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Abstract

Attribution of the origin of an illicit drug relies on identification of compounds indicative of its clandestine production and is a key component of many modern forensic investigations. The results of these studies can yield detailed information on method of manufacture, starting material source, and final product - all critical forensic evidence. In the present work, chemical attribution signatures (CAS) associated with the synthesis of the analgesic fentanyl, *N*-(1-phenylethylpiperidin-4-yl)-*N*-phenylpropanamide, were investigated. Six synthesis methods, all previously published fentanyl synthetic routes or hybrid versions thereof, were studied in an effort to identify and classify route-specific signatures. 160 distinct compounds and inorganic species were identified using gas and liquid chromatographies combined with mass spectrometric methods (GC-MS and LC-MS/MS-TOF) in conjunction with inductively coupled plasma mass spectrometry (ICP-MS). The complexity of the resultant data matrix urged the use of multivariate statistical analysis. Using partial least squares discriminant analysis (PLS-DA), 87 route-specific CAS were classified and a statistical model capable of predicting the method of fentanyl synthesis was validated and tested against CAS profiles from crude fentanyl products deposited and later extracted from two operationally relevant surfaces: stainless steel and vinyl tile. This work provides the most detailed fentanyl CAS investigation to date by using orthogonal mass spectral data to identify CAS of forensic significance for illicit drug detection, profiling, and attribution.

Introduction

Critical to law enforcement and related intelligence efforts to combat illicit drug abuse are methods that can assess the presence and the persistence of both the drug and its associated compounds: its chemical attribution signatures (CAS). These signatures often include synthesis precursors and byproducts, metabolites in various biological matrices, and degradation products after exposure to laboratory and other operational surfaces. Analytical tools for the characterization of such compounds generally include various forms of chromatographic separation combined with spectrometric detection schemes. Gas and high-pressure liquid chromatography (GC and HPLC, respectively) combined with mass spectrometric (MS) detection have traditionally been the workhorses of such studies.[1] Though they each have specific merits and drawbacks, both are geared exclusively towards organic speciation and are generally used independently of one another. Another mass spectrometric technique that has found considerable success in forensic applications, particularly when coupled to statistical chemometric analyses, is isotope ratio mass spectrometry using, for example, time-of-flight secondary ion mass spectrometry (TOF-SIMS), accelerator mass spectrometry (AMS), or elemental analyzers coupled to isotope ratio mass spectrometers.[2-4]

Fentanyl, or *N*-(1-phenylethylpiperidin-4-yl)-*N*-phenylpropanamide, is a synthetic opioid originally designed for anesthesia and analgesia. With a potency roughly 100 times that of morphine[5], its strong euphoric effects have bred a significant potential for misuse. Since its designation as a Schedule II narcotic, multiple domestic clandestine fentanyl laboratories were seized by law enforcement. Between 2000 and 2010, there

were more than 1,000 confirmed or suspected fentanyl-related overdoses and deaths in the United States.[6]

In almost all clandestine laboratory raids in the 2000s, manufacture of fentanyl was found to rely on *N*-phenethyl-4-piperidone (NPP) as the starting reagent.[6] The use of NPP is driven by its commercial availability and the ease of its manufacture from smaller, readily available compounds. Various sophisticated chromatographic and spectrometric techniques[7-11] have been previously exploited to obtain information on fentanyl signatures. In a particularly detailed study by Lurie et al. two fentanyl synthesis pathways were profiled using UHPLC-MS/MS.[9] A substantial number of compounds was detected and identified in this work, but unique CAS were readily classified due in part to the small number of routes studied and the significant differences between them (one route did not use NPP as an intermediate). Forensic chemical attribution of synthetic schemes that rely on a common intermediate, however, are often difficult to discriminate amongst due to a small number of unique signatures, particularly when present at trace levels. The work of Lurie thus prompted us to investigate the degree to which highly similar synthetic routes can be unambiguously discriminated.

In the present work, six synthetic methods were selected to prepare fentanyl in a manner believed to most closely mimic conditions used by a clandestine manufacturer. All methods were found either in the open literature[12,13], on illicit drug synthesis websites[14], or were hybrids of such methods. Intermediate purification was limited to simple solvent extractions; and silica gel chromatography, crystallization, etc. were eliminated both to retain unique CAS and to simulate the often more novice skills of illicit drug manufacturers. Taking a cue from samples previously seized from clandestine

laboratories, all of the routes chosen here relied on NPP as the critical intermediate compound.

Studies similar to that reported presently have sought to use a variety of analytical techniques for chemical signal attribution and forensics related to acutely toxic chemicals. In particular these studies have sought to link relevant signatures to synthetic origin, particularly in work by Fraga and coworkers.[15,16] The current work, however, employs *several* orthogonal, but complementary techniques for the identification of fentanyl CAS. Electron impact (EI) and chemical ionization (CI) GC-MS and HPLC combined with time-of-flight mass spectrometry (MS/MS-TOF) were used to profile the organic content in the crude fentanyl reaction mixtures. In addition, inorganic content was profiled by inductively coupled plasma mass spectrometry (ICP-MS), which has, to the best of our knowledge, never been employed in previous fentanyl CAS studies.

