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Project Title: Evaluating the Robustness and Ruggedness of a Statistical Model for Comparison of Mass Spectral Data for Seized Drug Identification

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1. Summary of the Project

Identification of seized drugs in forensic laboratories typically involves analysis of the submitted sample by gas chromatography-mass spectrometry (GC-MS) as part of the analytical scheme. The corresponding mass spectrum is then compared to a reference spectrum for identification, evaluating correspondence between the two spectra in the molecular ion (if present), the base peak, dominant fragment ions, and ratios between and among fragment ions. However, in recent years, the increase in submissions of novel psychoactive substances (NPS) has made identifications based on visual evaluation of spectra substantially more challenging. Many novel psychoactive substances are structurally similar, with new analogs varying only in the identity or the position of a substituent. With the conventional electron-ionization (EI) sources and single quadrupole mass spectrometers used in benchtop GC-MS instruments, the mass spectra of these structurally similar compounds are highly similar, with the result that definitive distinction among analogs and isomers is often not feasible.

Over the last several years, many researchers have developed methods to improve confidence in the identification of NPS [1-14]. Solutions primarily include instrument modification, development of chemometric classification models, and development of software tools to improve library searches. In terms of instrument modification, successful differentiation of analogs and isomers has been demonstrated by modifying the ionization method (*e.g.*, low-energy EI and cold EI), modifying the mode of mass analysis (primarily tandem MS), or by modifying the detector (*e.g.*, infrared or vacuum ultraviolet detectors) [1-7]. While successful differentiation of analogs or isomers was demonstrated in each case, the modified instrumentation is not routinely available in forensic science laboratories for seized drug analysis.

Statistical and chemometric methods to distinguish structurally similar analogs based on EI spectra have also gained momentum in the last several years [6, 8-10]. These approaches generally involve an unsupervised approach such as principal component analysis (PCA) followed by a supervised approach such as discriminant analysis for classification. With these methods, the successful distinction of positional isomers has been demonstrated, as well as the identification of seized drugs in case samples. However, the continued success of classification approaches requires that the training set upon which the model is developed is representative of the compounds of interest. As such, the emergence of new analogs will require that the training set is re-evaluated to ensure sufficient representation of the compounds of interest.

As new NPS analogs appear on the market, reference materials and corresponding reference spectra are not immediately available to aid in identification. To that end, several researchers have developed software tools to assist in the identification of emerging substances [11-14]. Examples include the application of machine-learning methods to indicate the presence of specific substructures as well as enhancements to library search algorithms to highlight highly similar spectra in the library [11-14]. While these tools can certainly be used to gain more information on the likely identity of a new analog, the actual identification will still come down to a visual comparison of the sample spectrum and the corresponding library or reference spectrum.

Our group previously developed a method by which two mass spectra, the sample spectrum and the reference spectrum, are statistically compared [15-19]. The method uses the unequal variance form of the *t*-test (also known as Welch's *t*-test) to compare corresponding ion intensities between the two spectra for all mass-to-charge (*m/z*) values in the scan range. For each comparison, the null hypothesis (H_0) states that the difference in ion intensity is equal to zero whereas, the alternative hypothesis (H_a) states that the difference in ion intensity is not equal to zero. If H_0 is accepted at all *m/z* values, then the two spectra are statistically indistinguishable. In such cases, the compound represented by the sample spectrum is identified as that represented by the reference spectrum. In contrast, if H_0 is not accepted for at least one *m/z* value, then the two spectra are statistically distinguishable. The *m/z* values for which H_0 is not accepted are defined as discriminating ions and in these cases, the sample spectrum and the reference spectrum do not represent the same compound.

The success of the statistical comparison method has been demonstrated for the association and discrimination of amphetamine-type stimulants, salvinorins extracted from the plant material *S. divinorum*, and positional isomers of ethylmethcathinone, fluoromethamphetamine, fluorobutyryl fentanyl, and fluoroisobutyryl fentanyl [15-19]. However, these comparisons primarily used relatively small data sets with spectra collected on one instrument over a short period of time.

1.1 Major Goals and Objectives

The focus in this work was to further evaluate the robustness and ruggedness of the statistical comparison method, which is an essential step in moving toward implementation in forensic laboratories. Compounds representing different NPS classes were selected for this evaluation. These compounds included structural and positional isomers previously documented as being difficult to distinguish based only on EI mass spectra [20]. The specific research goals were defined as follows:

Goal 1. Assess the effect of sample concentration on statistical association and discrimination of positional isomers (Robustness)

Goal 2. Assess the effect of different instruments on statistical association and discrimination of positional isomers (Ruggedness)

Goal 3. Develop and implement testing of the statistical comparison method in operational forensic science laboratories (Testing)

Goal 4. Develop and host training sessions to provide recommendations for implementing the method in forensic laboratories (Training)

1.2 Research Design, Methods, and Data Analysis Techniques

1.2.1 Research design overview

Reference materials representing structurally similar analogs and positional isomers of compounds of interest were selected for evaluation. Electron-ionization mass spectra were collected for each compound over a period of up to 12 months, on different instruments, and at different concentrations. For each spectral collection, compounds within each set were statistically compared to evaluate association and discrimination of the spectra. Spectra collected over time were primarily used to evaluate the effects of inherent experimental and instrumental variation on the association and discrimination of structurally similar compounds. Further, these data were used to identify ions that were reliable for the discrimination of these compounds. Additional spectra collected on a second GC-MS instrument were used to further evaluate the reliability of ions responsible for discrimination of the compounds, thereby enabling an evaluation of method ruggedness. Finally, samples were prepared at different concentrations to evaluate the effect of spectral intensity on association and discrimination and to provide recommendations for accurate comparisons.

1.2.2 Methods

Sets of compounds representing synthetic cathinones, fentanyl analogs, synthetic cannabinoids, fluorobutyryl fentanyl (FBF) positional isomers, and fluoroisobutyryl fentanyl (FIBF) positional isomers were purchased from Cayman Chemical (Ann Arbor, MI). Many of these compounds were selected as they were previously identified by the Seized Drugs Subcommittee of the OSAC as having EI spectra that were difficult to distinguish based on visual assessment alone [20]. Structures of compounds included in this work are shown in Figure 1.

Each reference material was initially prepared at a concentration of 1 mg/mL in methanol (ACS grade, Sigma Aldrich, St. Louis, MO) prior to analysis. Additionally, serial dilutions were performed to generate concentrations of 0.5, 0.25, and 0.1 mg/mL in methanol for the synthetic cathinones, fentanyl analogs, and synthetic cannabinoids. The FBF and FIBF isomers were prepared at concentrations of 0.5 and 0.1 mg/mL in methanol.

Each reference standard was analyzed by GC-MS using an Agilent 6890 gas chromatograph coupled to an Agilent 5975C mass spectrometer, equipped with an Agilent 7683A autosampler (Instrument 1, Agilent Technologies, Santa Clara, CA). Instrument 1 was a well-maintained instrument with limited user access and, prior to each spectral collection, a dedicated 5%-diphenyl-95%-dimethylpolysiloxane column (Rtx-5ms, 30 m x 0.25 mm internal diameter x 0.25 μm film thickness, Restek Corporation, Bellefonte, PA) was installed. Ultra-high purity helium (Airgas, Independence, OH) was used as the carrier gas, with a nominal flow rate of 1 mL/min, and a 1-μL aliquot was injected. There were slight differences in the injection mode and oven temperature program for the different compound sets analyzed on this instrument, which are detailed in the Appendix, **Table A1**. For all compound sets, the transfer line temperature was set to the final oven temperature, and the mass spectrometer was operated in electron-ionization mode (70 eV), with a

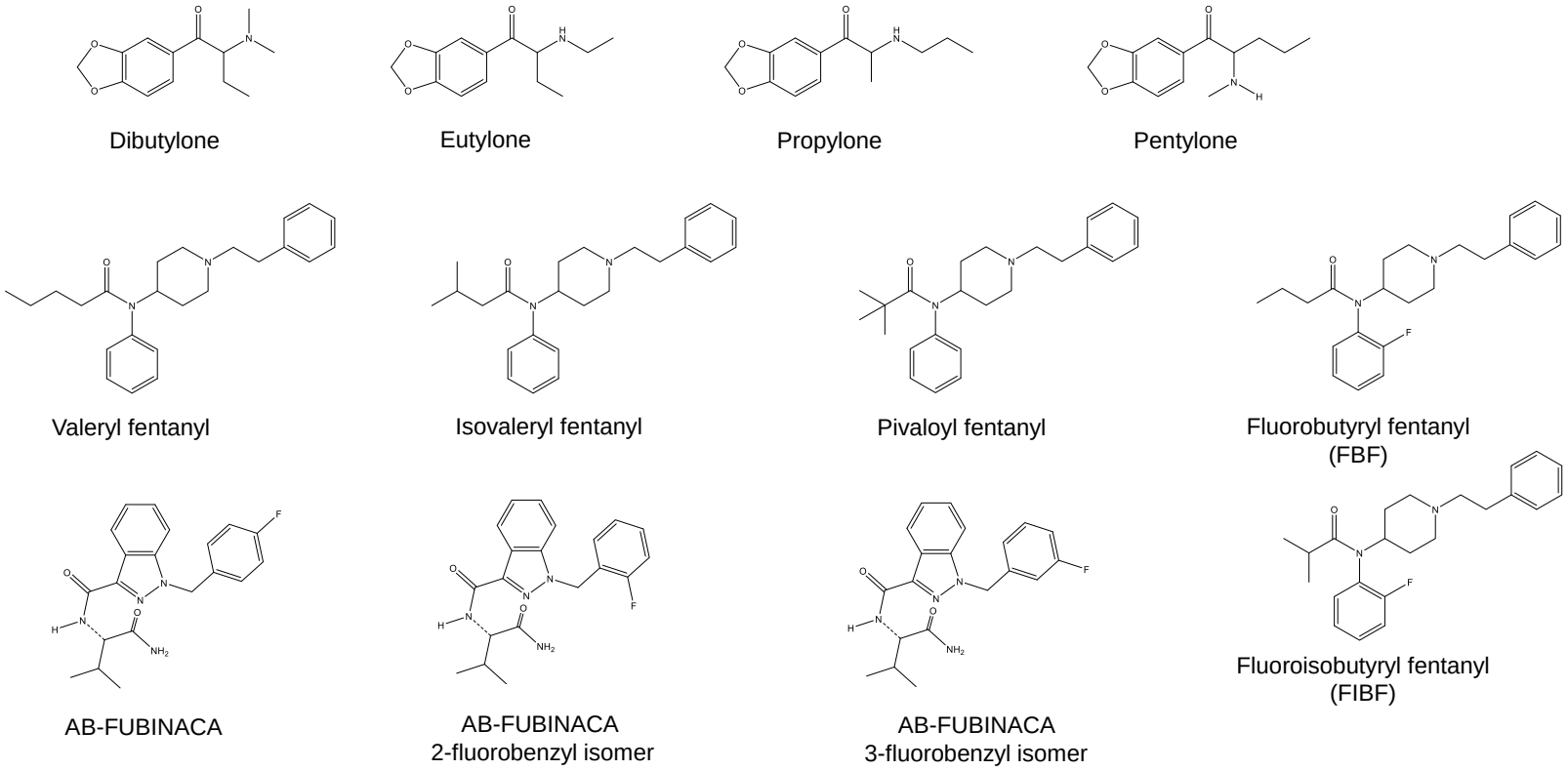

Figure 1. Structures of compounds representing synthetic cathinones, fentanyl analogs, synthetic cathinones, and fluorobutyryl fentanyl (FBF) and fluoroisobutyryl fentanyl (FIBF) positional isomers that were analyzed throughout this work. Only structures of the *ortho*-isomer of FBF and FIBF are shown; however, in each case, both the *meta*- and *para*-isomers were also included in the compound set.

scan range of *m/z* 40 – 450, and a scan rate of 2.83 scans/s. All reference standards were analyzed in triplicate during each collection.

Spectra were collected on this instrument multiple times over a period of up to 12 months (**Table 1**). For each collection, the 1 mg/mL reference solutions were analyzed in triplicate over a period of two days. Spectra collected on the first day were labeled as "Spectrum 1" and compared to spectra collected on the second day, which were labeled as "Spectrum 2." In this manner, spectral comparisons within a collection correspond to different spectra and are not comparisons of instrument replicates.

