Updates to the Inverted Library Search Algorithm for Mixture Analysis

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Abstract

Identifying mixture components is a well-known challenge in analytical chemistry. The Inverted Library Search Algorithm (ILSA) is a recently proposed method for identifying mixture components using in-source collision induced dissociation (is-CID) mass spectra of a query mixture and a reference library of pure compound is-CID mass spectra (*J. Am. Soc. Mass Spectrom.* 2021, 32, 7, 1725–1734). This article presents several subtle but important advances to the algorithm, including updated compound matching strategies that improve result explainability, and spectral filtering to better handle noisy mass spectra as is often observed with real-world samples such as seized drug evidence.

Keywords: ILSA; Mass Spectrometry; Mixture Analysis; Search Algorithms; Seized Drug Analysis.

1. Introduction

Identifying compounds in a mixture is a standard challenge in analytical chemistry. A common way to approach this challenge is by separating the mixture into its components using chromatography, and then determining each component's molecular structure using electron ionization (EI) or electrospray ionization (ESI) mass spectrometry. Often, a pure compound mass spectral library is used to aid an analyst in determining a components molecular structure (i.e., compound identification); the mass spectrum of a component is compared to reference mass spectra of known compounds. Several algorithms have been developed in the context of compound identification using mass spectral libraries, including but not limited to probability-based matching [1, 2], and pattern similarity-based methods like the Simple and Hybrid Similarity Search [3, 4].

This coupled chromatography-mass spectrometry workflow allows for the detailed characterization of most mixtures, but chromatography can take on the order of tens of minutes per run, in addition to the tens of minutes required for sample preparation. In application areas where minimizing analysis time is more heavily weighted than the completeness of characterization, like the pre-screening of seized drug evidence to presumptively identify compounds, it can be prudent to implement ambient ionization mass spectrometry platforms like Direct Analysis in Real Time Mass Spectrometry (DART-MS) that are capable of collecting multiple mass spectra at in-source collision induced dissociation (is-CID) voltages [5, 6]. However, the resulting is-CID mass spectra are measurements of the complete mixtures instead of separated mixture components, and so traditional library search algorithms are not suitable for analyzing these mass spectra.

The inverted library-search algorithm (ILSA) is a method for screening is-CID mass spectra of mixtures. Broadly speaking, the algorithm searches a set of mixture is-CID mass spectra for *partial patterns* that are similar to pure compound is-CID mass spectra (as opposed to *complete pattern* matches as are used in traditional mass spectral library search algorithms). In doing so, the ILSA can identify multiple partial patterns in the same mixture spectrum—the logical equivalent to identifying compounds in a mixture. We recently demonstrated that the ILSA could accurately identify components of eleven simple laboratory mixtures containing between two and four drugs using is-CID mass spectra collected with Direct Analysis in Real Time Mass Spectrometry (DART-MS) [7] and the National Institute of Standards and Technology (NIST) DART-MS Forensics Database [8, 9].

Since its initial publication, we have continued to explore the utility of the ILSA. We have experimented with algorithm parameters and configurations while challenging the ILSA with more complex mixtures. We have also heard from other scientists who have tested the ILSA with their use-cases. Through this experimentation and discussion, we have identified several subtle but non-trivial ways to improve the performance of the ILSA. In this note, we provide a revised description of the ILSA that includes these advancements. A simple software implementation of the ILSA to supplement this note is available for review [10], and an interactive implementation of the ILSA is included with the NIST/NIJ DART-MS Data Interpretation Tool [11].

2. The ILSA

The ILSA is a three-step method for interpreting a set of mixture is-CID mass spectra using a library of pure compound is-CID mass spectra (see Figure 1). The algorithm requires as input a low-fragmentation is-CID mass spectrum and can handle as many additional fragmentation is-CID mass spectra as is reasonable for the considered search library. In our work, we employ the NIST DART-MS Forensics Database (v.5 Earthworm) that contains three is-CID mass spectra representing low-, mid-, and high-fragmentation levels (measured at orifice 1 voltages of +30, +60 and +90 V, respectively), for 953 compounds of interest to the forensic community.