The three analytical techniques provided 160 unique synthesis-related signatures. The complexity of the resultant data, however, demanded the use of statistical techniques to extract relevant CAS. Therefore, a multivariate statistical model is presented that highlights the main sources of variance among the six synthetic routes. These statistical results directly relate to route-specific CAS and provide a model that can also be extended to the prediction of synthesis method for samples extracted from common laboratory surfaces, namely stainless steel ducting and vinyl tile flooring. Forensic samples are typically collected from a wide variety of surfaces, so the applicability of such statistical models towards data from traditional sampling strategies is therefore highly important, particularly when linking a compound to a particular laboratory is critical. Ultimately, we demonstrate the power of statistical methods for CAS analysis

using multiple orthogonal analytical techniques, and we provide what we believe to be the most detailed investigation to date for organic and inorganic signatures of fentanyl manufacture.

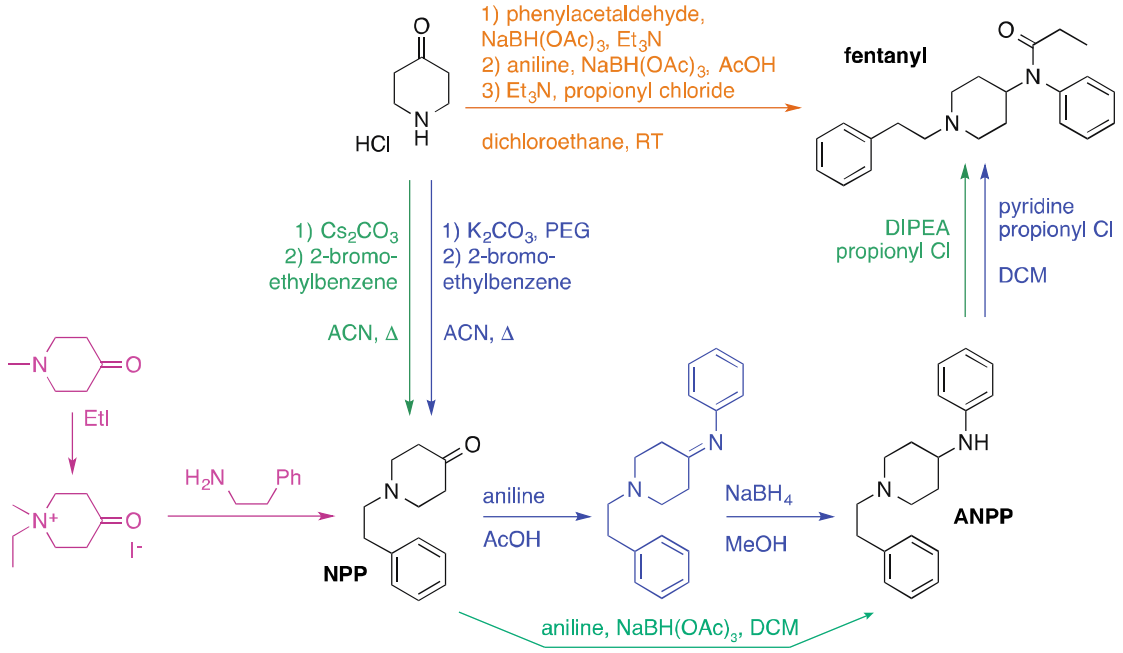
Experimental

Synthetic Approach

The synthesis of fentanyl-like compounds dates back to the 1960s in work by Janssen.[17] The procedure patented in 1965 involved techniques believed to be too complicated or expensive for clandestine laboratories (e.g. hydrogenations with precious metal catalysts). As discussed above it is likely that the illicit manufacture of fentanyl would rely on NPP, and so the six routes chosen presently use this intermediate. In-house synthesis of NPP was preferred, however, as use of commercially available NNP is unlikely due to its controlled status (Drug Enforcement Administration (DEA) Schedule I compound).

The six chosen methods offer variations on the attachment of the different fentanyl functional groups on the piperidine ring. 1-phenethyl-*N*-phenylpiperidine-4-amine (ANPP), the direct precursor to fentanyl, was formed either directly from NPP via reductive amination (Methods 1, 3, and 5) or by a two-step condensation-reduction process (Methods 2, 4, and 6). ANPP was transformed into fentanyl using propionyl chloride (an unscheduled compound) using either pyridine or *N,N*-diisopropylethylamine (DIPEA). With few modifications of the published routes, all synthetic methods successfully yielded fentanyl with a range of purities. Due to the nature of the routes, complete synthetic details are not given in the present work, though they may be found in

the literature cited. We have, however, provided some information and observations of the six routes studies in Section 1 of the Supporting Information. In all, three replicate syntheses were performed for each of the six methods yielding a total of 18 crude reaction samples. Scheme 1 gives the overall synthetic strategy for this work.