A series of normal (*n*-) alkanes at different concentrations was also analyzed and used to model the electron multiplier response to predict the standard deviation associated with ion intensities, which is necessary for the *t*-test calculation (*vide infra*) [16, 17]. A stock solution was prepared by transferring 0.5 mL each of *n*-heptane (C_7), *n*-decane (C_{10}), *n*-dodecane (C_{12}), and *n*-pentadecane (C_{15}) (all Sigma Aldrich) into a 25-mL volumetric flask and diluting to volume with methylene chloride (ACS grade, Macron Fine Chemicals, Darmstadt, Germany). The final stock solution contained the *n*-alkanes at the following concentrations: 0.14 M C_7, 0.10 M C_{10}, 0.082 M C_{12}, and 0.065 M C_{15}. The stock solution was further diluted to 75%, 50%, 25%, and 10% v/v in methylene chloride prior to GC-MS analysis.

The alkane solutions were analyzed in triplicate albeit with slight modifications to the GC-MS method. Specifically, a 100:1 split injection was used, the initial oven temperature was 50 °C, which was held for 3 min, then ramped to 280 °C at a rate of 10 °C/min, with a final hold of 4 min, and the transfer line temperature was reduced to 280 °C. The mass spectrometer parameters were as described above.

Spectra of the synthetic cathinones, fentanyl analogs, synthetic cannabinoids, and *n*-alkanes were also collected on two additional GC-MS instruments. One of these instruments (Instrument 2) was housed in a multi-user facility and was used for a wide variety of different applications. There were numerous issues with this instrument, primarily related to sensitivity, high electron multiplier gain, and contamination issues. As such, no data collected on this instrument are included in this report. The third instrument used in this work (Instrument 3) was housed in an analytical teaching laboratory also with multi-user access but with more limited applications. Instrument 3 was an Agilent 7890 GC coupled to an Agilent 5795 MSD equipped with a general use DB-5 column (*i.e.*, a dedicated column was not installed for this work). However, as Instrument 3 was primarily a teaching instrument, access for this work was more limited, meaning that fewer spectral collections were generated compared to Instrument 1 (**Table 1**). As before, there were slight differences in the oven temperature programs for the different sets of compounds, as detailed in the Appendix, **Table A2**.

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Table 1. Summary of spectral collections for the synthetic cathinones, fentanyl analogs, and synthetic cannabinoids

Compound Class	Instrument	Number of Spectral Collections	Collection Time Period
Synthetic Cathinones	1	10	July 2021 – August 2022
	3	3	July 2021 – August 2022
Fentanyl Analogs	1	7	September 2021 – June 2022
	3	2	June 2022 – August 2022
Synthetic Cannabinoids	1	9	July 2021 – August 2022
	3	3	July 2021 – August 2022
FBF and FIBF Isomers	1	4	December 2022 – July 2023
	Previously collected	3	September 2019 – November 2019

1.2.3 Data analysis

Spectra for each reference compound in each collection were collected at the apex of the chromatographic peak. The spectral data (*m/z* value and intensity) were exported from the Agilent ChemStation software (version #E.01.00237, Agilent Technologies) into Microsoft Excel (version 2301, Microsoft® Excel® for Microsoft 365 MSO, Microsoft Corp., Redmond, WA) for further processing.

Spectra corresponding to the *n*-alkanes were exported into a spreadsheet set to automate the generation of the regression line used to model the electron multiplier response. In the spreadsheet, the imported data are first automatically zero-filled and the mean intensity and standard deviation associated with each ion based on triplicate injections are calculated. A natural logarithm plot of standard deviation versus mean intensity is automatically generated and the regression analysis is automatically performed to generate the coefficients (slope and intercept) necessary to predict standard deviation.

Spectra corresponding to the reference standards were exported into a separate spreadsheet that was formatted to automatically perform the statistical comparison. In this case, the imported data are again zero-filled and each *m/z* value is rounded to the nearest integer. A series of logical functions is included to highlight any *m/z* values that round to the same integer. When this occurs, the residuals are calculated and, if the residuals exceed ±0.5 or if the residuals are of opposite sign, the *m/z* values are considered to represent different ions and these ions are not statistically compared.

The spectral data are then automatically averaged per set of triplicates and normalized to the base peak intensity. The regression coefficients determined through analysis of the *n*-alkanes are inserted into the spreadsheet and the standard deviations associated with the mean intensity of each *m/z* value are automatically predicted. The user selects the confidence level at which to perform the *t*-tests and, for each *m/z* value, the t_{calc} value and the degrees of freedom (ν) are automatically calculated, according to Equations 1 and 2, respectively,

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$$t_{calc} = \frac{|\mu_1 - \mu_2|}{\sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}} \qquad \text{Eq. 1}$$

$$\nu = \frac{\left(\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}\right)^2}{\frac{1}{n_1 - 1}\left(\frac{\sigma_1^2}{n_1}\right)^2 + \frac{1}{n_2 - 1}\left(\frac{\sigma_2^2}{n_2}\right)^2} \qquad \text{Eq. 2}$$

where μ_1 and μ_2 represent the mean intensities of the ions, σ_1 and σ_2 represent the predicted standard deviations associated with those intensities, and n_1 and n_2 represent the number of spectra used to calculate the mean intensities in the sample spectrum and the reference spectrum, respectively.

At each *m/z* value, the t_{crit} value is populated based on the selected confidence level and the degrees of freedom. Within the spreadsheet, the t_{calc} values are automatically compared to the t_{crit} values and a summary of the comparison is returned. If the t_{calc} value is less than or equal to the t_{crit} value (*i.e.*, H_0 accepted) at every *m/z* value, then the two spectra are associated and the spreadsheet returns "statistically indistinguishable." If the t_{calc} value exceeds the t_{crit} value for at least one *m/z* value (*i.e.*, H_0 not accepted), the two spectra are discriminated and the spreadsheet returns "statistically distinguishable." In these cases, the list of ions for which H_0 is not accepted (defined as discriminating ions) is also returned in the summary output.

For each pair of compounds compared, there were differences in the number and identity of discriminating ions in spectra collected over time, which is expected due to inherent experimental and instrumental variation. A ranking system was employed to identify those *m/z* values with consistently large t_{calc} values. A large t_{calc} value indicates a *m/z* value for which there is a greater difference in ion intensity between the two spectra being compared relative to the uncertainty in those measurements. A third Microsoft Excel spreadsheet was prepared to automate the ranking of ions according to t_{calc} value. For a given pair of compounds, the previously calculated t_{calc} values at each *m/z* value in all spectral collections were imported into the spreadsheet. The t_{calc} values are ranked in order of decreasing magnitude within each collection and the average rank is calculated across collections. By definition, *m/z* values with the lowest average rank are those that consistently have high t_{calc} values and, therefore, greater difference in intensity between the spectra being compared. The spreadsheet provides a summary output of the ten lowest-ranked ions for each comparison; however, this number is easily adjusted to return any desired number.

1.3 Expected Applicability of the Research

Due to the rapid rise in submissions of NPS in recent years, distinction of structurally similar analogs, including positional isomers, is now necessary within forensic laboratories. However, as GC-MS with electron ionization remains the gold standard in seized drug analysis, distinction and definitive identification of these analogs remains challenging. The statistical method described and demonstrated in this work provides an objective method to statistically compare mass spectra. The method is an extension of current methods to compare spectra, with a statistical evaluation of the

reference spectrum and the sample spectrum rather than a visual assessment. The spectra are compared at a user-defined confidence level to determine if they are statistically indistinguishable (*i.e.*, associated) or statistically distinguishable (*i.e.*, discriminated). In cases of association, the submitted sample can be identified as the compound represented by the reference spectrum. In cases of discrimination, the compound is not the same as that represented in the reference spectrum and the specific ions that are statistically different in intensity between the two spectra are identified.

2. Outcomes

2.1 Activities

Spectra of compounds within each set (synthetic cathinones, fentanyl analogs, synthetic cannabinoids, FBF isomers, and FIBF isomers) were collected over time, on different instruments, and at different concentrations to evaluate the association and discrimination within each set and to identify reliable ions for discrimination. The synthetic cannabinoids in the sample set were initially selected as examples of positional isomers to provide a robust test of the comparison method. However, throughout this work, spectra of these compounds were not as reproducible as spectra of the synthetic cathinones and fentanyl analogs collected on the same instrument. The poorer reproducibility may be due to instrument sensitivity, concentration, and compound stability issues for the cannabinoids. Thus, two additional sets of positional isomers were later included in the compound set: the *ortho*-, *meta*-, and *para*-isomers of fluorobutyryl fentanyl (*o*-, *m*-, and *p*-FBF, respectively) and of fluoroisobutyryl fentanyl (*o*-, *m*-, and *p*-FIBF, respectively). These were primarily selected as we had previously collected spectra of the isomers on a different GC-MS instrument over a three-month period in 2019. Thus, inclusion of the FBF and FIBF isomers allows a further evaluation of method robustness.

In this report, the focus is on the synthetic cathinones and the FBF isomers to demonstrate association and discrimination of structurally similar compounds and of positional isomers, respectively. Statistical comparisons of the other compound classes (fentanyl analogs, synthetic cannabinoids, and FIBF isomers) are summarized in the Appendix.

2.2 Statistical Comparison of Synthetic Cathinones

2.2.1 Visual evaluation of synthetic cathinone mass spectra

Representative spectra of the synthetic cathinones considered in this work are shown in **Figure 2**. Given the structural similarity among the compounds, a high degree of similarity in the mass spectra is expected. The molecular ion at *m/z* 235 is not present in any of the spectra. All four cathinones display a base peak at *m/z* 86 and fragment ions at *m/z* 121 and *m/z* 149. However, some visual differences are apparent in the spectra. For example, *m/z* 71 is present at higher intensity in dibutylone compared to the other three cathinones, *m/z* 58 is more prevalent in eutylone compared to the other cathinones, and *m/z* 44 is present in both propylone and pentylone but is not present in dibutylone and eutylone. Based on a visual comparison of the spectra, it is not clear how

significant these differences are and whether they are sufficient to distinguish the cathinones of interest.

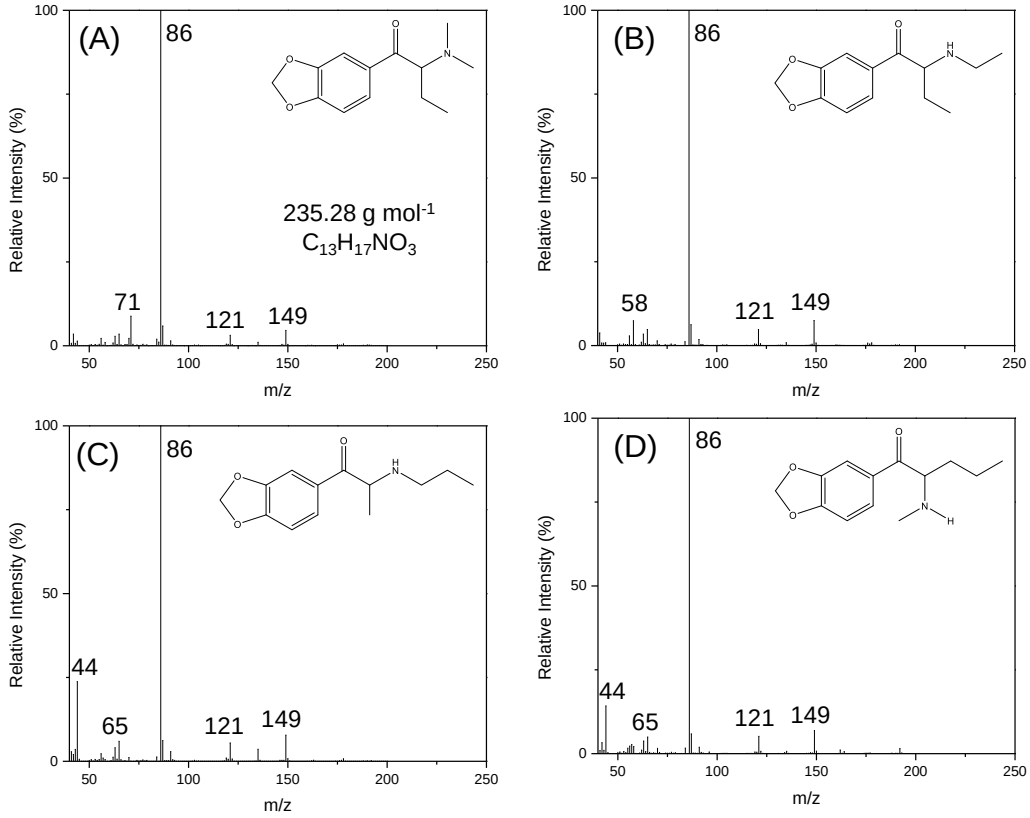

Figure 2. Representative electron-ionization mass spectra of the four synthetic cathinones considered in this work (A) dibutylone, (B) eutylone, (C) propylone, and (D) pentylone.