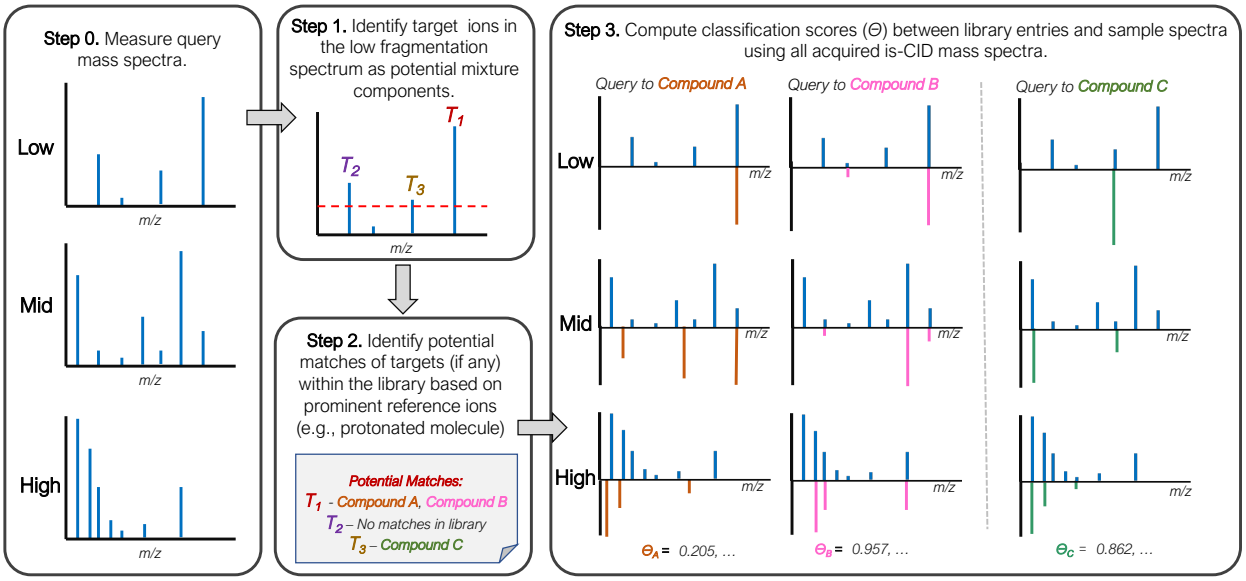


Figure 1: A visual summary of the Inverted Library Search Algorithm (ILSA) for identifying components of a mixture using in-source collision induced dissociation (is-CID) mass spectra and a library of is-CID mass spectra of pure compounds. Classification scores are described in Step 3 and summarized in Table 1.

Step 1: Target Identification. In this step, the low-fragmentation is-CID mass spectrum of the mixture is used to identify a set of *target* mass-to-charge ratios (*m/z*) with relative intensities greater than a user prescribed threshold (e.g., 5 % relative intensity). Each of these target peaks are considered representative of one or more potential pure components in the mixture. There have been no updates to this step of the algorithm.

Step 2: Compound Matching. For each target *m/z* value, compounds from the reference library are selected as potential matches. In the preliminary implementation of the ILSA, we generated the list of reference *m/z* values to match against by considering either the (i) calculated protonated molecule *m/z* of all library compounds, or (ii) observed base peak *m/z* from the low-fragmentation is-CID library spectrum of all library compounds; but not both simultaneously. The library entries with reference *m/z* values that fell within a user prescribed mass tolerance of the target *m/z* (e.g., ±0.005 Da) were considered potential matches and proceeded to step 3 for scoring. From experimentation, we observed that target *m/z* values from the mixture were occasionally not identified as a protonated molecule or base peak but could be explained as either the major isotope of one of these peaks or as a major fragment ion from the library compound that was not the base peak. Accordingly, we now generate the list of reference *m/z* values to include (i) protonated molecule, (ii) low-fragmentation spectrum base peak, (iii) prominent isotope (most abundant between M+1 or M+2) of the protonated molecule, (iv) prominent isotope of the base peak, and (v) second highest intensity ion with relative intensity at least 5 % (referred to as the major fragment ion, if present)—all reference *m/z* values are calculated during the library building process and all target-types are identified simultaneously. Note that for many compounds in the considered database, the protonated molecule and low-fragmentation spectrum base peak are the same. This larger reference *m/z* list can provide a broader set of potential matches for every *target*

m/z and explain more peaks in the mixture, without adding appreciable computing time given the library sizes thus far considered.