Scheme 1. Fentanyl synthesis summary. Black compounds are those common to a majority of the synthetic routes. Specific compounds in Orange: One Pot[12], Method 1; Blue: Siegfried[14], Methods 2, 4, 6; Green: Valdez[13], Method 3, 5; Purple: *N*-methyl-4-piperidinone route[18,19], Method 6. Methods 4, 5, and 6 are hybrid routes exploiting certain parts of the Valdez and Siegfried syntheses. ACN = acetonitrile, AcOH = acetic acid, Et_3N = triethylamine, MeOH = methanol.

Method 1 was a so-called "One Pot" method taken from the open literature[12] and was slightly modified for the current research. Method 2 was taken from a drug enthusiast website and is generally referred to as the "Siegfried" method due to the webpage's authorship.[14] Method 3 was taken from the open literature and was reported to

be an efficient, high yielding synthesis route.[13] It is referred to as the "Valdez" method. Method 4 uses the Valdez method of generating NPP and continues on to fentanyl using Siegfried's synthesis (named "Valdez→Siegfried") . Method 5 is the complement to Method 4, using Siegfried NPP to generate fentanyl via the Valdez route ("Siegfried→Valdez"). Method 6 is the only route that uses *N*-methylpiperidone to generate NPP ("Alt NPP→Siegfried").[18,19] The reaction then uses the Siegfried synthesis to form fentanyl.

Materials

Unless otherwise stated, all reagents and solvents were obtained from commercial suppliers (Sigma-Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), J.T. Baker (Avantor Performance Materials, Center Valley, PA), Fisher Chemical (Fairlawn, NJ)) and used as received.

Surface Sample Preparation

Coupons for the surface study were made out of either 0.01" thick 304 stainless steel or small pieces of 1/8" thick vinyl tile (Standard Excelon vinyl composition tiles, Pattern 51858, Imperial Texture, sandrift white, 1/8 inch thick, Armstrong Commercial Flooring, Lancaster, PA). The coupons were tared on an analytical balance, and a small droplet of each crude fentanyl mixture was deposited via a clean metal wire. The mass of the droplet was measured to within a tenth of a milligram. The spiked coupons were then allowed to sit for two hours.

After exposure was complete, the spiked coupon was then placed in a clean 20 ml glass vial and 10 ml of solvent was added. The solvents chosen were acetonitrile, dichloromethane, and 3 M nitric acid for LC-, GC-, and ICP-MS, respectively. The vials for GC and LC analysis were then shaken using a mechanical mixer for 15 minutes at 600 rpm, while the samples for ICP-MS analysis were instead sonicated due to safety reasons (i.e. to prevent gas build up). The extracts were then transferred to clean glass vials. Vinyl tile extractions were filtered through 0.45 μm filter cartridges and directly analyzed. Stainless steel extracts showed no evidence of solid debris and were used as is.

Instrumentation

GC-MS

A quantitative weight of each crude fentanyl product was transferred to a 2 mL glass vial and diluted in 1 mL dichloromethane. Dilutions were then performed in dichloromethane to yield a series of 20 μg/mL solutions. An Agilent Technologies (Santa Clara, CA) 7890A GC equipped with an Agilent HP-5MS column (5%-Phenyl-methylpolysiloxane, 30 m x 0.25 mm x 0.25 μm) was used for the chromatographic separation. A carrier gas of helium (99.999%, Praxair, Inc., Danbury CT) was used, and the GC was operated in constant flow mode (2.0 mL/min). One microliter of the liquid sample was introduced via an autosampler (Agilent 7890 series) to the injection port held at 230°C, splitless injection. The oven temperature was held at 40°C for 3 minutes, then ramped at 10°C/min to 300°C and held for 5 minutes. Detection was performed with an Agilent 5975C MS detector and operated in EI (70 eV) and CI (positive, NH_3) modes. For both ionization modes, the system was operated in scan mode (m/z 29-600, 2.57

scans/s) with the source and quadrupole mass analyzer held at 230°C and 150°C, respectively. A solvent delay of 3 minutes was used. The detector was auto-tuned using the standard tune capability of the ChemStation software, and the tune was confirmed before each set of experiments. Compounds were identified based on spectral comparison to the National Institute of Standards and Technology (NIST) Mass Spectral Library (NIST 08 MS Search 2.0, NIST, Gaithersburg, MD) as well as manual comparison to mass spectra published in the literature. Peak areas were calculated from extracted ion chromatograms of the base peak identified for each compound.

LC-MS/MS

A quantitative weight of each crude fentanyl product was transferred to a 4 mL glass vial and diluted in 1 mL 50:50 acetonitrile:water. From each solution, dilutions were performed in 50:50 acetonitrile:water to yield a series of 20 μg/mL solutions. An Agilent 1260 LC equipped with an Atlantis T3 reverse phase column (C18, 150 mm x 2.1 mm, 3 μm particle size, Waters, Milford, MA) was used. Time-of-flight mass spectrometric detection was performed in positive ion mode with a Bruker micrOTOF-Q III (Bruker Daltonics, Billerica, MA) equipped with an electrospray ionization (ESI) source and operated in Auto MS/MS mode (m/z 50-1000). Three precursor ions were monitored at a given time (m/z 50-450) with active exclusion after three spectra. MS was performed with a capillary voltage of 4500 V, a dry gas flow rate of 8 L/min at 200°C, quadrupolar ion and collision energies of 4.0 eV and 8.0 eV, respectively, and a spectral acquisition rate of 2 Hz.