2.2.2 Evaluating the effect of inherent experimental and instrumental variation on statistical association and discrimination of synthetic cathinones

To evaluate the effect of inherent experimental and instrumental variation on association and discrimination of the synthetic cathinones, a total of ten spectral collections were generated between July 2021 and August 2022 (**Table 1**). Spectra within each collection were statistically compared and the association and discrimination among the cathinones is summarized in **Table 2**. Spectra of corresponding isomers are statistically associated at the 99.9% confidence level (*i.e.*, zero discriminating ions) with two exceptions: dibutylone in Collection 5 and pentylone in Collection 8 (**Table 2**). However, for each of these incorrect discriminations, only one ion is responsible for discrimination and that ion is only present in one of the six spectra compared (triplicate spectra for each isomer compared). For our purposes, discrimination is recorded in these cases; however, we anticipate that individual laboratories would develop their own threshold for discrimination (*e.g.*, ion must be present in all three replicate spectra of a given sample to be recorded as a discriminating ion).

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In terms of discrimination, all cathinones are statistically discriminated from the other cathinones across all collections at the 99.9% confidence level, with 7 – 46 ions responsible for discrimination. For a given comparison, the number of discriminating ions does vary across collections; for example, for the comparison of dibutylone and eutylone, the number of discriminating ions ranges from 9 in Collection 4 to 38 in Collection 10 (**Table 2**). It is worth noting that new samples were prepared between Collections 4 and 5 and that the ion source was cleaned between Collections 8 and 9.

Table 2. Statistical comparison of dibutylone, eutylone, propylone, and pentylone for ten spectral collections on Instrument 1

Spectrum A	Spectrum B	Number of Discriminating Ions in Each Collection (C)*									
		C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
Dibutylone	Dibutylone	0	0	0	0	*m/z* 85[1]	0	0	0	0	0
	Eutylone	10	21	23	9	24	17	22	12	33	38
	Propylone	12	29	29	18	30	26	26	16	33	40
	Pentylone	14	26	28	20	31	21	25	13	41	46
Eutylone	Dibutylone	10	25	23	12	25	21	25	17	32	38
	Eutylone	0	0	0	0	0	0	0	0	0	0
	Propylone	7	15	13	10	16	12	16	9	25	35
	Pentylone	10	18	18	12	22	15	20	13	24	34
Propylone	Dibutylone	13	27	29	20	33	29	28	19	27	44
	Eutylone	7	16	13	9	17	10	13	10	21	31
	Propylone	0	0	0	0	0	0	0	0	0	*m/z* 101[1]
	Pentylone	8	19	21	17	25	16	22	11	25	34
Pentylone	Dibutylone	13	26	28	26	28	23	28	16	37	43
	Eutylone	11	19	18	12	20	13	18	10	26	33
	Propylone	7	19	21	15	21	15	19	8	28	36
	Pentylone	0	0	0	0	0	0	0	*m/z* 81[1]	0	*m/z* 84[6]

* 99.9% confidence level. Entries in red font indicate false discrimination, with the superscript indicating the number of spectra the ion is present in (from six total spectra being compared).

From **Table 2**, it is clear that association and discrimination is generally maintained across multiple spectral collections, albeit with differences in the number of discriminating ions. Such variation is expected in large data sets collected over time, due to inherent experimental and instrumental variation. As an example, variation in the electron multiplier (EM) voltage and gain, along with the intensity of three of the perfluorotributylamine (PFTBA) calibration gas ions (*m/z* 69, 219, and 502) across the 10 spectral collections are plotted in **Figure 3**. The EM voltage varies from 1617 V in Collection 4 to 1812 V in Collections 2 and 7. The gain varies more substantially from 1.59×10^4 in Collection 9 to 3.35×10^4 in Collection 2 (**Figure 3A**). While the intensities of the calibration gas ions vary, the ratio of intensities across the three ions is generally retained across Collections 1 – 7 (**Figure 3B**). In each of the first seven collections, the ratio of *m/z* 69: *m/z* 219 is 0.8:1; however, in Collections 8 and 9, this ratio is closer to 1:1 and, in Collection 10, the intensity of *m/z* 69 is greater than that of *m/z* 219 (1.1:1 ratio). Instrumental variations such as these

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influence the mean intensity and the predicted standard deviation of the ions being compared. In turn, variation in mean intensity and standard deviation influences the t_{calc} value (Eq. 1), the degrees of freedom (Eq. 2), and the t_{crit} value.

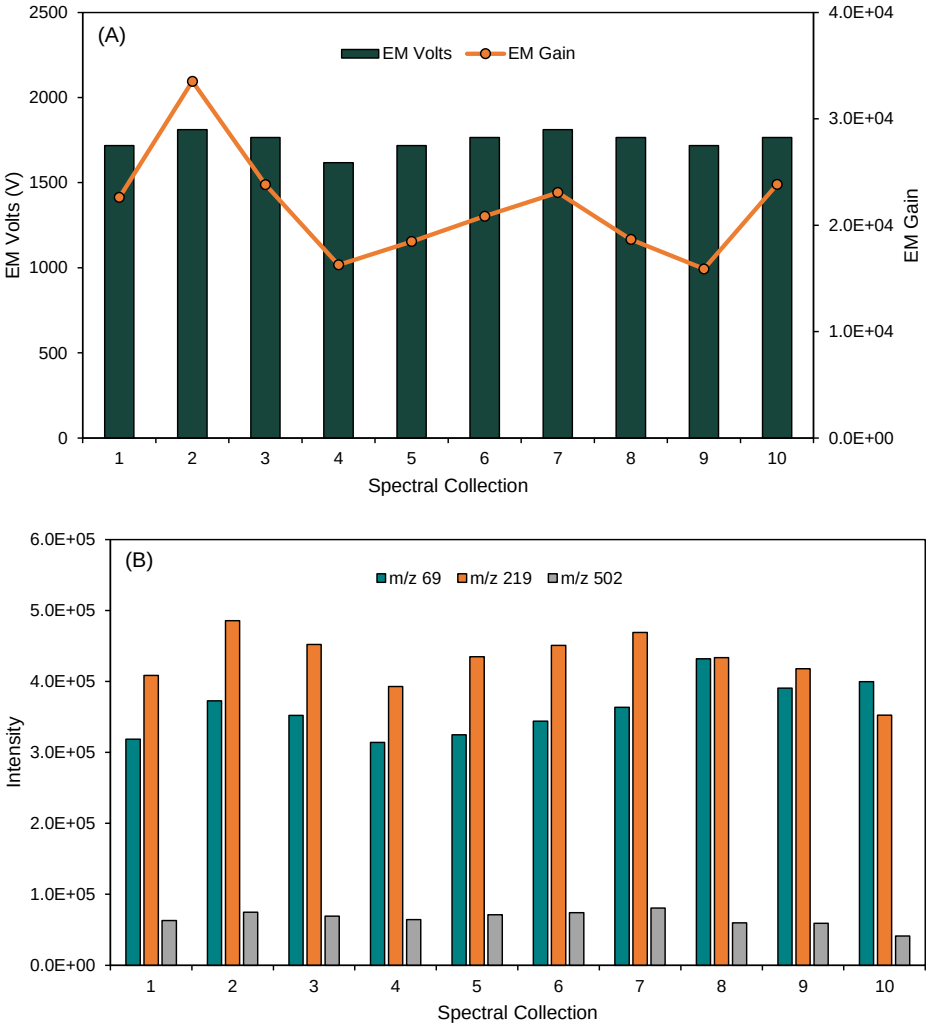

Figure 3. Variation in (A) electron multiplier (EM) voltage and gain and (B) perfluorotributylamine (PFTBA) calibration gas ion intensity following mass spectral autotunes prior to each spectral collection of the synthetic cathinones.

In each statistical comparison of two spectra, an ion is defined as a discriminating ion when H_0 is not accepted. This occurs when the t_{calc} value is greater than the t_{crit} value, the latter of which is dependent on the calculated degrees of freedom (ν) and the selected confidence level for comparison. As such, in each collection, ions identified as discriminating are true discriminating ions for that comparison. However, some ions are identified as discriminating in all collections while other ions are identified as discriminating ions less frequently. In these cases, the main contributing factor is differences in the calculated degrees of freedom (ν), which impacts the t_{crit} value. For example, at the 99.9% confidence level, for ν = 2, t_{crit} = 31.599 whereas, for ν = 3, t_{crit}

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= 12.924. This means that a given ion may have a similar t_{calc} value across collections but, due to differences in the calculated degrees of freedom and, therefore, the t_{crit} value, the ion may be defined as discriminating in one collection but not in the other.

While all ions defined as discriminating ions are in fact discriminating ions for that comparison, there is variability in the discriminating ions across collections due to inherent experimental and instrumental variation. However, the ideal behavior of true or reliable discriminating ions should be consistent, irrespective of instrument variation. To further evaluate the reliability of ions for discrimination for each pairwise comparison of the cathinones, the absolute t_{calc} values for a given comparison were ranked in order of decreasing magnitude and the average rank across all corresponding comparisons was determined. Higher magnitude t_{calc} values indicate greater difference in intensity of a given ion in the two spectra being compared. Ranking in order of decreasing magnitude means that ions with low average ranks consistently yield high magnitude t_{calc} values and hence, greater difference in intensity between the two spectra. As such, the lowest ranked ions can be considered reliable for discrimination due to the consistently high t_{calc} values. For comparisons of dibutylone to the other three cathinones, the top 10 ranked ions are summarized in **Table 3**, along with the most frequently occurring discriminating ions across the 20 comparisons. All other pairwise comparisons are shown in the Appendix (**Table A3**).

For the comparison of dibutylone to eutylone, five of the ten lowest ranked ions (*m/z* 71, 58, 41, 42, and 149) are the same ions that were identified as discriminating ions in all 20 comparisons. That is, these five ions display the t_{calc} values with the greatest magnitude, which indicates greatest difference in intensity between the two spectra. As such, these ions can be considered reliable for the discrimination of dibutylone and eutylone. Ranking ions in this manner provides a means to identify reliable discriminating ions that are least affected by inherent experimental and instrumental variation. It is worth noting here that ranking ions is not necessary in routine forensic applications in which only two spectra (*e.g.*, sample and reference spectra) are to be compared: in those cases, discriminating ions determined *via* comparison of t_{calc} to t_{crit} values are responsible for differentiation of the two spectra. However, in cases where spectra collected over time are to be compared and, therefore, instrumental variation becomes a factor, ranking ions based on the magnitude of the t_{calc} value can be used to evaluate the reliability of ions identified as discriminating across the collections. Additionally, identifying reliable ions for discrimination of structurally similar compounds is important for our future work in which we aim to understand the chemical reasons why these ions are discriminating.

2.2.3 Evaluating the ruggedness of the statistical comparison method for association and discrimination of synthetic cathinones

Throughout this work, spectra of the synthetic cathinones were also collected on a different GC-MS instrument (Instrument 3) to evaluate the ruggedness of the method. Given time constraints, fewer spectral collections were generated on Instrument 3; however, the data collected thus far demonstrate the wider applicability of the statistical comparison method. The synthetic cathinones were collected a total of three times on the second instrument, resulting in six

comparisons for each pair of cathinones. Association and discrimination of the cathinones for each collection are summarized in **Table 4**.

Table 3. Comparison of ranked ions and most frequently occurring discriminating ions for comparison of dibutylone to eutylone, propylone, and pentylone

Spectrum 1	Spectrum 2	*m/z* Value of Ranked Ions*	*m/z* Value of Most Frequent Discriminating Ions**
Dibutylone	Eutylone	**71** **58** **41** **42** 72 176 204 59 **149** 206	**41** **42** **58** **71** **149**
Dibutylone	Propylone	**44** **71** **43** 41 135 72 45 57 206 69	42 **43** **44** 58 65 68 70 **71**
Dibutylone	Pentylone	**71** **44** **57** **192** 162 72 55 96 178 164	**44** **57** **71** 121 149 150 **192**

*All ions in the scan range were ranked but only the 10 lowest-ranked ions are shown.
**Defined as those ions being defined as discriminating ions in all 20 comparisons (for each spectral collection, there are two comparisons of each pair of cathinones (*e.g.*, dibutylone versus eutylone and eutylone versus dibutylone), resulting in a total of 20 comparisons)
Entries in bold font are ions that are both highly ranked and frequently observed as discriminating ions.