Step 3: Compound Scoring. For each library compound identified as a potential match for a target *m/z*, we compute several reference metrics that can be used to support the presumptive identification of the target. Since its initial publication, there have been several subtle changes in the scoring and presentation of scores. For ease of reading, we will discuss two subtle data preprocessing steps that occur prior to scoring, and a major philosophical change in the presentation of computed scoring metrics. A separate summary of each scoring metric is provided in Table 1.

One of the first things we observed when moving away from laboratory examples was that mixture mass spectra often contained low level noise peaks. This was especially true for low-resolution mass spectra where random matching of a peak in the library spectrum to a peak in the query spectrum is more likely. These noise peaks would occasionally inflate scoring metrics and complicate interpretation. To address this limitation, we now include a rudimentary "noise" filter that discards all peaks below a user-prescribed threshold (e.g., 1 % relative intensity) in both query and library spectra when computing scoring metrics. Finding an alternative method capable of filtering only true noise peaks is ongoing work, with one such alternative being the recently published method of mass defect filtering [12]. Secondly, we now score spectra using peaks in the range of *m/z* 80 to the five mass units beyond the protonated molecule (as opposed to stopping the scoring at protonated molecule). This ensures that the major peaks in the isotopic envelope around the protonated molecule are accounted for in scoring. The lower bound on the mass range was chosen as representative of the minimum *m/z* value commonly employed in DART-MS for seized drug screening and can be changed based on application. The upper bound can also be adjusted for other applications where adduct formation is more likely.

In the first implementation of the ILSA, the algorithm output was a pair of composite scores titled "FPIE" (fraction of peak intensity explained) and "RevMF" (reverse match factor) for every potential library match. These composite scores were non-linear combinations of several underlying metrics. Through discussion with other ILSA users, it became clear that presenting composite scores actually complicates the interpretation process and thus an analyst's confidence when making presumptive identifications. With explainability being important in science and engineering, and vital to our primary application area (forensic seized drug analysis), we have updated the ILSA to now present a complete set of up to 14 metrics depending on the number of fragmentation spectra available for the mixture. Five of the 14 metrics are described in Table 1, with the remaining 9 metrics being the values that underlie the 'average' metrics (i.e., FPIE, RevMF, and spread). For example, the ILSA computes up to three individual FPIE values between the appropriate mixture and library spectra, and the "Average FPIE" is the arithmetic average of those values—up to a total of 4 FPIE values are computed.

Of the five metrics summarized in Table 1, two are new to this version of the ILSA: the average spread and Low-Fragmentation Protonated Molecule (LFPM) Isotope Ratio Difference (IRD). For a given spectral comparison, peaks from the library spectra are matched to a corresponding peak in the mixture. The spread between the largest and smallest *m/z* differences between library peaks

and their corresponding peak in the mixture is recorded for each spectral comparison, and the average spread is the arithmetic mean of the spread for all available comparisons. Computed spread values are only discriminatory when considering high-resolution mass spectra. The LFPM-IRD, which we colloquially refer to as the IRD, is the difference between the calculated relative intensity isotope ratio between the reference protonated molecule and its major isotope and relative intensity isotope ratio observed with corresponding peaks in the low-fragmentation mixture mass spectrum.

A metric referred to as weighted mass bias was described in [7] but is no longer included in the ILSA scoring stage, being replaced with the more interpretable average spread.

Table 1: *Summary of scoring metrics produced by the updated ILSA. Values for mass tolerance (ϵ) are prescribed by user for high-resolution mode, and $\epsilon = 0$ for low-resolution mode. All metrics assume spectra have been filtered for noise peaks prior to scoring.*