The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient profile started with 95% A for 2 min, ramped to 5% A at 18 minutes, held for 13.5 minutes, ramped quickly back to 95% over 0.5 min, and held for 10 min for column regeneration. This method was used for all samples except those in the vinyl tile surface study, for which the final 5% A was held for 18.5 min to allow background contaminants to be completely removed from the column. Ten microliters of the liquid sample were introduced via an autosampler (Agilent B1329B) to the injection port. The detector was tuned and calibrated using the 20 μL injection loop of a 6-port valve on the MS using Agilent's ESI-L Low Concentration Tuning Mix (G1969-85000). Compounds relevant to each synthetic route were identified based on computer-aided identification of MS/MS peaks using Bruker's Compass for otofSeries 1.5 software. Detailed analysis of each sample was done manually with peak areas calculated by manually integrating the extracted ion chromatogram of the base peak. After route-specific compounds were identified for each route, a target table was created and searched against all 18 samples.

ICP-MS

Elemental analysis was performed using an Agilent Technologies (Santa Clara, CA) 8800 Triple Quadrupolar ICP-MS (ICP-QQQ). An initial semi-quantitative scan was performed to determine elements of interest within the sample sets. Down-selected analytes were then measured quantitatively with the following parameters: carrier gas (0.65 L/min), nebulizer pump (0.50 rps), spray chamber temperature (15°C), and dilution gas (0.40 L/min). Argon was used as plasma, carrier, and dilution gas. In the collision

cell, a helium flow was used as follows: 0.0 mL/min in No Gas tuning mode and 4.0 mL/min in He mode. The measurements were performed as three replicates, with 50 sweeps per replicate. Integration time per mass was held at 0.10 sec. The rinse time was set to 30 sec at 0.3 rps of the nebulizer pump, followed by 10 sec at 0.3 rps. Sample introduction was performed using an ASX-500 autosampler (Cetac, Omaha, NE).

Chemometric Data Analysis

Data analysis was performed using Solo (V8.0, Eigenvector Research Inc., Wenatchee, WA). Partial Least Squares Discriminant Analysis (PLS-DA), a supervised technique that facilitates classification of unknown samples against a known calibration data set was then performed on the entire data set. All processed data sets (LC, GC, and ICP-MS) were mean-centered by sample and range scaled by compound for each analytical method *separately* (technique by technique). Range scaling was performed by normalizing the data for each compound by the difference between its maximum and minimum mean-centered responses. This process ensures that 1) all compounds are given equal weight and 2) responses from each analytical technique are given equal importance. This preprocessing procedure has been employed previously for such fused datasets to remove response factors and to express response as "concentrations" independent of analytical technique and experimental conditions.[20]

Results and Discussion

PLS-DA Discrimination of Fentanyl CAS from Crude Reaction Mixtures

From the analytical data, 126 unique compounds were identified by LC-MS/MS, and GC-MS identified 29 compounds. Sample LC- and GC-MS chromatograms from each route are given in Section 2 of the Supporting Information to demonstrate chromatographic ability of the methods chosen. Unabridged lists of LC- and GC-MS compounds are given in Section 4 of the Supporting Information. LC and GC compounds were given a numerical or alphabetical designation, respectively, in order of retention time. LC- and GC-MS detected the same compound if it is labeled by both a number and a letter. Doubly charged compounds detected by LC-MS are designated with a "D" followed by its retention time. Initial semi-quantitative ICP-MS runs were performed and five elements were subsequently quantified for statistical analysis based on relevance to the synthetic methods and signal over background: ^{23}Na, ^{39}K, ^{127}I, ^{133}Cs, and ^{137}Ba. In total, 160 species were identified from analytical data.

Statistical techniques are available that can make analysis of large, complex data sets objective and procedurally much simpler. These methods seek to describe the observed experimental data with a reduced set of new "latent" variables whose goal is a more efficient description of the underlying sources of sample variance. For this study partial least-squares discriminant analysis (PLS-DA) was chosen as the multivariate statistical methodology. In PLS-DA a compromise is struck between describing the set of explanatory variables and predicting the response variables. General descriptive discriminant analysis (DA) techniques allow one to identify variables that best discriminate among various classes. Predictive DA techniques, however, extend the concept of class assignment to classification of "unknown" data.[21] The current work exploits such a statistical model to ultimately predict synthesis routes of "unknown"

samples taken from operational surfaces. First, we address the use of PLS-DA to identify important compounds that are highly route-specific and to highlight routes that produce similar CAS profiles.