The trends observed previously are retained here; that is, association of corresponding cathinones is observed at the 99.9% confidence level and discrimination among the cathinones is observed, with 11 – 36 ions responsible for discrimination. There is one exception in terms of association: spectra of eutylone are discriminated in the first collection. However, only one ion is

responsible for this discrimination (*m/z* 85) and the ion is only present in one of the six spectra compared. Again, for our purposes, the ion is listed here as a discriminating ion although individual laboratories may define their own thresholds for discrimination.

Table 4. Statistical comparison of dibutylone, eutylone, propylone, and pentylone for three spectral collections on Instrument 3

Spectrum 1	Spectrum 2	Number of Discriminating Ions in Each Collection* (C)		
		C1	C2	C3
Dibutylone	Dibutylone	0	0	0
	Eutylone	15	18	33
	Propylone	19	19	33
	Pentylone	17	21	37
Eutylone	Dibutylone	16	17	31
	Eutylone	*m/z* 85[1]	0	0
	Propylone	12	14	27
	Pentylone	12	14	30
Propylone	Dibutylone	26	23	36
	Eutylone	11	12	28
	Propylone	0	0	0
	Pentylone	14	14	27
Pentylone	Dibutylone	18	18	36
	Eutylone	15	15	30
	Propylone	13	14	29
	Pentylone	0	0	0

* 99.9% confidence level. Entries in red font indicate false discrimination, with the superscript indicating the number of spectra the ion is present in (from six total spectra being compared).

For each comparison (six total comparisons), the ions were also ranked in order of decreasing t_{calc} value to evaluate the reliability of ions for discrimination. The ranked ions based on spectra collected on Instrument 3 were compared to the corresponding ranked ions for spectra collected on Instrument 1. This comparison is summarized in Table 5 for comparison of dibutylone to the other synthetic cathinones and all comparisons are summarized in the Appendix (**Table A4**).

There is a remarkable degree of consistency in the ranked ions for each comparison between the two instruments. In fact, for the comparison of dibutylone and eutylone, the ten lowest ranked ions are the same between the two instruments while for comparison of dibutylone to propylone and to pentylone, there is only one ion difference between the two instruments. These comparisons provide further evidence that ions previously deemed to be reliable for discrimination of these compounds are indeed reliable as they are retained on a second instrument. Again, ranking ions in this manner is not necessary for routine implementation but rather, demonstrates that ions responsible for discrimination are reliably observed over time and on different instruments.

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Table 5. Comparison of ranked ions for comparison of dibutylone to eutylone, propylone, and pentylone on two different instruments

Spectrum 1	Spectrum 2	*m/z* Value of Ranked Ions*	
		Instrument 1	Instrument 3
Dibutylone	Eutylone	71	71
		58	58
		41	41
		42	42
		72	72
		176	176
		204	204
		59	149
		149	59
		206	206
Dibutylone	Propylone	44	44
		71	71
		43	85
		41	43
		135	135
		72	41
		45	72
		57	45
		206	57
		69	206
Dibutylone	Pentylone	71	71
		44	44
		57	57
		192	192
		162	162
		72	72
		55	96
		96	55
		178	206
		164	164

*All ions in the scan range were ranked but only the 10 lowest-ranked ions are shown.
Entries in red font are ions that are different between the two instruments.

2.2.4 Evaluating the effect of spectral intensity on the statistical association and discrimination of the synthetic cathinones

A summary of the spectral comparison of dibutylone to all synthetic cathinones prepared at different concentrations is shown in **Table 6** and the full concentration study is summarized in the Appendix (**Tables A5 – A7**). In general, association of corresponding isomers is achieved at the 99.9% confidence level, irrespective of concentration albeit with three exceptions. In two cases (comparison of corresponding dibutylone spectra at 1 mg/mL and comparison of eutylone at 1.0 mg/mL to eutylone at 0.25 mg/mL), one discriminating ion (*m/z* 85) is identified although the ion is only present in one out of six spectra. In the third case (pentylone at 1.0 mg/mL to pentylone at 0.5 mg/mL), again, one discriminating ion is identified although the ion (*m/z* 84) is present in all six spectra.

In terms of discrimination, each cathinone is successfully discriminated from the other cathinones at the 99.9% confidence level, irrespective of concentration. For each comparison, the number of discriminating ions decreases as concentration decreases, which is expected due to less intense spectra containing fewer ions at lower concentrations.

Overall, association and discrimination of the synthetic cathinones was demonstrated with spectra collected over time, on different instruments, and at different concentrations thereby demonstrating the robustness and ruggedness of the statistical comparison method.

Table 6. Effect of spectral intensity on statistical comparison of dibutylone to dibutylone, eutylone, propylone, and pentylone

Spectrum 1				Spectrum 2				Number of Discriminating Ions
Synthetic Cathinone	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	Synthetic Cathinone	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	
Dibutylone	1.0	3.54 ± 0.03	98 ± 1	Dibutylone	1.0	3.58 ± 0.04	98 ± 0	1 (*m/z* 85)
					0.5	1.71 ± 0.05	78 ± 3	0
					0.25	0.87 ± 0.03	52 ± 3	0
					0.1	0.1927 ± 0.0004	19 ± 2	0
				Eutylone	1.0	2.85 ± 0.09	96 ± 2	24
					0.5	1.78 ± 0.09	78 ± 2	17
					0.25	0.71 ± 0.01	48 ± 1	8
					0.1	0.17 ± 0.01	20 ± 1	3
				Propylone	1.0	2.43 ± 0.04	83 ± 2	30
					0.5	1.16 ± 0.05	61 ± 2	20
					0.25	0.40 ± 0.01	36 ± 0	8
					0.1	0.11 ± 0.01	16 ± 1	3
				Pentylone	1.0	2.2 ± 0.2	90 ± 4	31
					0.5	1.09 ± 0.08	61 ± 4	16
					0.25	0.59 ± 0.01	43 ± 1	11
					0.1	0.105 ± 0.001	16 ± 2	2

* Mean base peak intensity in triplicate spectra ± standard deviation

† Mean total number of ions in triplicate spectra ± standard deviation

‡ 99.9% confidence level. Entries in red font indicate false discrimination.

2.3 Statistical Comparison of Fluorobutyryl Fentanyl (FBF) Positional Isomers

2.3.1 Visual evaluation of FBF mass spectra

Representative spectra of the fluorobutyryl fentanyl (FBF) positional isomers are shown in **Figure 4**. The molecular ion at *m/z* 368 is not visible in the spectra and the base peak at *m/z* 277 is due to cleavage of the α-β bond of the phenethyl group on the piperidine ring [21, 22]. The fragment ion at *m/z* 207 results from cleavage of the C-N amide bond from the base peak, with *m/z* 164 formed *via* subsequent cleavage along the piperidine ring. It is worth noting that while *m/z* 207 is a known column bleed ion in GC-MS, the ion is chemically relevant in the FBF isomers.

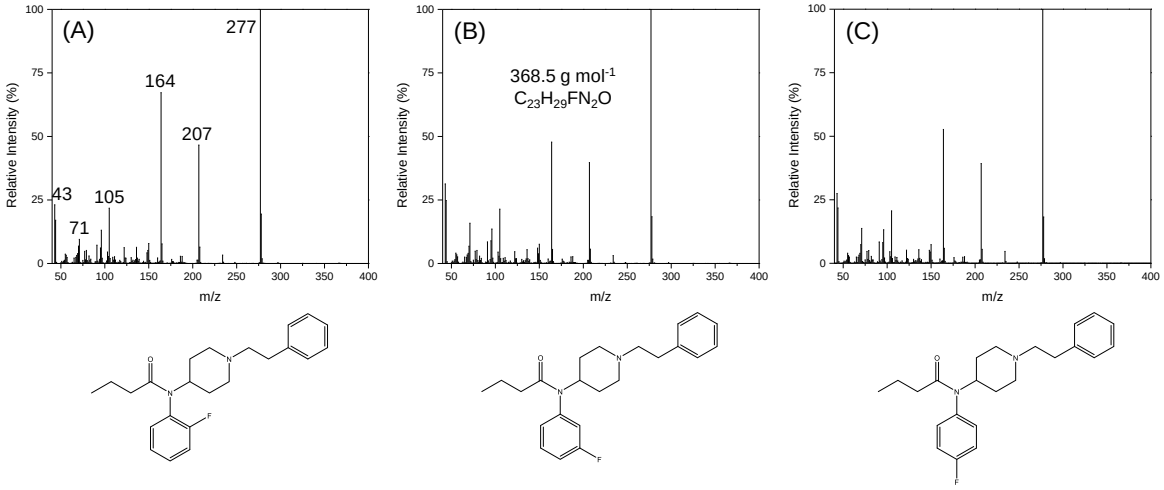

Figure 4. Representative electron-ionization mass spectra of (A) *ortho*-fluorobutyryl fentanyl (*o*-FBF), (B) *meta*-fluorobutyryl fentanyl (*m*-FBF), and (C) *para*-fluorobutyryl fentanyl (*p*-FBF). Structures corresponding to each isomer are shown below the spectra.

The spectra are highly similar as expected although there are slight differences in ion intensity that may afford distinction. For example, *m/z* 43 is present at similar intensity in both *m*-FBF and *p*-FBF (28% and 31% relative intensity, respectively) but is present at lower intensity in *o*-FBF (23% relative intensity). Distinction of *o*-FBF from *m*-FBF and *p*-FBF may also be possible based on differences in intensity of *m/z* 71 and *m/z* 164. The ion at *m/z* 71 is present at lower intensity in *o*-FBF (10% relative intensity) compared to *m*-FBF and *p*-FBF (16% and 14%, respectively) while *m/z* 164 is present at higher intensity (67% relative intensity) in *o*-FBF compared to the other two isomers (48% and 53% relative intensity in *m*-FBF and *p*-FBF, respectively). While there are visual differences in the spectra, the repeatability, the reproducibility, and the significance of such differences are not known.

2.3.2 Evaluating the effect of inherent experimental and instrumental variation on statistical association and discrimination of FBF positional isomers

The FBF positional isomers were analyzed four times over a seven-month period (December 2022 – July 2023) and statistical comparisons of each pair of isomers in each spectral collection are summarized in **Table 7**.

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Table 7. Statistical comparison of fluorobutyryl fentanyl (FBF) positional isomers at 1 mg/mL for four spectral collections on Instrument 1.

Spectrum 1	Spectrum 2	Number of Discriminating Ions in Each Collection (C)*			
		C1	C2	C3	C4
o-FBF	*o*-FBF	0	0	*m/z* 111[6]	*m/z* 111[6], 118[6]
	m-FBF	6	14	10	1
	p-FBF	3	12	5	**0**
m-FBF	*o*-FBF	10	14	10	1
	m-FBF	0	0	0	*m/z* 111[6]
	p-FBF	1	6	7	1
p-FBF	*o*-FBF	7	13	11	1
	m-FBF	1	4	4	**0**
	p-FBF	0	0	*m/z* 111[6]	*m/z* 111[6]

* 99.9% confidence level. Entries in bold font indicate false association. Entries in red font indicate false discrimination, with the superscript indicating the number of spectra the ion is present in (from six total spectra being compared).

In Collections 1 and 2, spectra of corresponding isomers are statistically associated at the 99.9% confidence level, with zero discriminating ions. However, in Collections 3 and 4, false discrimination of corresponding isomers is observed. Interestingly, the same two ions are responsible for this discrimination: *m/z* 111 and *m/z* 118. Both ions are also present in all six spectra being compared. The chemical relevance of these ions is not yet known; however, *m/z* 111 is present in the background in all collections. This ion is present at relatively low background intensity (~1000 counts) in Collections 1 and 2 but at higher intensities in Collections 3 and 4 (~2500 counts in Collection 3 and ~7000 counts in Collection 4). As such, the identification of *m/z* 111 as a discriminating ion in Collections 3 and 4 may be due to high background levels rather than due to variability in a chemically relevant ion.

