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

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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

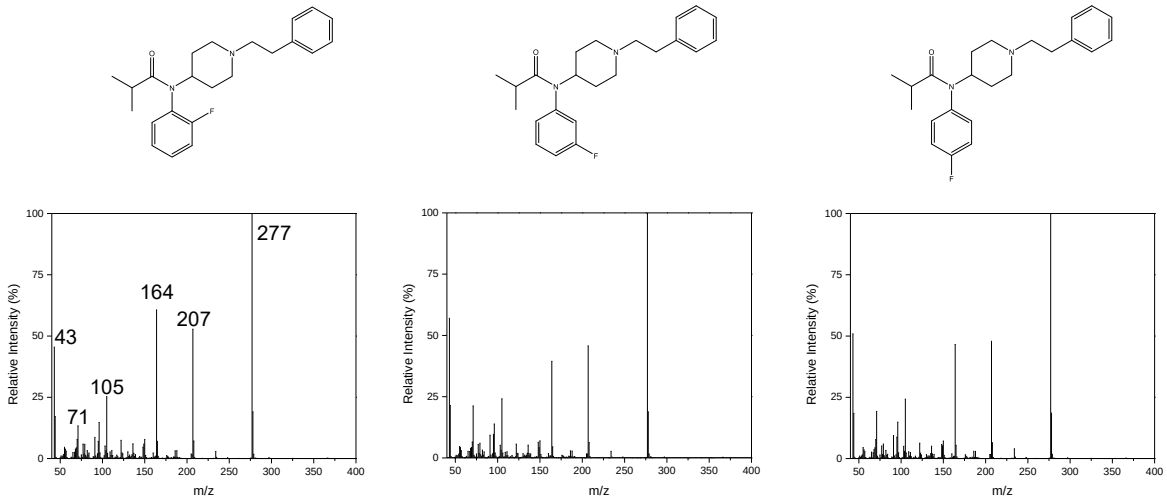

Figure A3. Representative electron-ionization mass spectra of (A) *ortho*-fluoroisobutyryl fentanyl (*o*-FIBF), (B) *meta*-fluoroisobutyryl fentanyl (*m*-FIBF), and (C) *para*-fluoroisobutyryl fentanyl (*p*-FIBF).

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).