The selectivity ratio (SR) is a ratio of explained to residual variance of a given variable (i.e. chromatogram peak area) and is useful in the objective identification of important CAS.[22] High SR values indicate the spectral variable (i.e. compound) contributes much towards discrimination of samples (i.e. reaction mixtures or routes). Selecting an appropriate SR threshold results in a reduced data set containing the most important compounds, i.e., a purely statistically determined list of CAS.

Choosing a SR threshold for CAS determination reflects a balance between identifying real markers and excluding those that do not bear significance on describing sample variance. Though various strategies such as the *F*-test or so-called nonparametric DIVA plots[23] exist to more objectively determine SR thresholds, our initial goal was simply to generate a minimum of 2 signatures for each route, and a global value $SR_{min} = 1$ was shown to be sufficient. Table 1 gives compounds that were identified as CAS for which their SR values were greater than unity. Table S-1 of Section 3 of the Supporting Information connects the identified compounds to their retention times, formulae, and tentative names when possible.

Table 1. *Route specific CAS identified through PLS-DA using a selectivity ratio, $SR_{min} = 1$. Letters and numbers refer to GC- and LC-MS detected compounds, respectively. Compounds represented as both a letter and a number were detected by both techniques. CAS with a "D" followed by a number are doubly charged compounds detected by LC-MS. See the Tables S-1 through S-3 in the Supporting Information for more details, particularly assignments based on LC-MS/MS data.*

Method (Class)	LC-MS	GC-MS	ICP-MS
1, One Pot	2, 3, 4, 6, 7-S, 8, 9, 15-S, 20, 23, 24-I, 26-AB, 31, 32-AA, 33-AC, 36, 48, 52, 54, 59, 64, 81, 87, 89, 95, 98, 99, 102, 103, 104, 105, D2.7, D2.8, D2.8-2	A, G, 24-I, 7-S, U, V, Z, 32-AA, 26-AB	--
2, Siegfried	19, 27, 28, 43, 50, 53, 58, 77, 82, 91, 94, 97, D13.7, D13.8, D14.1, D14.4, D14.8, D14.8-3, D15, D15.4, D15.7, D17.3, D18	--	--
3, Valdez	73	67-E	--
4, Valdez→Siegfried	11, 35, 60, 70, 84, 100	B, H, Y	--
5, Siegfried→Valdez	39-K, 71, D15.3, D15.3-2	J, 39-K	--
6, Alt NPP→Siegfried	45, 51, 83, 107	--	--

The vast majority of the 87 PLS-DA derived CAS (SR_{min} = 1) belongs to Methods 1 and 2. Many of the SR values for these compounds are high, indicating large discriminatory ability. Considering the top 25% CAS in terms of SR value, almost all belong to Method 1. Only four of these variables were classified to other routes, namely Methods 2 and 6. The large SR values of these variables imply the model should discriminate among these three synthesis methods. Conversely, the CAS identified for Methods 3, 4, and 5 are low in number and/or have low SR values. These facts indicate the chosen threshold may be too high to confidently classify samples from these methods.

A Chemical Perspective of Fentanyl CAS

Though extremely powerful, statistical methods of data analysis are often of a "black box" nature and require a contextualization from a chemical perspective if a true understanding of the underlying data is desired. To that end we now turn to a brief discussion of some of the statistically determined CAS given in Table 1 and their

relevance to specific synthetic routes. We emphasize here that if structures/chemical names are given, they have been *tentatively* assigned through MS/MS data in the case of LC data or through GC library comparisons. They have not, however, been verified by authentic reference standards. The synthetic endeavor that would require is outside of the scope of the current work, especially considering the statistical focus. Work is on-going to unambiguously assign structures to compounds considered highly important to statistical discrimination. For compounds that were part of the synthesis (reaction components, isolatable intermediates, etc.) their identity was easily confirmed through comparing MS/MS spectra and retention times.

Fentanyl synthesized via Method 1 contained many early eluting compounds in the LC-MS analyses. Many of these were alcohols that did not apparently undergo further reaction. Under the LC analytical conditions used, most of these compounds are not well retained on the column, and therefore elute at or near the system dead volume. This observation was shown to result from the mismatch in initial mobile phase and sample solvent compositions. This poor separation was deemed acceptable, however, as the high organic component of the solvent system is required to preserve sample stability and to ensure complete sample dissolution.

Due to the high resolution and exact mass capabilities of the LC-MS/MS-TOF, all compounds could be deconvolved from the data. One alcohol in particular, 1-phenethylpiperidin-4-ol, was detectable in relatively large amounts by both LC- (89% relative to fentanyl) and GC-MS (~200-300% relative to fentanyl). Method 1 also produced a large amount of acetylfentanyl (roughly 3.6x that of the desired fentanyl product by LC-MS). In fact, greater than 25% of the total LC-MS base peak

chromatogram area was due to this byproduct. This finding agrees reasonably well with the GC data, which showed roughly a 4.5-fold increase over fentanyl. Also present in large quantities were unreacted ANPP and aniline – two compounds found in extremely low amounts, if at all, in the other routes.