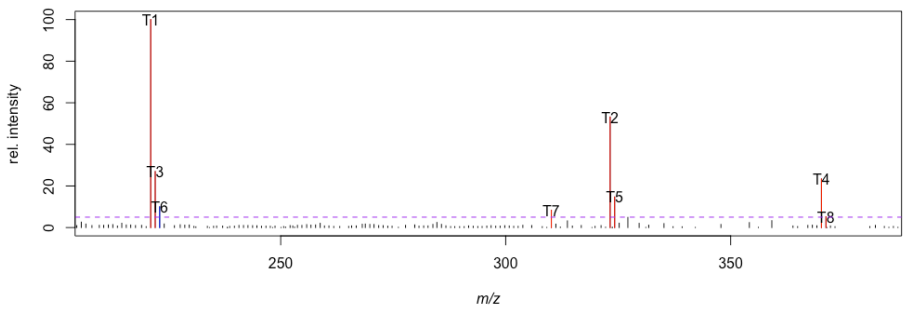
Metric	Short Description	Output Range	Ideal Output	Updates from previous ILSA
$\Delta m/z$	Mass difference between the observed target *m/z* and the calculated library reference *m/z.*	$[-\epsilon, +\epsilon]$	0	Previously included in composite FPIE and RevMF scores, now displayed as independent metric.
Average FPIE[1]	The fraction of total peak intensity from the library spectrum that have a 'matching' peak in the corresponding mixture spectrum, where matching implies a peak in the mixture spectrum within a mass tolerance window ($\pm 2\epsilon$) and with signal intensity above the noise threshold. The reported number is a simple arithmetic average computed based on number of available fragmentation spectra compared.	[0,1]	1	Wider mass tolerance window ($\pm 2\epsilon$) to account for measurement-to-measurement mass drift differences. Original window was ($\pm\epsilon$).
Average RevMF[2]	The cosine similarity between the vector constructed of relative intensities above the noise threshold from the library spectra and the vector constructed with relative intensities from 'matching' in the corresponding mixture spectrum, where matching implies the peak in the mixture closest (in *m/z*) to the library spectrum within a mass tolerance window ($\pm 2\epsilon$). Note that the vector representing the mixture could have zero valued entries. The reported number is a simple arithmetic average computed based on number of available fragmentation spectra compared.	[0,1]	1	Wider mass tolerance window ($\pm 2\epsilon$) to account for measurement-to-measurement mass drift differences. Original window was ($\pm\epsilon$).
Average spread[3]	The spread between the largest and smallest *m/z* differences observed between reference spectrum peaks and 'matching' peaks in the mixture spectrum, where matching implies the mixture closest (in *m/z*) to the reference peak. If there are less than two matching peaks between the reference and mixture, the spread is reported as NA. The reported number is a simple arithmetic average computed based on number of available fragmentation spectra compared.	$[0,4\epsilon]$[5]	0	New to this version of the ILSA.
LFPM[4] Isotope Ratio Difference (IRD)	The difference in calculated relative intensity isotope ratio $\left(\frac{M+i}{M}\right)$ between the reference protonated molecule and its major isotope ($i = 1$ or $i = 2$) and the observed intensity values in the matching peaks in the mixture spectrum, where matching implies a peak in the mixture spectrum within a mass tolerance window ($\pm\epsilon$) and with signal intensity above the noise threshold. If there is no peak matching the protonated molecule in the mixture spectrum, the LFPM Isotope Ratio Difference is reported as NA.	$[-9.99, +9.99]$[5]	0	New to this version of the ILSA. Mass tolerance window ($\pm\epsilon$) as measured values are compared to computed values.

[1]FPIE: Fraction of reference spectrum Peak Intensity Explained
[2]RevMF: Reverse Match Factor
[3]Average spread only provides useful information when comparing high-resolution mass spectra
[4]LFPM: Low-Fragmentation mass spectrum Protonated Molecule
[5]Values of <NA> are possible for average mass error spread if there are less than 2 matching peaks in any one of the spectral pairs considered during averaging or with LFPM Isotope Ratio Difference if the protonated molecule of the matching compound is not present in the mixture spectrum above the noise threshold.

3. Example Analysis

To highlight the new modifications to the ILSA, we present an example analysis using both the original and updated algorithms followed by a possible interpretation of the results. Consider the low-fragmentation (+30 V) is-CID mass spectrum shown in Figure 2. This spectrum is a DART-MS measurement of a methanol solution containing equal masses of xylazine, acetyl fentanyl and heroin (purchased from Cayman Chemical, Ann Arbor, MI). We collected the spectra using identical instrumentation and method parameters as [7], including parameter switching to simultaneously collect three is-CID fragmentation spectra at +30 V, +60 V, and +90 V orifice 1 voltages, representing low-, mid-, and high-fragmentation mass spectra, respectively.

Using a peak intensity threshold of 5 % relative intensity, step 1 of the ILSA identifies 8 peaks (targets) for compound matching and scoring.