Discrimination of different isomers is generally achieved, with the number of discriminating ions ranging from 1 – 14 ions (**Table 7**). In general, discrimination of *m*-FBF and *p*-FBF is more difficult with less ions responsible for discrimination (1 – 7 ions, **Table 7**). There are two instances of false association, both of which occur in Collection 4. Here, *o*-FBF and *p*-FBF are statistically associated, as are *p*-FBF and *m*-FBF. It is also worth noting that overall, the fewest discriminating ions are observed for comparisons in Collection 4.

To further investigate instrumental variation, the electron multiplier voltage and gain for the four collections were evaluated (**Figure 5**). The EM voltage ranges from 1718 V in Collection 2 to 2047 V in Collection 4. Further, the EM gain ranges from 1.5 x 10^5 in Collection 2 to 3.6 x 10^5 in Collection 4. The high EM gain and voltage in Collection 4 results in higher spectral intensity. For example, for *o*-FBF, the mean base peak intensities for Spectrum 1 and Spectrum 2 are 3.1 x 10^5 and 3.4 x 10^5, respectively, in Collection 4 compared to 1.8 x 10^5 and 1.6 x10^5, respectively, in Collection 2. In addition to higher spectral intensity, the regression slope used to predict standard deviation (**Table 8**) is relatively high in Collection 4. This combination of increased intensity and higher regression slope results in higher predicted standard deviations. For the same difference in ion intensity, higher predicted standard deviations will result in lower t_{calc} values (Eq. 1). With low t_{calc} values, discrimination is more difficult as it is more likely that the low t_{calc} value will be less

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than the t_{crit} value. As a result, there are fewer discriminating ions in Collection 4 and a greater occurrence of false associations and false discriminations.

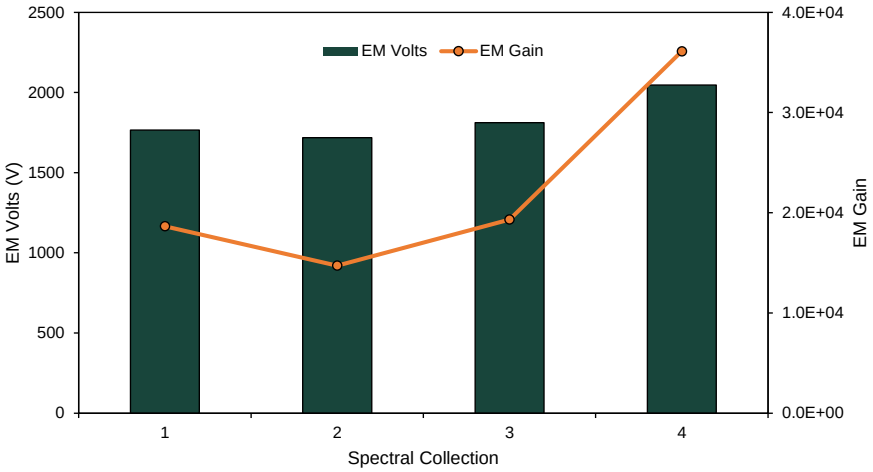

Figure 5. Variation in electron multiplier (EM) voltage and gain following mass spectral autotunes prior to each spectral collection of the fluorobutyryl fentanyl (FBF) isomers.

Table 8. Regression coefficients to model electron multiplier response prior to each spectral collection of the fluorobutyryl fentanyl (FBF) isomers

Collection	Electron Multiplier		Regression Coefficients	
	Voltage (V)	Gain (x10^4)	Slope	Intercept
1	1765	1.87	0.8143	-0.7271
2	1718	1.47	0.6825	-0.4713
3	1215	1.93	0.7017	-0.4699
4	2047	3.61	0.7996	-0.4698

Given the differences in the number and identity of discriminating ions, the ions were ranked according to t_{calc} magnitude and compared to the ions most frequently identified as discriminating ions in the eight comparisons (**Table 9**). Due to the spectral similarity among the isomers, "frequently discriminating" was defined as an ion appearing as a discriminating ion in at least 6 of the 8 comparisons.

For each pairwise comparison of isomers, only one to two ions are defined as frequently occurring discriminating ions. These ions appear within the top four ions when ranked according to t_{calc} value. As such, taking experimental and instrumental variation across multiple spectral collections into account, ions at *m/z* 102, 164, and 234 can be considered reliable for discrimination among the FBF isomers (**Table 9**). While the chemical relevance of *m/z* 102 is not yet known, the ions at *m/z* 164 and *m/z* 234 are likely due to cleavage along the piperidine ring and cleavage of the C-N amide bond, as shown in **Figure 6**. Both ions are statistically higher in intensity in *p*-FBF compared to the other two isomers, likely due to the increased resonance stabilization with fluorine in the *para*-position.

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Table 9. Comparison of ranked ions and most frequently occurring discriminating ions for comparison of the FBF positional isomers at 1 mg/mL on Instrument 1

Spectrum 1	Spectrum 2	*m/z* Value of Ranked Ions*	*m/z* Value of Most Frequent Discriminating Ions**
o-FBF	*m*-FBF	**164** **102** 71 118 90 95 144 148 190 122	**102** **164**
o-FBF	*p*-FBF	90 **102** 118 **234** 71 176 144 95 235 130	**102** **234**
m-FBF	*p*-FBF	**234** 176 235 109 111 70 164 84 181 248	**234**

*All ions in the scan range were ranked but only the 10 lowest-ranked ions are shown.
**Defined as those ions being defined as discriminating ions in 6 of 8 comparisons (for each spectral collection, there are two comparisons of each pair of isomers (*e.g.*, *o*-FBF versus *m*-FBF and *m*-FBF versus *o*-FBF), resulting in a total of 8 comparisons)
Entries in bold font are ions that are both highly ranked and frequently observed as discriminating ions.

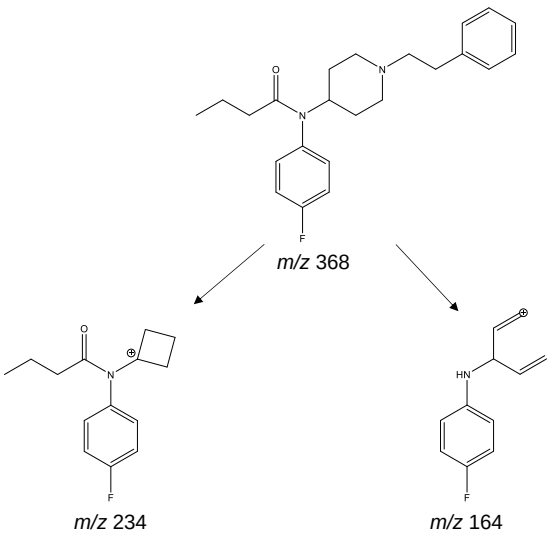

Figure 6. Proposed structures of two reliable ions for discrimination among the fluorobutyryl fentanyl (FBF) positional isomers.

2.3.3 Evaluating the ruggedness of the statistical comparison method for association and discrimination of FBF positional isomers

Spectra of the FBF isomers previously collected over three-month period in 2019 on a different GC-MS instrument were used to evaluate the ruggedness of the method for these isomers. The 2019 spectra were previously compared to demonstrate the potential to associate and discriminate the isomers [18]. In this project, the spectra were further probed to first determine the most frequently occurring discriminating ions and second, to rank the ions in order of decreasing t_{calc} magnitude (**Table 10**).

For comparisons of *o*-FBF with *m*- and *p*-FBF, there are more frequently occurring discriminating ions in the 2019 spectral collections than for Instrument 1. Nonetheless, discriminating ions defined on Instrument 1 were also defined as such in the 2019 spectra. The frequently occurring discriminating ions defined in the 2019 spectra are all also among the 10 ions with the greatest magnitude t_{calc} value for this instrument. The ranked ions for the FBF comparisons on both instruments are shown in **Table 11**. For each comparison, at least seven of the ten ranked ions appear on both instruments. Thus, despite being collected on different instruments, four years apart, and with different batches of reference materials, there is a high degree of correspondence among the ranked ions.

Table 10. Comparison of ranked ions and most frequently occurring discriminating ions for comparison of the FBF positional isomers at 1 mg/mL for data collected in 2019

Spectrum 1	Spectrum 2	*m/z* Value of Ranked Ions*	*m/z* Value of Most Frequent Discriminating Ions**
o-FBF	*m*-FBF	**164**	
		102	**44**
		71	**90**
		118	**102**
		171	**118**
		44	122
		90	**144**
		165	**164**
		144	**165**
		95	
o-FBF	*p*-FBF	171	
		164	
		118	
		102	**90**
		71	**118**
		73	**144**
		234	**164**
		144	
		130	
		90	
m-FBF	*p*-FBF	**234**	
		176	
		164	
		109	
		235	**234**
		70	
		182	
		84	
		98	
		110	

*All ions in the scan range were ranked but only the 10 lowest-ranked ions are shown.
**Defined as those ions being defined as discriminating ions in 5 of 6 comparisons (for each spectral collection, there are two comparisons of each pair of isomers (*e.g.*, *o*-FBF versus *m*-FBF and *m*-FBF versus *o*-FBF), resulting in a total of 6 comparisons)
Entries in bold font are ions that are both highly ranked and frequently observed as discriminating ions.

Table 11. Ranked ions on two different instruments for comparison of fluorobutyryl fentanyl (FBF) positional isomers

Spectrum 1	Spectrum 2	*m/z* Value of Ranked Ions*	
		Instrument 1	2019 Spectral Collections
o-FBF	*m*-FBF	164	164
		102	102
		71	71
		118	118
		90	171
		95	44
		144	90
		148	165
		190	144
		122	95
o-FBF	*p*-FBF	90	171
		102	164
		118	118
		234	102
		71	71
		176	73
		144	234
		95	144
		235	130
		130	90
m-FBF	*p*-FBF	234	234
		176	176
		235	164
		109	109
		111	235
		70	70
		164	182
		84	84
		181	98
		248	110

*All ions in the scan range were ranked but only the 10 lowest-ranked ions are shown.
Entries in red font are ions that are different between the two instruments.

2.3.4 Evaluating the effect of spectral intensity on the statistical association and discrimination of the FBF positional isomers

The FBF positional isomers were prepared at three concentrations (1.0, 0.5, and 0.1 mg/mL) and, within a given collection, spectra corresponding to each concentration were collected in replicate over multiple days to enable pairwise comparisons at each concentration (*e.g.*, spectra collected at 0.5 mg/mL compared to spectra collected at 0.5 mg/mL and spectra collected at 0.1 mg/mL compared to spectra collected at 0.1 mg/mL). This contrasts with our previous concentration studies in which spectra collected at different concentrations were all compared to

the spectrum of the 1 mg/mL standard. Statistical comparisons of spectra collected for the 0.5 mg/mL standards and the 0.1 mg/mL standards are summarized in **Tables 12 and 13**, respectively.

Table 12. Statistical comparison of fluorobutyryl fentanyl (FBF) positional isomers at 0.5 mg/mL for four spectral collections on Instrument 1.

Spectrum 1	Spectrum 2	Number of Discriminating Ions in Each Collection (C)*			
		C1	C2	C3	C4
o-FBF	*o*-FBF	0	*m/z* 341[6]	0	*m/z* 267[2], 346[1]
	m-FBF	4	7	4	**0**
	p-FBF	2	8	3	**0**
m-FBF	*o*-FBF	2	6	4	**0**
	m-FBF	0	0	0	0
	p-FBF	2	9	**0**	**0**
p-FBF	*o*-FBF	**0**	10	4	1
	m-FBF	2	5	4	**0**
	p-FBF	0	0	*m/z* 44[6], 111[6], 181[6]	*m/z* 111[6]

* 99.9% confidence level. Entries in bold font indicate false association. Entries in red font indicate false discrimination, with the superscript indicating the number of spectra the ion is present in (from six total spectra being compared).

Table 13. Statistical comparison of fluorobutyryl fentanyl (FBF) positional isomers at 0.1 mg/mL for four spectral collections on Instrument 1.

Spectrum 1	Spectrum 2	Number of Discriminating Ions in Each Collection (C)*			
		C1	C2	C3	C4
o-FBF	*o*-FBF	0	0	*m/z* 44[6]	0
	m-FBF	**0**	1	1	**0**
	p-FBF	**0**	**0**	1	**0**
m-FBF	*o*-FBF	**0**	1	2	**0**
	m-FBF	0	0	0	0
	p-FBF	**0**	**0**	1	**0**
p-FBF	*o*-FBF	**0**	1	1	**0**
	m-FBF	**0**	2	**0**	**0**
	p-FBF	0	0	*m/z* 44[6]	0

* 99.9% confidence level. Entries in bold font indicate false association. Entries in red font indicate false discrimination, with the superscript indicating the number of spectra the ion is present in (from six total spectra being compared).