Additional unique attribution signatures of the one pot method are large amounts of other acetate- or acetamide-based compounds. For example, 1-phenethylpiperidin-4-yl acetate, *N*-phenylacetamide and *N*-phenethyl-*N*-phenylacetamide were deemed CAS by PLS-DA. Also unique to this reaction method was a series of bipiperidine compounds presumably formed through reductive amination of the phenethylpiperidinone and unreacted piperidine hydrochloride. Again, it is important to keep in mind, as with most assignments, these tentative identities are based merely on MS/MS data, GC-MS library matches, and most probable chemical reaction pathways. They have not been confirmed by authentic reference standards or in-house syntheses.

Method 2 only had two unique CAS based on LC-MS/MS and GC-MS data: *N*-phenethyl-*N*-phenylpropionamide and *N*-phenethyl-*N*-propionylpropionamide, both derivatives of 2-bromoethylbenzene. Also, the Siegfried method is the only one to use a potassium-containing base (K_2CO_3) in the formation of NPP. Only one potassium adduct, $C_{10}H_{23}KN_3$, was deemed a CAS, though a structure was not proposed. ICP-MS data was expected to reflect potassium use, as well, but no statistical importance was observed from the PLS-DA analysis. Lastly, fentanyl synthesized via Method 2 yielded a significant number of LC CAS that are doubly charged. Due to inconclusive MS/MS fragmentation patterns we were not able to posit structures. Their presence, however, was found to be indicative of the Siegfried method.

Only two compounds were classified as unique CAS for the Valdez synthesis method, and only one structure was determined. A library match of GC-MS data identified this compound as *N*,*N*-diisopropylpropionamide. This compound most likely forms as a result of reaction between propionyl chloride and DIPEA impurities. Exclusive to the Siegfried route is a biphasic reaction mixture where water-soluble compounds are removed into an aqueous phase via polyethylene glycol. The Valdez method lacks such a phase transfer catalyst, so one may expect additional signatures to appear, particularly charged quaternary amines. In fact, two such CAS were revealed by LC-MS, namely 1,1-diphenethyl-4-(*N*-phenylpropionamido)piperidin-1-ium and 1-phenethylpyridin-1-ium. These compounds were classified as CAS for Method 4, Valdez→Siegfried.

PLS-DA identified two propionamides as specific to Method 5. Based on MS/MS fragmentation patterns, these compounds were tentatively identified as *N*-phenylpropionamide and *N*-ethyl-*N*-phenylpropionamide. A GC mass spectral library match confirmed the assignment of *N*-phenylpropionamide, but the assignment of the latter compound was not confirmed. Finally, several CAS were associated with Method 6 but none was assigned a specific structure. This method's alternative route to making NPP should result in a variety of byproducts, but few expected compounds were detected by any means. Iodine was expected to be a relevant CAS for this route as well, considering its exclusive presence in this route. The PLS-DA analysis, however, did not consider it important. This may result from iodine being dominated by compounds with higher selectivity ratios. In all, quantitative ICP-MS data affected data analysis very little. ^{139}I seemed to have some influence on discrimination of Method 6, but its SR value was

relatively low (SR = 0.47). Overall, it was observed that ICP-MS data was not useful in the current study. That fact does not discount it as useful for other synthetic methods or forensic studies, however.

PLS-DA Model Validation and Application to Surface Samples

From the complete data set of 160 variables, five components were chosen for the PLS-DA calibration model, covering an explained variance of 95.0% of the analytical data. The calibration data from the 18 samples derived from crude reaction mixtures are displayed as a scores plot of the two dominant components given in Figure 1. The graph reveals Method 1 can be separated well with the first component. The second component, in turn, can separate Method 2 well; but there exists poor separation between Methods 3 and 5 and Methods 4 and 6. Plotting these data in conjunction with the third component's scores (Figure 2), though, allows for reasonable separation for all but Methods 3 and 5.

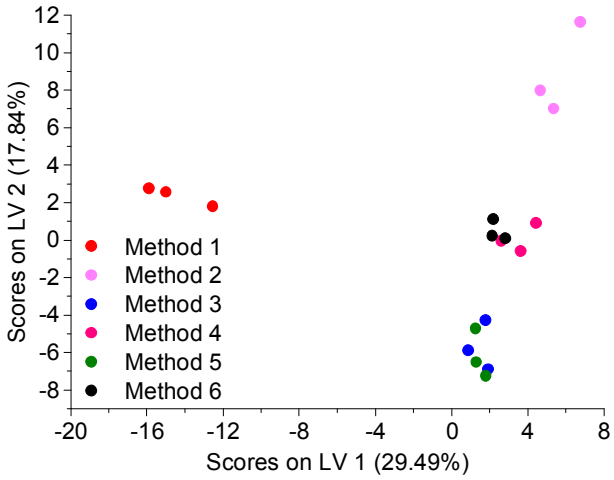


Figure 1. Scores plot for the first and second components for the crude fentanyl mixture calibration data.