Target	m/z
1	221.1140
2	323.2131
3	222.1150
4	370.1664
5	324.2155
6	223.1090
7	310.1442
8	321.1688

Figure 2: *Example section of a low-fragmentation mixture in-source collision induced dissociation mass spectrum analyzed with the updated Inverted Library Search Algorithm (ILSA) and the NIST DART-MS Forensics Database (v5 Earthworm). Peaks outside of the illustrated range had relative intensities less than 5 % and were not identified as targets in Step 1 of the ILSA. Target peaks (marked T#) that are red indicate targets having potential matches in the NIST DART-MS Forensics Database (Earthworm version) based on the matchable set of reference m/z values (Step 2 of the ILSA). Target peaks that are blue (for example, T6) do not have any possible matches.*

With the original ILSA, assuming target *m/z* values are protonated molecules and scoring matches with all three fragmentation is-CID mass spectra of the mixture, Targets 1 (*m/z* 221.1140), 2 (*m/z* 323.2131) and 4 (*m/z* 370.1664) are correctly identified as xylazine (protonated molecule *m/z* 221.1112), acetyl fentanyl (protonated molecule *m/z* 323.2123) and heroin (protonated molecule *m/z* 370.1654), respectively, all with high composite FPIE and RevMF scores. Note that if we only use the low-fragmentation spectrum in scoring, it would be unclear whether Target 2 was acetyl fentanyl or its positional isomer, benzylfentanyl, using any of our implemented scoring metrics. Targets 5 (*m/z* 324.2155), 6 (*m/z* 223.109), and 8 (*m/z* 371.1688) do not match any reference protonated molecules in the library. Target 3 (*m/z* 222.1150) matches several synthetic cathinone isomers (protonated molecule *m/z* 222.1130), albeit with low (< 0.6) scores using all three fragmentation is-CID mass spectra. And Target 7 (*m/z* 310.1442) matches with fluoxetine (protonated molecule *m/z* 310.1419), again with a low score, using all available is-CID mass spectra. If instead we assume targets were base peaks from low-fragmentation mass spectra, Target 7 is correctly identified as heroin, but Target 4 is now unmatched—all other results are unaffected.

With the updated ILSA, which allows matching of targets with the expanded reference *m/z* lists, all Targets except 6 and 7 are easily identified. For example, Target 3 still matches several synthetic cathinone isomers with low scores, but also matches xylazine as an M+1 isomer with much higher scores than the cathinones. The identification of this target as the M+1 isomer of xylazine is further supported by an isotope ratio difference close to zero (0.14)—if a cathinone or another compound with a major ion at *m/z* 222 was in the mixture, this intensity ratio would be further from zero in the positive direction. A complete comparison of results using the original and updated ILSA is provided in Table 2. Summary head-to-tail plots comparing the low-fragmentation is-CID mass spectrum of the query to the low-fragmentation library mass spectra of acetyl fentanyl, heroin and xylazine is provided as Figure 3. Additional summary head-to-tail plots comparing all potential library match spectra to the mixture spectra collected at all fragmentation levels are provided as Figures S1 through S3 of the Supporting Information.

One example of how the ILSA modifications negatively interfere with result interpretation is with Target 7. Since the matching window ϵ was widened to account for two measured mass spectra drifting in opposing directions, the computed average RevMF score is comparable with both heroin and fluoxetine. Taking all metrics and results into consideration, an analyst should notice that the mass difference ($\Delta m/z$) between the target *m/z* and the reference *m/z* is smaller for heroin than it is for fluoxetine, and that there are several other explained targets in the mixture that support the notion that heroin is a mixture component. That said, there is still room for improvement with ILSA scoring metrics and this is work we continue to pursue.

The only target that went unmatched with the updated ILSA in this example is Target 6. To most analysts, it will be clear that Target 6 is the minor isotope of xylazine. Adding minor isotope matching to the ILSA was discussed, but deemed unnecessary for our current application space—we have not seen many minor isomers as targets in our evaluation of seized drug evidence. It may be worth re-visiting additional targeting approaches, such as minor isotope matching or dimer matching, in application areas outside of seized drug analysis.

Table 2: Comparison of results from searching the example query mass spectrum with the original (italicized) and updated ILSA and the NIST DART-MS Forensics Database (v5 Earthworm). Match types include protonated molecule (PM), base peak (BP), protonated molecule isotope (M+1 Iso). Base peak isotope and major fragment matches are possible, but did not occur in this example search. The mixture contained xylazine, acetyl fentanyl, and heroin.