In general, lower concentration results in less intense spectra with fewer ions available for comparison. While correct association and discrimination is observed for many of the comparisons, there are increasing occurrences of false associations and false discriminations particularly at 0.1 mg/mL and in Collection 4. Particularly at the lower concentration, discrimination becomes more difficult as evidenced by the number of false associations. To some extent, this is expected given less intense spectra with fewer ions available for comparison, coupled with the high degree of similarity among the spectra. Overall, for accurate comparisons, spectra should be sufficiently intense to provide a spectrum representative of the compound in question and spectra for comparison should be of similar intensities.

2.4 Limitations

The primary limitation throughout this work was availability and access to additional GC-MS instruments. Instrument 1 was housed in our laboratory and for each set of compounds, spectra were collected over a relatively long time period. Given limited availability of the other instruments, substantially fewer spectral collections were possible. The initial data collected on the additional instruments do demonstrate the reliability of ions for discrimination given the high degree of correspondence that is observed between two instruments. However, moving forward additional spectra should be collected on these instruments. In addition, all instruments in this work were Agilent GC-MS systems albeit with slight differences in the specific GC or MSD model. To demonstrate wider applicability of the method, instruments from different manufacturers should be evaluated. Finally, while the effect of spectral intensity was demonstrated in this work, a more in-depth study is warranted to evaluate a wider range of concentrations and potentially, to define minimum threshold intensities to ensure accurate comparisons.

3. Artifacts

3.1 List of Products

3.1.1 Published manuscripts

Sacha AM, Willis IC, McGuffin VL, Waddell Smith R. Identifying Reliable Ions for the Statistical Differentiation of Structurally Similar Fentanyl Analogs. *Journal of Forensic Sciences* **2023**, *68*, 1527 – 1541. DOI:10.1111/1556-4029.15300

3.1.2 Manuscripts in preparation

Sacha AM, McGuffin VL, Waddell Smith R. Statistical Discrimination of Synthetic Cathinone Structural Isomers based on EI Mass Spectra. In preparation for submission to *Journal of Forensic Sciences* or *Forensic Chemistry* (anticipated submission February 2024).

Willlis IC, McGuffin VL, Waddell Smith R. Demonstrating the Robustness of a Statistical Method to Distinguish Structural and Positional Isomers of Fentanyl Analogs. In preparation for submission to *Journal of Forensic Sciences* or *Forensic Chemistry* (anticipated submission April 2024).

3.1.3 Conference presentations

Denotes invited presentation; †denotes graduate student; presenter underlined

*†Andrew Sacha, Victoria L. McGuffin, Ruth Waddell Smith. Addressing the Rate of False Positive and False Negative Associations in the Mass Spectral Comparison of Structurally Similar Seized Drugs. Oral presentation at the International Chemical Congress of Pacific Basin Societies (Pacifichem, virtual). December 2021.

†Andrew Sacha, Victoria L. McGuffin, Ruth Waddell Smith. Evaluating the Robustness and Ruggedness of a Statistical Method to Compare Mass Spectra. Oral presentation at the 74th

American Academy of Forensic Sciences Annual Meeting, Seattle, WA (hybrid). February 2022.

†Andrew Sacha, Victoria L. McGuffin, Ruth Waddell Smith. Distinction of Cathinone Isomers and Fentanyl Isomers based on Statistical Comparison of Mass Spectra. Oral presentation at the Northeastern Association of Forensic Scientists Annual Meeting, Niagara Falls, NY. October 2022.

*†Andrew M. Sacha, Victoria L. McGuffin, Ruth Waddell Smith. Statistical Evaluation of Mass Spectral Data for Seized Drug Identification. Oral presentation at the 73rd Annual Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (Pittcon), Philadelphia, PA. March 2023.

†Isaac C. Willis, Victoria L. McGuffin, and Ruth Waddell Smith. Statistical Tools to Identify Reliable Discriminating Ions of Structurally Similar Fentanyl Analogs. Oral presentation at the SciX Conference, Sparks, NV. October 2023.

3.1.3 Conference abstracts accepted for presentation

†Isaac C. Willis, Victoria L. McGuffin, and Ruth Waddell Smith. Effect of Spectral Intensity on Mass Spectral Discrimination of Fentanyl Positional Isomers. Accepted for oral presentation at the American Academy of Forensic Sciences Annual Meeting to be held in Denver, CO, February 2024.

*†Andrew Sacha, Isaac C. Willis, Victoria L. McGuffin, Ruth Waddell Smith. Evaluating the Robustness and Ruggedness of a Statistical Method for Comparison of Mass Spectral Data for Seized Drug Identification. Accepted for oral presentation at the 7th Annual National Institute of Justice Forensic Science Symposium at Pittcon 2024 to be held in San Diego, CA, March 2024.

3.2 Data sets generated

For each spectral collection (**Table 1**), the following data sets were generated:

- ChemStation files containing the raw chromatographic and mass spectral data.
- Microsoft Excel files containing the imported mass spectral data.
- Microsoft Excel files containing the spectral comparison of each pair of compounds within the set.
- Microsoft Excel file containing a summary of all spectral comparisons for a set of compounds within the collection.
- Microsoft Excel files containing rankings for each pairwise comparison of the synthetic cathinones, fentanyl analogs, and FBF and FIBF positional isomers.
- Microsoft Excel files containing ranking summaries and frequently occurring discriminating ions.

3.3 Dissemination activities

The research has primarily been disseminated *via* conference presentations and a published manuscript (please see section 3.1 above). At least two more conference presentations have been accepted for presentation in 2024 and two more manuscripts are currently in preparation (details including in section 3.1).

We also presented a training workshop on the statistical comparison method at the Midwestern Association of Forensic Scientists Fall Meeting in August 2023 (Detroit, MI), titled "*Statistically Distinguishing NPS Positional Isomers based on Electron-Ionization Mass Spectra*." The workshop was attended by 29 forensic scientists from across the Midwest. The majority of participants were seized drug analysts although there were also one or two forensic toxicologists in the audience. The four-hour workshop included the rationale behind the need for statistical evaluation of spectra, the theory of the statistical methods used, and a hands-on section in which analysts were given a version of the automated Excel spreadsheet to use for themselves with data provided by us. The workshop was well received, and several participants indicated that they would be willing to beta-test the method as we move toward that stage in the research.

We are also working with the Forensic Technology Center of Excellence to present a webinar on applications of the statistical comparison method for seized drug analysis. We initially hoped to present the webinar in early 2024; however, given the scheduling of AAFS and Pittcon, the webinar is now planned for March/April 2024.

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5. Appendices

5.1 Instrument Parameters

Table A1. Parameters for GC-MS analysis of each compound set on Instrument 1

Compound Set	Injection Parameters	Oven Temperature Program
Synthetic Cathinones	Splitless, 280 °C	50 °C for 1 min 20 °C/min to 300 °C 10 min hold
Fentanyl Analogs	Splitless, 250 °C	100 °C for 1 min 30 °C/min to 300 °C 8 min hold
Synthetic Cannabinoids	Splitless, 280 °C	250 °C for 1 min 20 °C/min to 300 °C 10 min hold
FBF and FIBF Isomers	Split (100:1), 220 °C	200 °C for 1 min 30 °C/min to 300 °C 8 min hold

Table A2. Parameters for GC-MS analysis of each compound set on Instrument 3

Compound Set	Injection Parameters	Oven Temperature Program
Synthetic Cathinones	Split (50:1), 250 °C	150 °C for 1 min 20 °C/min to 280 °C 2 min hold
Fentanyl Analogs	Split (50:1), 250 °C	100 °C for 1 min 30 °C/min to 300 °C 5 min hold
Synthetic Cannabinoids	Split (50:1), 250 °C	250 °C for 1 min 20 °C/min to 300 °C 7 min hold

5.2 Association and Discrimination of Synthetic Cathinones

Table A3. Comparison of ranked ions and most frequently occurring discriminating ions for comparisons of eutylone, propylone, and pentylone.

Spectrum 1	Spectrum 2	*m/z* Value of Ranked Ions*	*m/z* Value of Most Frequent Discriminating Ions**
Eutylone	Propylone	**44** **58** **43** **206** 135 45 176 42 57 59	**43** **44** **58** 91 119 **206**
Eutylone	Pentylone	**44** **58** **57** **41** **206** **42** **162** 55 192 178	**41** **42** **44** **57** **58** **162** **206**
Propylone	Pentylone	**135** **192** 43 58 **162** 57 178 41 **44** 96	42 **44** 119 **135** **162** **192**

*All ions in the scan range were ranked but only the 10 lowest-ranked ions are shown.
**Defined as those ions being defined as discriminating ions in all 20 comparisons (for each spectral collection, there are two comparisons of each pair of cathinones (*e.g.*, dibutylone versus eutylone and eutylone versus dibutylone), resulting in a total of 20 comparisons)
Entries in bold font are ions that are both highly ranked and frequently observed as discriminating ions.

Table A4. Comparison of ranked ions for comparisons of eutylone, propylone, and pentylone on two different GC-MS instruments.

Spectrum 1	Spectrum 2	*m/z* Value of Ranked Ions*	
		Instrument 1	Instrument 3
Eutylone	Propylone	44	44
		58	58
		43	206
		206	43
		135	135
		45	45
		176	176
		42	42
		57	59
		59	57
Eutylone	Pentylone	44	44
		58	57
		57	58
		41	41
		206	206
		42	42
		162	162
		55	192
		192	55
		178	96
Propylone	Pentylone	135	135
		192	192
		43	162
		58	58
		162	57
		57	43
		178	96
		41	178
		44	55
		96	41

*All ions in the scan range were ranked but only the 10 lowest-ranked ions are shown.
Entries in red font are ions that are different between the two instruments.

Table A5. Effect of spectral intensity on statistical comparison of eutylone to dibutylone, eutylone, propylone, and pentylone

Spectrum 1				Spectrum 2				Number of Discriminating Ions[‡]
Synthetic Cathinone	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)[*]	Mean Number Ions/Spectrum[†]	Synthetic Cathinone	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)[*]	Mean Number Ions/Spectrum[†]	
Eutylone	1.0	2.79 ± 0.01	93 ± 1	Dibutylone	1.0	3.58 ± 0.04	98 ± 0	25
					0.5	1.71 ± 0.05	78 ± 3	22
					0.25	0.87 ± 0.03	52 ± 3	16
					0.1	0.1927 ± 0.0004	19 ± 2	7
				Eutylone	1.0	2.85 ± 0.09	96 ± 2	0
					0.5	1.78 ± 0.09	78 ± 2	0
					0.25	0.71 ± 0.01	48 ± 1	1 (*m/z* 85)
					0.1	0.17 ± 0.01	20 ± 1	0
				Propylone	1.0	2.43 ± 0.04	83 ± 2	16
					0.5	1.16 ± 0.05	61 ± 2	13
					0.25	0.40 ± 0.01	36 ± 0	6
					0.1	0.11 ± 0.01	16 ± 1	2
				Pentylone	1.0	2.2 ± 0.2	90 ± 4	22
					0.5	1.09 ± 0.08	61 ± 4	16
					0.25	0.59 ± 0.01	43 ± 1	10
					0.1	0.105 ± 0.001	16 ± 2	3

[*] Mean base peak intensity in triplicate spectra ± standard deviation
[†] Mean total number of ions in triplicate spectra ± standard deviation
[‡] 99.9% confidence level. Entries in red font indicate false discrimination.