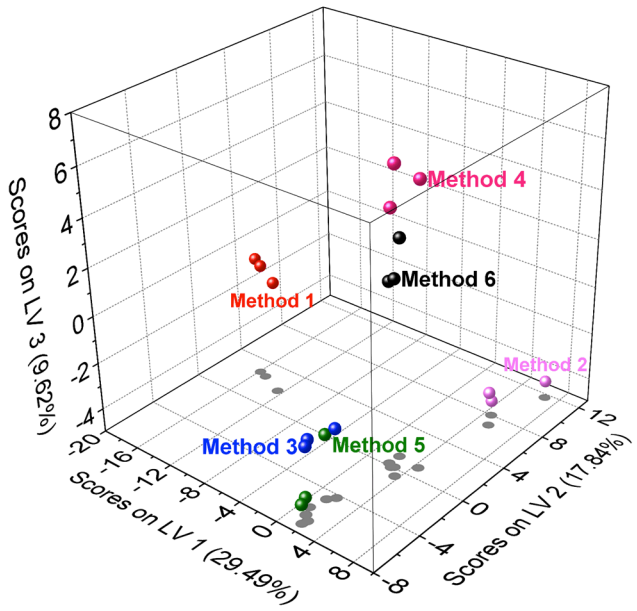


Figure 2. Three-dimensional scores plot for components 1, 2, and 4 showing good separation of all classes except for data from synthesis Methods 3 and 5. Color-coding was done according to class membership. The X-Y projection is equivalent to those data plotted in Figure 2.

The observed statistical results can be easily rationalized in terms of the reaction chemistries. Method 1 generated quite a number of CAS with large SR values and was therefore easily separated by the first component. The same discriminatory ability was observed for Method 2 by a second component. Methods 4 and 6, which share the Siegfried method of ANPP→fentanyl synthesis, could not be distinguished by two components alone, and a third component was required for acceptable discrimination. Particularly problematic was the separation by scores values of Methods 3 and 5, routes that both share the Valdez method of ANPP→fentanyl synthesis. Since these methods share various precursors and byproducts in relatively low amounts, common signatures result in few CAS, which is reflected in similar component scores. Note the close clustering of replicates for a given route. This is due to the consistency in reaction

profiles between replicates for both the GC- and LC-MS data sets. Section 5 of the Supporting Information gives sample raw peak areas for the One Pot route to demonstrate the excellent reproducibility between syntheses.

Though scores plots are convenient ways to conceptualize the statistical results, PLS-DA also provides a quantitative analysis of the data and can classify unknown samples based on their similarity to calibration data sets. PLS-DA model generation begins with cross-validation, which provides objective quantitation of the model's ability to generalize to independent data sets (i.e. those resulting from the follow-up surface study).

The current model was shown to perform very well in the discrimination of samples into their corresponding classes during cross-validation. In other words, the likelihood that a given validation set corresponds to its known specific synthesis route is extremely high. In fact, for almost all cases there was no significant probability of misclassification. Only a single sample from Method 3 had any significant non-zero probability of belonging to another method (Sample M3-3 as Method 5, $p = 0.094$). This observation is understandable, though, as Method 5 (Siegfried NPP/Valdez ANPP→fentanyl) is a hybrid method of Methods 2 and 3 (Siegfried and Valdez, respectively) and therefore shares a variety of common signatures with those routes. Nevertheless, correct classification for all 18 samples was observed since the probability of correct classification outweighs any underlying similarities to other methods. A heat map of classification data is shown in Figure S-3 in Section 6 of the Supporting Information.

It is important to recognize that the ability to discriminate amongst different syntheses is in part reflective of the different nature of the routes themselves. Minor changes in synthesis, whether in the form of material source, reaction conditions, or synthetic chemist (novice or professional), may have a significant impact on the end discriminatory ability. Unfortunately, the matrix of such conditions that can be tweaked and/or investigated is infinite and can therefore not be fully addressed, particularly with regards to the scope of the current work. We reemphasize the fact that the routes chosen were intended to reflect clandestine synthesis routes, but that sample-to-sample variability (though shown to be small) was inherently incorporated via completely independent syntheses from start to finish. It is part of on-going work to assess the influence of the abovementioned factors towards ultimate route discrimination. For not, we feel it is sufficient to use the current reaction matrix to demonstrate the proof-of-concept nature of the statistical CAS methodology.

With a validated PLS-DA calibration model, we can now attempt classification of samples that have been exposed to stainless steel and vinyl tile. These materials aim to mimic protocols commonly found in forensic investigations, namely surface-based sampling from laboratories, warehouses, containers, etc. It is important to demonstrate that any additional signals associated with surface swipes or extractions especially for porous and/or organic matrices do not interfere with those from compounds of interest. Determination of the presence of these signatures on such surfaces is critical for linking a batch of seized fentanyl to its synthesis location, even if elucidating the synthetic pathway from surface data is not required or possible.

