration of 10 ppm and two-component mixtures were prepared at a total concentration of 20 ppm in methanol. All analogs were purchased as Certified Reference Materials from Cayman Chemical or Cerilliant.

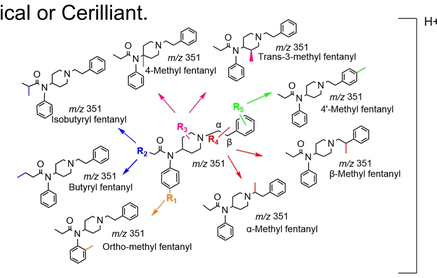


**Figure 1.** The core fentanyl structure, summarizing the isobaric methyl-substituted fentanyl analogs, including ortho-methyl fentanyl ($R_1$); butyryl fentanyl and isobutyryl fentanyl ($R_2$); trans-3-methyl fentanyl and 4-methyl fentanyl ($R_3$); α-methyl fentanyl and β-methyl fentanyl ($R_4$); and 4'-methyl fentanyl ($R_5$).

## RESULTS & DISCUSSION

**Table 1.** Average FPIE and RevMF scores from 10 replicates when 4'-methyl fentanyl was searched against an internal library.

| Methyl-substituted fentanyl analog | Average FPIE | Average RevMF |
|---|---|---|
| Ortho-methyl fentanyl | 0.677 | 0.392 |
| Butyryl fentanyl | 0.668 | 0.399 |
| Isobutyryl fentanyl | 0.674 | 0.388 |
| Trans-3-methyl fentanyl | 0.815 | 0.644 |
| 4-methyl fentanyl | 0.891 | 0.686 |
| α-methyl fentanyl | 0.977 | 0.921 |
| β-methyl fentanyl | 0.992 | 0.783 |
| **4'-methyl fentanyl** | 0.999 | 0.999 |
| Standard deviation | 0.148 | 0.243 |
| Difference between the top 2 scores | 0.007 | 0.078 |

- Greater variability for the RevMF was observed for all the methyl-substituted fentanyl analogs analyzed individually and in mixtures.

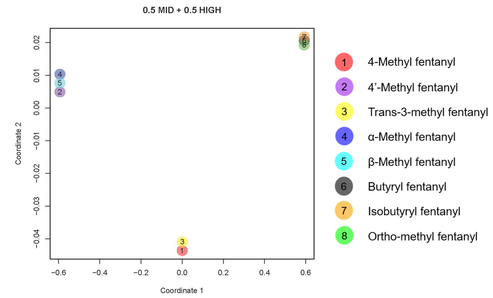


**Figure 2.** Mass spectral similarity space using 50:50 weighting of the medium and high activation energy spectra.

- 50:50 weighting enables differentiation, except for butyryl fentanyl, isobutyryl fentanyl, and ortho-methyl fentanyl.

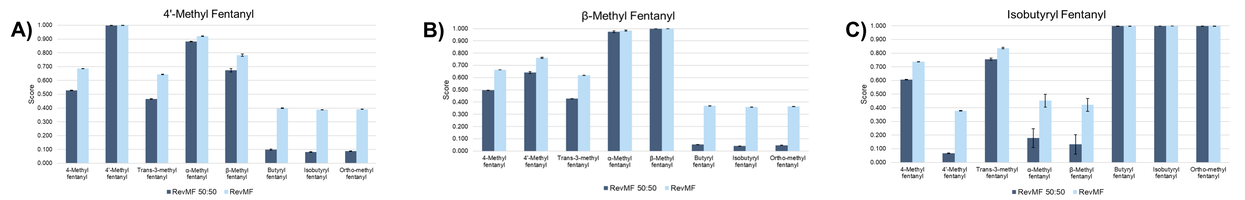


**Figure 3.** Comparison of the average computed RevMF 50:50 and RevMF scores for a) 4-methyl fentanyl, b) β-methyl fentanyl, and c) isobutyryl fentanyl. Error bars represent ±1 standard deviation (n=10).

- The average RevMF 50:50 produced a larger separation between true positive and true negative results compared to the average RevMF, although the same R-group substitution location produced smaller decreases in score.