Table A6. Effect of spectral intensity on statistical comparison of propylone to dibutylone, eutylone, propylone, and pentylone

Spectrum 1				Spectrum 2				Number of Discriminating Ions‡
Synthetic Cathinone	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	Synthetic Cathinone	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	
Propylone	1.0	2.78 ± 0.09	89 ± 2	Dibutylone	1.0	3.58 ± 0.04	98 ± 0	33
					0.5	1.71 ± 0.05	78 ± 3	31
					0.25	0.87 ± 0.03	52 ± 3	27
					0.1	0.1927 ± 0.0004	19 ± 2	10
				Eutylone	1.0	2.85 ± 0.09	96 ± 2	17
					0.5	1.78 ± 0.09	78 ± 2	13
					0.25	0.71 ± 0.01	48 ± 1	11
					0.1	0.17 ± 0.01	20 ± 1	6
				Propylone	1.0	2.43 ± 0.04	83 ± 2	0
					0.5	1.16 ± 0.05	61 ± 2	0
					0.25	0.40 ± 0.01	36 ± 0	0
					0.1	0.11 ± 0.01	16 ± 1	0
				Pentylone	1.0	2.2 ± 0.2	90 ± 4	25
					0.5	1.09 ± 0.08	61 ± 4	21
					0.25	0.59 ± 0.01	43 ± 1	12
					0.1	0.105 ± 0.001	16 ± 2	4

* Mean base peak intensity in triplicate spectra ± standard deviation
† Mean total number of ions in triplicate spectra ± standard deviation
‡ 99.9% confidence level.

Table A7. Effect of spectral intensity on statistical comparison of pentylone to dibutylone, eutylone, propylone, and pentylone

Spectrum 1				Spectrum 2				Number of Discriminating Ions[‡]
Synthetic Cathinone	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)[*]	Mean Number Ions/Spectrum[†]	Synthetic Cathinone	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)[*]	Mean Number Ions/Spectrum[†]	
Pentylone	1.0	2.09 ± 0.01	87 ± 1	Dibutylone	1.0	3.58 ± 0.04	98 ± 0	28
					0.5	1.71 ± 0.05	78 ± 3	25
					0.25	0.87 ± 0.03	52 ± 3	19
					0.1	0.1927 ± 0.0004	19 ± 2	10
				Eutylone	1.0	2.85 ± 0.09	96 ± 2	20
					0.5	1.78 ± 0.09	78 ± 2	16
					0.25	0.71 ± 0.01	48 ± 1	13
					0.1	0.17 ± 0.01	20 ± 1	4
				Propylone	1.0	2.43 ± 0.04	83 ± 2	21
					0.5	1.16 ± 0.05	61 ± 2	16
					0.25	0.40 ± 0.01	36 ± 0	8
					0.1	0.11 ± 0.01	16 ± 1	1
				Pentylone	1.0	2.2 ± 0.2	90 ± 4	0
					0.5	1.09 ± 0.08	61 ± 4	1 (*m/z* 84)
					0.25	0.59 ± 0.01	43 ± 1	0
					0.1	0.105 ± 0.001	16 ± 2	0

[*] Mean base peak intensity in triplicate spectra ± standard deviation
[†] Mean total number of ions in triplicate spectra ± standard deviation
[‡] 99.9% confidence level. Entries in red font indicate false discrimination.

5.3 Association and Discrimination of Fentanyl Analogs

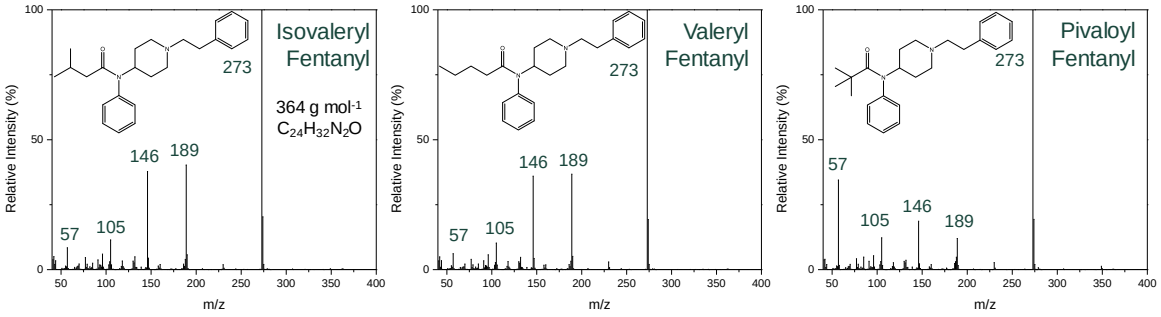

Figure A1. Representative electron-ionization mass spectra of the three fentanyl analogs considered in this work: valeryl fentanyl, isovaleryl fentanyl, and pivaloyl fentanyl.

Table A8. Effect of spectral intensity on statistical comparison of isovaleryl fentanyl to valeryl fentanyl, isovaleryl fentanyl, and pivaloyl fentanyl

Spectrum 1				Spectrum 2				Number of Discriminating Ions‡
Fentanyl Analog	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	Fentanyl Analog	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	
Isovaleryl Fentanyl	1.0	3.1 ± 0.5	135 ± 6	Valeryl Fentanyl	1.0	2.5 ± 0.1	130 ± 2	3
					0.5	1.0 ± 0.1	109 ± 5	1
					0.25	0.42 ± 0.04	86 ± 4	**0**
					0.1	0.13 ± 0.03	64 ± 6	**0**
				Isovaleryl Fentanyl	1.0	1.8 ± 0.1	121 ± 3	0
					0.5	0.7 ± 0.1	102 ± 10	0
					0.25	0.28 ± 0.03	84 ± 2	0
					0.1	0.09 ± 0.02	57 ± 6	0
				Pivaloyl Fentanyl	1.0	3.34 ± 0.01	137 ± 3	15
					0.5	1.4 ± 0.2	116 ± 2	10
					0.25	0.5 ± 0.1	92 ± 5	7
					0.1	0.12 ± 0.02	65 ± 1	4

* Mean base peak intensity in triplicate spectra ± standard deviation
† Mean total number of ions in triplicate spectra ± standard deviation
‡ 99.9% confidence level. Entries in bold font indicate false association of isomers.

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Table A9. Effect of spectral intensity on statistical comparison of pivaloyl fentanyl to valeryl fentanyl, isovaleryl fentanyl, and pivaloyl fentanyl

Spectrum 1				Spectrum 2				Number of Discriminating Ions‡
Fentanyl Analog	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	Fentanyl Analog	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	
Pivaloyl Fentanyl	1.0	2.8 ± 0.4	130 ± 6	Valeryl Fentanyl	1.0	2.5 ± 0.1	130 ± 2	18
					0.5	1.0 ± 0.1	109 ± 5	13
					0.25	0.42 ± 0.04	86 ± 4	4
					0.1	0.13 ± 0.03	64 ± 6	4
				Isovaleryl Fentanyl	1.0	1.8 ± 0.1	121 ± 3	20
					0.5	0.7 ± 0.1	102 ± 10	11
					0.25	0.28 ± 0.03	84 ± 2	5
					0.1	0.09 ± 0.02	57 ± 6	4
				Pivaloyl Fentanyl	1.0	3.34 ± 0.01	137 ± 3	0
					0.5	1.4 ± 0.2	116 ± 2	0
					0.25	0.5 ± 0.1	92 ± 5	0
					0.1	0.12 ± 0.02	65 ± 1	0

* Mean base peak intensity in triplicate spectra ± standard deviation
† Mean total number of ions in triplicate spectra ± standard deviation
‡ 99.9% confidence level. Entries in bold font indicate false association of isomers.

5.4 Association and Discrimination of Synthetic Cannabinoids

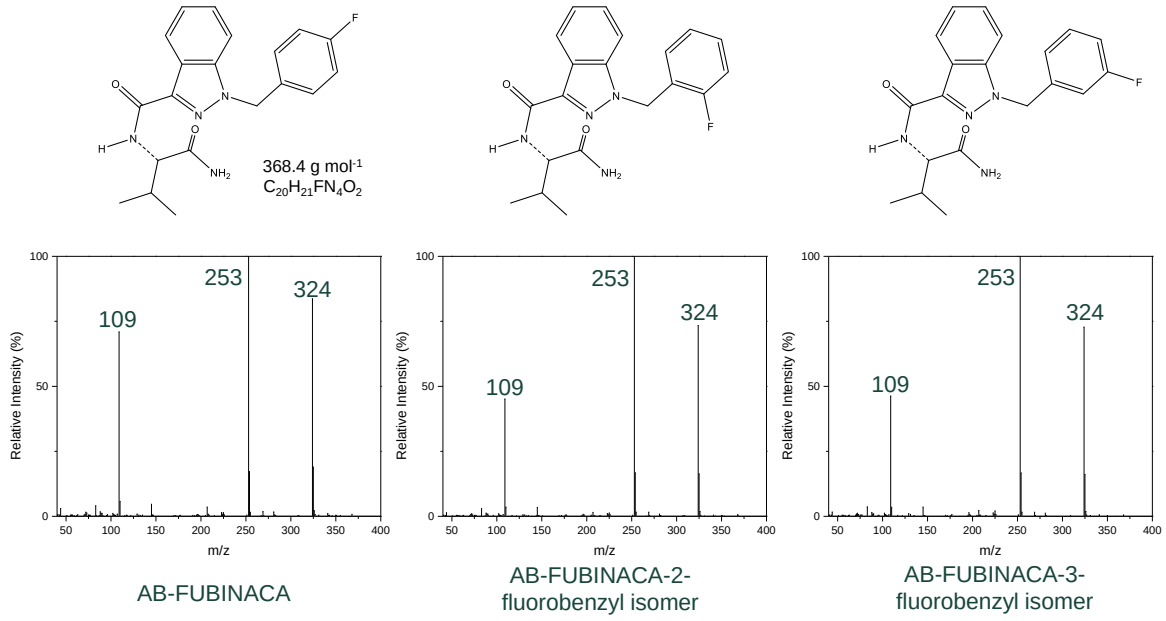

Figure A2. Representative electron-ionization mass spectra of the three synthetic cannabinoid isomers considered in this work: AB-FUBINACA, AB-FUBINACA-2-fluorobenzyl isomer, and AB-FUBINACA-3-fluorobenzyl isomer

Table A10. Statistical comparison of AB-FUBINACA positional isomers for seven spectral collections on Instrument 1.

Spectrum 1	Spectrum 2	Number of Discriminating Ions in Each Collection (C)*						
		C1	C2	C3	C4	C5	C6	C7
AB-FUBINACA	AB-FUBINACA	*m/z* 208[6], 265[5]	*m/z* 44[6]	*m/z* 44[6]	0	*m/z* 252[1]	*m/z* 44[6]	0
	2-fluorobenzyl	3	5	6	4	4	6	2
	3-fluorobenzyl	2	8	6	3	5	7	4
AB-FUBINACA-2-fluorobenzyl isomer	AB-FUBINACA	1	5	5	2	7	6	2
	2-fluorobenzyl	*m/z* 323[1]	*m/z* 44[6], 108[1]	*m/z* 44[6], 108[3], 252[1]	0	0	0	0
	3-fluorobenzyl	1	3	3	2	1	4	1
AB-FUBINACA-3-fluorobenzyl isomer	AB-FUBINACA	3	4	5	4	4	7	5
	2-fluorobenzyl	**0**	3	4	1	**0**	3	1
	3-fluorobenzyl	0	*m/z* 44[6], 252[1]	*m/z* 44[6]	*m/z* 108[1]	0	*m/z* 252[1]	0

* 99.9% confidence level. Entries in bold font indicate false association. Entries in red font indicate false discrimination, with the superscript indicating the number of spectra the ion is present in (from six total spectra being compared).

Table A11. Effect of spectral intensity on statistical comparison of AB-FUBINACA to AB-FUBINACA, AB-FUBINACA-2-fluorobenzyl isomer, and AB-FUBINACA-3-fluorobenzyl isomer

Spectrum 1				Spectrum 2				Number of Discriminating Ions‡
Synthetic Cannabinoid	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	Synthetic Cannabinoid	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	
AB-FUBINACA	1.5	1.86 ± 0.23	151 ± 7	AB-FUBINACA	1.5	2.15 ± 0.26	160 ± 8	1
					1.0	1.04 ± 0.04	129 ± 2	2
					0.5	0.27 ± 0.02	81 ± 3	0
					0.25	0.08 ± 0.01	56 ± 2	1
					0.1	0.045 ± 0.005	49 ± 1	1
				AB-FUBINACA-2-fluorobenzyl isomer	1.5	2.01 ± 0.28	152 ± 9	4
					1.0	1.14 ± 0.19	122 ± 10	3
					0.5	0.35 ± 0.02	79 ± 4	3
					0.25	0.100 ± 0.002	46 ± 4	1
					0.1	0.037 ± 0.005	39 ± 1	2
				AB-FUBINACA-3-fluorobenzyl isomer	1.5	1.73 ± 0.09	154 ± 3	5
					1.0	0.87 ± 0.03	119 ± 2	3
					0.5	0.23 ± 0.02	74 ± 2	1
					0.25	0.09 ± 0.01	55 ± 2	2
					0.1	0.058 ± 0.007	49 ± 2	2

* Mean base peak intensity in triplicate spectra ± standard deviation
† Mean total number of ions in triplicate spectra ± standard deviation
‡ 99.9% confidence level.