Target (nominal *m/z*)	Possible Matches	Δ*m/z*	FPIE[1]	RevMF[1]	Spread	IRD	Match Type
1 (*m/z* 221)	*Xylazine*	--	*0.908*	*0.976*	--	--	--
	Xylazine	0.0002	0.898	0.973	0.005	0.14	PM
2 (*m/z* 323)	*Acetyl fentanyl*	--	*0.921*	*0.990*	--	--	--
	Benzyfentanyl		*0.530*	*0.651*			
	Acetyl fentanyl	0.0007	0.920	0.993	0.003	0.049	PM
	Benzyfentanyl	0.0007	0.518	0.666	0.002	0.049	PM
3 (*m/z* 222)	*Butylone*	--	*0.523*	*0.484*	--	--	--
	Ethylone		*0.428*	*0.479*			
	Dimethylone		*0.417*	*0.590*			
	Xylazine	0.0004	0.898	0.973	0.005	0.14	M+1 Iso
	Dimethylone	0.0020	0.408	0.596	0.006	-0.13	PM
	Ethylone	0.0020	0.401	0.483	0.005	-0.13	PM
	Butylone	0.0020	0.395	0.472	0.006	-0.13	PM
4 (*m/z* 370)	*Heroin*	--	*0.838*	*0.614*	--	--	--
	Heroin	0.0009	0.795	0.623	0.005	-0.003	PM
5 (*m/z* 324)	*No Hits*	--	--	--	--	--	--
	Acetyl fentanyl	0.0002	0.920	0.993	0.003	0.049	M+1 Iso
	Benzylfentanyl	0.0002	0.518	0.666	0.002	0.049	M+1 Iso
6 (*m/z* 223)	*No Hits*	--	--	--	--	--	--
	No Hits	--	--	--	--	--	--
7 (*m/z* 310)	*Fluoxetine*	--	*0.069*	*0.271*	--	--	--
	Heroin	-0.0001	0.795	0.623	0.005	-0.003	BP
	Fluoxetine	0.0024	0.404	0.692	0.005	0.015	PM
8 (*m/z* 371)	*No Hits*	--	--	--	--	--	--
	Heroin	0.0000	0.795	0.623	0.005	-0.003	M+1 Iso

[1] Note that output FPIE and RevMF scores using the original ILSA described in [7] were composite values that included other metrics like the Δ*m/z*. Here we present just the average FPIE and RevMF scores comparable to the updated ILSA (i.e., demonstrating the effect of implanting a noise filter and set mass range, and the widening of matching window when computing spectral similarity).

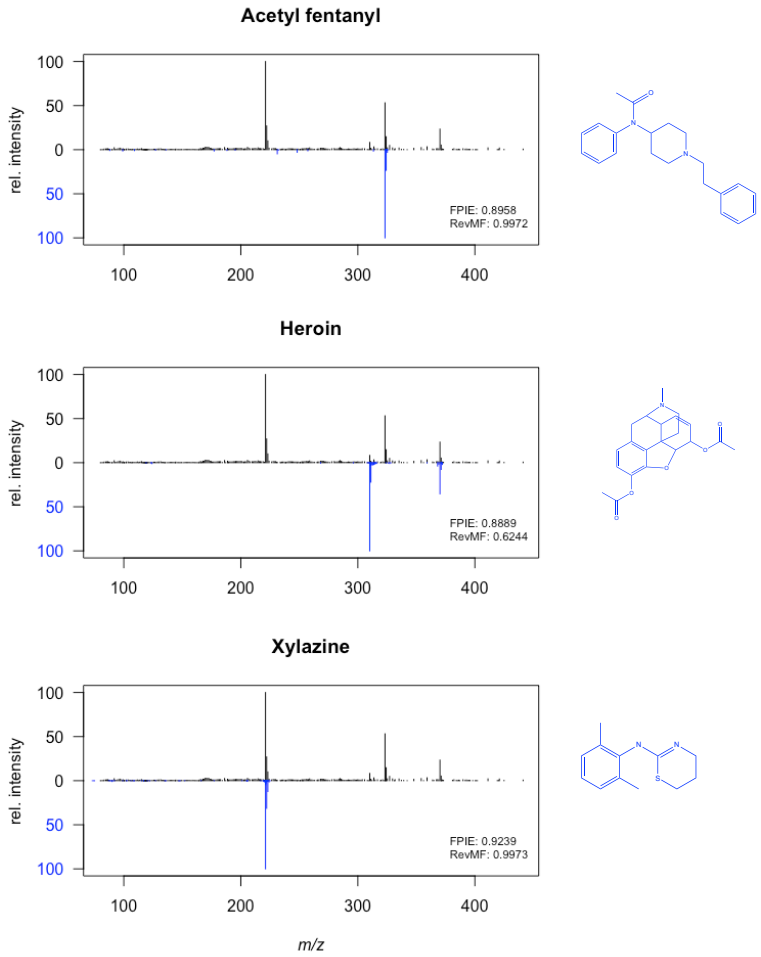


Figure 3: Head-to-tail comparisons of query in-source collision induced dissociation mass spectra collected at +30V (black/top) with library spectra of acetyl fentanyl, heroin and xylazine collected at +30V (bottom/blue). Molecular structures of each library compound included for reference.