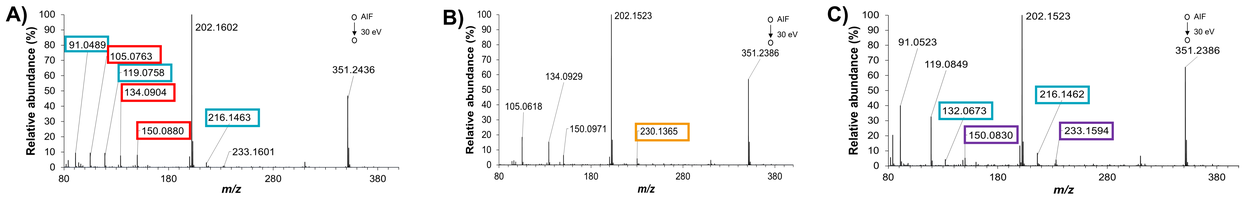


**Figure 4.** Exemplar AIF mass spectra of 2-component mixtures at a 1:1 ratio for a) 4-methyl fentanyl and β-methyl fentanyl, b) 4-methyl fentanyl and trans-3-methyl fentanyl, and c) α-methyl fentanyl and β-methyl fentanyl collected at 30 eV.

- Diagnostic ions can help differentiate methyl-substituted fentanyl analogs; however, this is further complicated when they are present in a mixture, since many of the analogs share similar diagnostic ions.

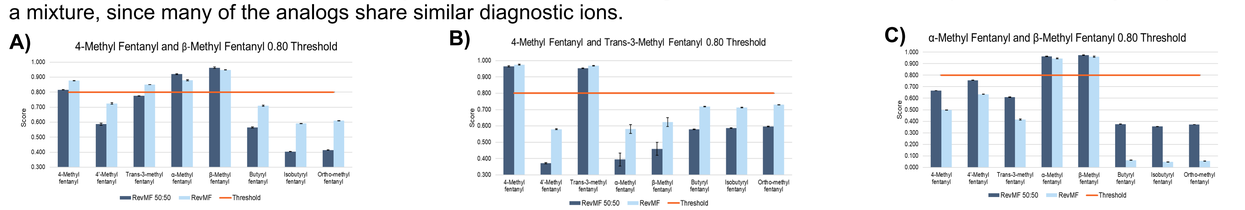


**Figure 5.** Average results for a 1:1 mixture of a) 4-methyl fentanyl and β-methyl fentanyl, b) 4-methyl fentanyl and trans-3-methyl fentanyl, and c) α-methyl fentanyl and β-methyl fentanyl with a 0.80 RevMF threshold. Error bars represent ±1 standard deviation (n=10).

- The combination of 50:50 weighing and a 0.80 RevMF threshold produced the highest correct presumptive identifications and minimized false positives.

## MATERIALS & METHODS

*Instrumentation*

A DART JumpShot® ionization source coupled to an Agilent 6530 quadrupole-time-of flight (Q-TOF) mass spectrometer was utilized in this study. Samples were introduced to the DART ionization source by depositing 5 µL of sample onto a QuickStrip™. All analyses were collected in positive ionization mode using helium as the source gas, heated to 350 °C. All ions fragmentation (AIF) and collision-induced dissociation (CID) activation were used to obtain low (i.e., 0 eV), medium (i.e., 30 eV), and high (i.e., 60 eV) fragmentation spectra.

*ILSA Processing*

RStudio was used to run a command-line function of the ILSA that enabled automated data processing. The centroid data from each replicate were converted into tab-delineated text files (.txt). Data was searched against an internal library containing only the methyl-substituted fentanyl analogs in this study, consisting of one exemplar MS/MS spectrum of the 0, 30, and 60 eV data. The spectral search parameters included a target threshold of 1% and an *m/z* tolerance of ±0.005 Da.

## CONCLUSIONS

- ❖ Differentiation of methyl-substituted fentanyl analogs with different R-group substitution locations is possible with the optimized ILSA weighting.
- ❖ Performance was improved for the RevMF in comparison to the FPIE.
- ❖ Utilizing the 50:50 RevMF achieved greater variation between potential matches.
- ❖ Implementing a 0.80 threshold minimized false positives when compounds were present in a mixture.
- ❖ Potential future applications to isomeric species from other compound classes.

## REFERENCES

[1] A.S. Moorthy, E. Sisco, A New Library-Search Algorithm for Mixture Analysis Using DART-MS, Journal of the American Society for Mass Spectrometry 32 (7) (2021) 1725-1734.
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[3] C. Liggins, A.N. Couch, A.S. Moorthy, J.T. Davidson, Differentiation of Methyl-Substituted Fentanyl Analogs Using Optimized Weighting Within the Inverted Library Search Algorithm (ILSA), Forensic Chemistry 46 (2025).

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