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Table A12. Effect of spectral intensity on statistical comparison of AB-FUBINACA-2-fluorobenzyl isomer to AB-FUBINACA, AB-FUBINACA-2-fluorobenzyl isomer, and AB-FUBINACA-3-fluorobenzyl isomer

Spectrum 1				Spectrum 2				Number of Discriminating Ions‡
Synthetic Cannabinoid	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	Synthetic Cannabinoid	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	
AB-FUBINACA-2-fluorobenzyl isomer	1.5	2.16 ± 0.28	161 ± 3	AB-FUBINACA	1.5	2.15 ± 0.26	160 ± 8	7
					1.0	1.04 ± 0.04	129 ± 2	3
					0.5	0.27 ± 0.02	81 ± 3	1
					0.25	0.08 ± 0.01	56 ± 2	1
					0.1	0.045 ± 0.005	49 ± 1	1
				AB-FUBINACA-2-fluorobenzyl isomer	1.5	2.01 ± 0.28	152 ± 9	0
					1.0	1.14 ± 0.19	122 ± 10	0
					0.5	0.35 ± 0.02	79 ± 4	0
					0.25	0.100 ± 0.002	46 ± 4	0
					0.1	0.037 ± 0.005	39 ± 1	1
				AB-FUBINACA-3-fluorobenzyl isomer	1.5	1.73 ± 0.09	154 ± 3	1
					1.0	0.87 ± 0.03	119 ± 2	0
					0.5	0.23 ± 0.02	74 ± 2	0
					0.25	0.09 ± 0.01	55 ± 2	1
					0.1	0.058 ± 0.007	49 ± 2	1

* Mean base peak intensity in triplicate spectra ± standard deviation
† Mean total number of ions in triplicate spectra ± standard deviation
‡ 99.9% confidence level.

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Table A13. Effect of spectral intensity on statistical comparison of AB-FUBINACA-3-fluorobenzyl isomer to AB-FUBINACA, AB-FUBINACA-2-fluorobenzyl isomer, and AB-FUBINACA-3-fluorobenzyl isomer

Spectrum 1				Spectrum 2				Number of Discriminating Ions‡
Synthetic Cannabinoid	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	Synthetic Cannabinoid	Concentration (mg/mL)	Mean Base Peak Intensity ($x10^5$)*	Mean Number Ions/Spectrum†	
AB-FUBINACA-3-fluorobenzyl isomer	1.5	1.70 ± 0.24	151 ± 0	AB-FUBINACA	1.5	2.15 ± 0.26	160 ± 8	4
					1.0	1.04 ± 0.04	129 ± 2	5
					0.5	0.27 ± 0.02	81 ± 3	0
					0.25	0.08 ± 0.01	56 ± 2	1
					0.1	0.045 ± 0.005	49 ± 1	1
				AB-FUBINACA-2-fluorobenzyl isomer	1.5	2.01 ± 0.28	152 ± 9	0
					1.0	1.14 ± 0.19	122 ± 10	1
					0.5	0.35 ± 0.02	79 ± 4	1
					0.25	0.100 ± 0.002	46 ± 4	0
					0.1	0.037 ± 0.005	39 ± 1	1
				AB-FUBINACA-3-fluorobenzyl isomer	1.5	1.73 ± 0.09	154 ± 3	0
					1.0	0.87 ± 0.03	119 ± 2	1
					0.5	0.23 ± 0.02	74 ± 2	0
					0.25	0.09 ± 0.01	55 ± 2	1
					0.1	0.058 ± 0.007	49 ± 2	1

* Mean base peak intensity in triplicate spectra ± standard deviation
† Mean total number of ions in triplicate spectra ± standard deviation
‡ 99.9% confidence level.

5.5 Association and Discrimination of Fluoroisobutyryl Fentanyl (FIBF) Positional Isomers

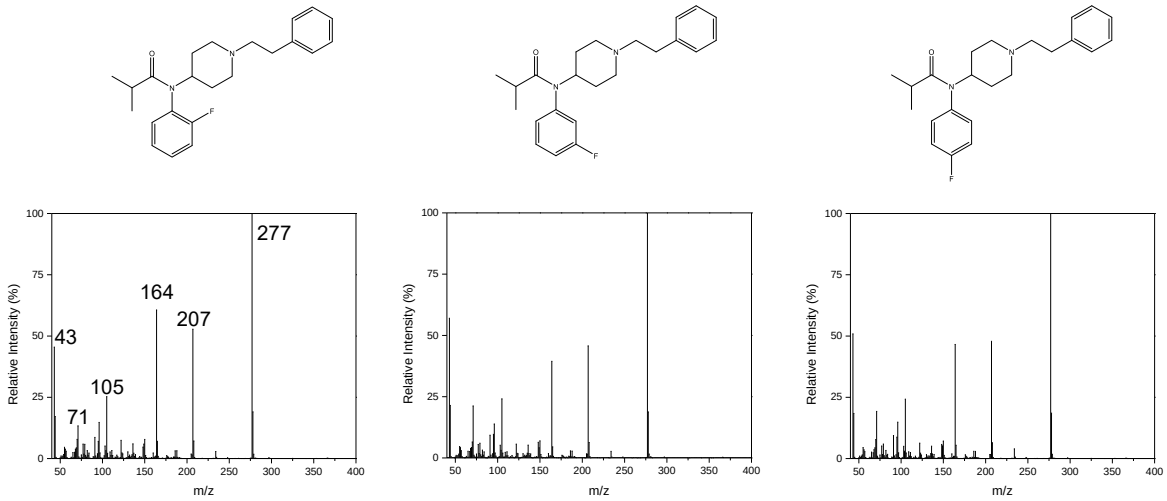

Figure A3. Representative electron-ionization mass spectra of (A) *ortho*-fluoroisobutyryl fentanyl (*o*-FIBF), (B) *meta*-fluoroisobutyryl fentanyl (*m*-FIBF), and (C) *para*-fluoroisobutyryl fentanyl (*p*-FIBF).

Table A14. Statistical comparison of fluoroisobutyryl fentanyl (FIBF) positional isomers at 1 mg/mL for four spectral collections on Instrument 1.

Spectrum 1	Spectrum 2	Number of Discriminating Ions in Each Collection (C)*			
		C1	C2	C3	C4
o-FIBF	*o*-FIBF	0	0	*m/z* 111[6], 118[6]	0
	m-FIBF	7	14	8	2
	p-FIBF	2	13	7	**0**
m-FIBF	*o*-FIBF	6	14	8	1
	m-FIBF	0	*m/z* 111[6], 118[6]	*m/z* 111[6]	*m/z* 111[6], 118[6]
	p-FIBF	1	3	2	**0**
p-FIBF	*o*-FIBF	4	10	6	**0**
	m-FIBF	**0**	9	5	1
	p-FIBF	0	0	0	*m/z* 111[6]

* 99.9% confidence level. Entries in bold font indicate false association. Entries in red font indicate false discrimination, with the superscript indicating the number of spectra the ion is present in (from six total spectra being compared).

Table A15. Comparison of ranked ions and most frequently occurring discriminating ions for comparison of the FIBF positional isomers at 1 mg/mL on Instrument 1.

Spectrum 1	Spectrum 2	*m/z* Value of Ranked Ions*	*m/z* Value of Most Frequent Discriminating Ions**
o-FIBF	*m*-FIBF	**164** 102 90 118 **165** **71** 122 144 149 110	**71** **164** **165**
o-FIBF	*p*-FIBF	102 **164** 118 90 130 71 234 144 112 165	**164**
m-FIBF	*p*-FIBF	**234** 176 235 164 111 70 149 122 165 182	**234*** **

*All ions in the scan range were ranked but only the 10 lowest-ranked ions are shown.
**Defined as those ions being defined as discriminating ions in 6 of 8 comparisons (for each spectral collection, there are two comparisons of each pair of isomers (*e.g.*, *o*-FIBF versus *m*-FIBF and *m*-FIBF versus *o*-FIBF), resulting in a total of 8comparisons)
***Present in 5 of 8 comparisons
Entries in bold font are ions that are both highly ranked and frequently observed as discriminating ions.

Table A16. Comparison of ranked ions and most frequently occurring discriminating ions for comparison of the FIBF positional isomers at 1 mg/mL on Instrument 2.

Spectrum 1	Spectrum 2	*m/z* Value of Ranked Ions*	*m/z* Value of Most Frequent Discriminating Ions**
o-FIBF	*m*-FIBF	**164** **71** **102** **165** 171 118 **90** 122 95 149	**71** **90** **102** **164** **165**
o-FIBF	*p*-FIBF	**164** 102 **71** 171 118 90 130 144 112 143	**71** **164**
m-FIBF	*p*-FIBF	**234** 235 84 164 149 283 176 265 73 98	**234*** **

*All ions in the scan range were ranked but only the 10 lowest-ranked ions are shown.
**Defined as those ions being defined as discriminating ions in 5 of 6 comparisons (for each spectral collection, there are two comparisons of each pair of isomers (*e.g.*, *o*-FIBF versus *m*-FIBF and *m*-FIBF versus *o*-FIBF), resulting in a total of 6 comparisons)
***Present in 4 of 6 comparisons
Entries in bold font are ions that are both highly ranked and frequently observed as discriminating ions.

Table A17. Ranked ions on two different instruments for comparison of fluoroisobutyryl fentanyl (FIBF) positional isomers.

Spectrum 1	Spectrum 2	*m/z* Value of Ranked Ions*	
		Instrument 1	Instrument 2
o-FIBF	*m*-FIBF	164	164
		102	71
		90	102
		118	165
		165	171
		71	118
		122	90
		144	122
		149	95
		110	149
o-FIBF	*p*-FIBF	102	164
		164	102
		118	71
		90	171
		130	118
		71	90
		234	130
		144	144
		112	112
		165	143
m-FIBF	*p*-FIBF	234	234
		176	235
		235	84
		164	164
		111	149
		70	283
		149	176
		122	265
		165	73
		182	98

*All ions in the scan range were ranked but only the 10 lowest-ranked ions are shown.
Entries in red font are ions that are different between the two instruments.

Table A18. Statistical comparison of fluoroisobutyryl fentanyl (FIBF) positional isomers at 0.5 mg/mL for four spectral collections on Instrument 1.

Spectrum 1	Spectrum 2	Number of Discriminating Ions in Each Collection (C)*			
		C1	C2	C3	C4
o-FIBF	*o*-FIBF	0	0	*m/z* 44[6]	*m/z* 267[2], 346[1]
	m-FIBF	3	9	4	**0**
	p-FIBF	2	6	3	**0**
m-FIBF	*o*-FIBF	5	9	5	**0**
	m-FIBF	*m/z* 276[1]	*m/z* 43[6]	*m/z* 44[6]	0
	p-FIBF	4	6	1	**0**
p-FIBF	*o*-FIBF	1	9	4	1
	m-FIBF	**0**	8	5	**0**
	p-FIBF	0	*m/z* 43[6], 44[6], 111[6], 341[6]	0	*m/z* 111[6]

* 99.9% confidence level. Entries in bold font indicate false association. Entries in red font indicate false discrimination, with the superscript indicating the number of spectra the ion is present in (from six total spectra being compared).

Table A19. Statistical comparison of fluoroisobutyryl fentanyl (FIBF) positional isomers at 0.1 mg/mL for four spectral collections on Instrument 1.

Spectrum 1	Spectrum 2	Number of Discriminating Ions in Each Collection (C)*			
		C1	C2	C3	C4
o-FIBF	*o*-FIBF	0	0	0	0
	m-FIBF	**0**	1	1	**0**
	p-FIBF	**0**	**0**	3	**0**
m-FIBF	*o*-FIBF	**0**	5	1	**0**
	m-FIBF	0	0	0	0
	p-FIBF	**0**	**0**	**0**	**0**
p-FIBF	*o*-FIBF	**0**	3	**0**	**0**
	m-FIBF	**0**	**0**	**0**	**0**
	p-FIBF	0	0	*m/z* 44[6]	0

* 99.9% confidence level. Entries in bold font indicate false association. Entries in red font indicate false discrimination, with the superscript indicating the number of spectra the ion is present in (from six total spectra being compared).