4. Discussion

To-date, the ILSA has been developed with is-CID mass spectra collected with DART-MS and with a focus on seized drug analysis. This is for two reasons: the NIST DART-MS Forensic Database is the only publicly available is-CID mass spectral database of which we are aware, and efficiently screening seized drug evidence has significant societal repercussions.

There are several recent reviews of analytical techniques in the seized drug analysis space (as an example, see [13]). The general consensus of these reviews is that "high tech" methods like gas chromatography mass spectrometry are highly effective but with pragmatic limitations (e.g., analysis time, cost, analyst training), while "low tech" methods like color tests having limited effectiveness. DART-MS rapidly produces informative mass spectra and the ILSA allows these spectra to be conveniently interpreted. That said, there are two limitations worth discussing with this analytical protocol.

Even with this update, the ILSA is a work in progress. In particular, we have yet to determine appropriate thresholds for either of the current scoring metrics (FPIE and RevMF). In pure compound analysis using EI-MS, we know that good spectral matches *generally* have similarity scores greater than 0.75 (i.e., 750), but can also use retention times and replicate mass spectra to further support any conclusions [14, 15]. With is-CID spectra of mixtures, there is less intuition about what makes a "good" score as there are significantly more variables to consider, such as the number of compounds in the mixture (which is unknown prior to the analysis). We are currently conducting a large-scale evaluation of DART-MS and the ILSA with a collection of casework samples that contained between 3 and 10 identifiable compounds (as determined with GC-MS), and a preliminary takeaway is that the scores (FPIE or RevMF) are much more discriminatory for prominent targets in the mixture spectrum. Note that the most prominent targets (by relative intensity) in the query mass spectrum are not necessarily representative of the most abundant compounds in the analyzed mixture.

In conjunction with algorithm shortcomings, there are limitations when using ambient ionization techniques like DART-MS to collect spectra of mixtures. For one, these platforms are not capable of discriminating many positional isomers. In the case of prescreening drugs, this is not too significant of a drawback—knowing that one or more positional isomers of an illicit compound is in a mixture is sufficient for pursuing further analysis. A more prominent issue is competitive ionization. Competitive ionization can occur when there is one or more major components in the mixture that have high proton affinities. In this instance, charge will be consumed by these compounds, reducing the likelihood of ionizing, and subsequently detecting, minor components. One known instance where this can happen in the analysis of seized drugs is in samples with high levels of fentanyl and low levels of heroin [16]. Understanding how we can algorithmically account for competitive ionization, either using minor signatures in the mass spectra or potentially using application specific prevalence data, are on-going work in our lab.

5. Conclusions

The ILSA has been shown as a useful method for identifying mixture components using is-CID mass spectra. Since its initial presentation, several subtle but meaningful updates to the ILSA have further improved its utility. This note summarized the updates and demonstrated the algorithm improvements with an example application using a laboratory mixture of xylazine, acetyl fentanyl and heroin. We expect that the ILSA will continue to evolve and find potential applications outside of seized drug analysis as well.

6. Disclaimer

7. Acknowledgments

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8. Associated Content

Supplemental figures to illustrate example search results

9. References

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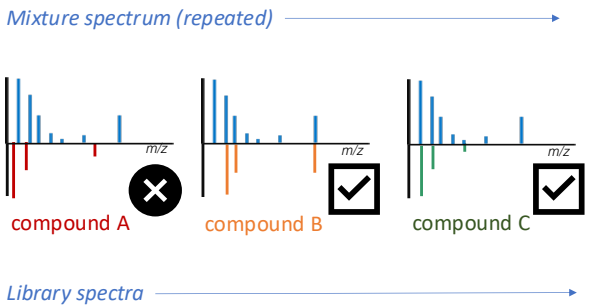
1 For Table of Contents Only



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