Figure 3 shows scores results of the PLS-DA analysis for the "unknown" stainless steel surface data and compares those to results from the calibration set. It is evident that despite having been exposed to the metal surface, the relevant signatures are persistent enough that correct classification is likely possible. In other words, surface sample and calibration scores values cluster qualitatively well. As additional evidence of CAS persistence over a given period of exposure, Section 7 of the Supporting Information gives LC-MS chromatographic traces of the time dependence of the CAS profile over a 24 hour period. CAS intensity invariance over this period suggests that this model should be useful even if sampling is delayed.

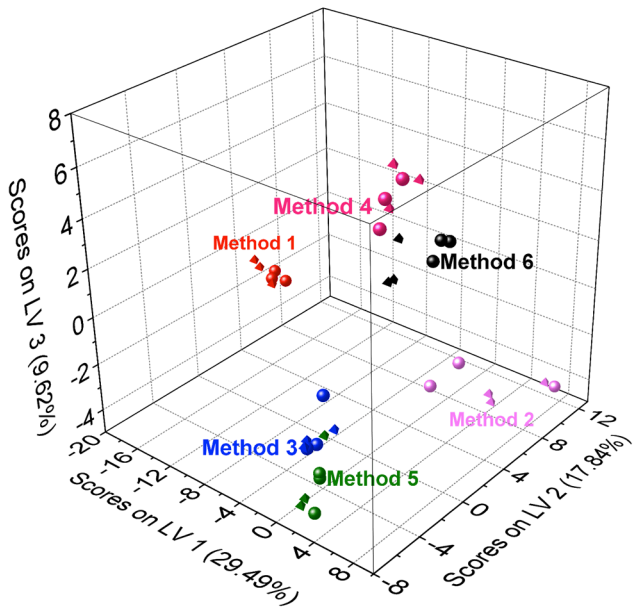


Figure 3. PLS-DA scores from stainless steel data color-coded by the predicted "most probable" class. Spheres are the surface data sets, whereas pyramids represent data from the calibration sets taken from Figure 2. Vinyl tile data given in Figure S-6 of the Supporting Information.

Figure 4a presents quantitative results from predicted class membership through a heat map for the stainless steel samples. Ideally there would only be on-diagonal intensity indicating complete confidence in correct class assignment. Indeed, for samples within a class (i.e. synthesis route), classification probability is essentially unity. The model does, however, predict that several samples bare similarities to others outside of its class (i.e. synthesis route). This fact is reflected by off-diagonal intensity. That these methods are all interrelated (i.e. permutations of the Valdez and Siegfried routes) may account for the multiple classifications. However, because the probability of making the correct assignment is generally higher than that of an incorrect classification, the PLS-DA analysis assigns the "most-probable" class correctly for every sample except for one. Only one Method 3 sample, M3-SS2, was classified as belonging to Method 5 but the probability was relatively low ($p = 0.680$). Despite surface matrix effects, incubation time and conditions, potential volatilization of compounds, etc., the statistical analysis still is able to assign the large majority of the stainless steel surface samples to their corresponding known synthetic origins.

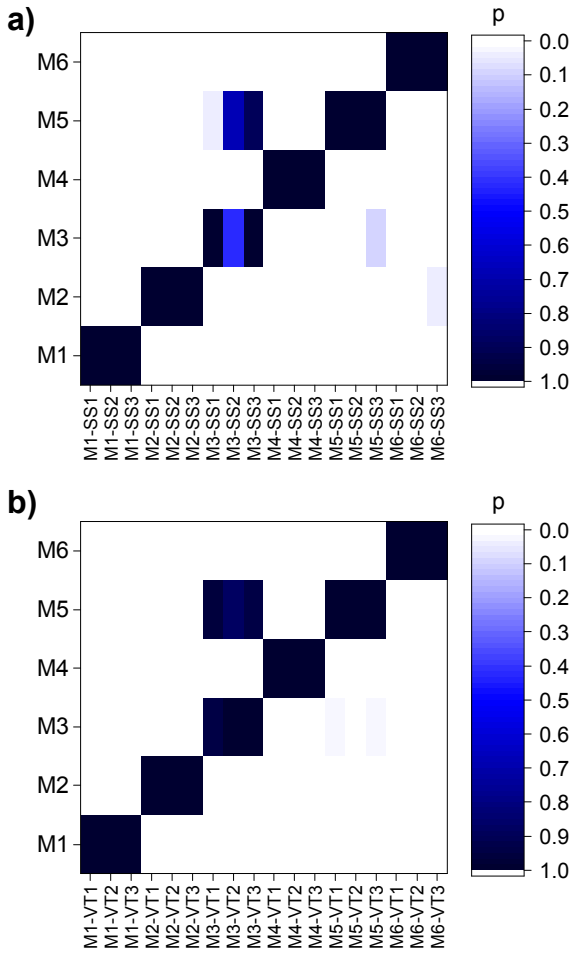


Figure 4. PLS-DA probabilities of class membership for Method 1 (M1, bottom) to Method 6 (M6, top) for stainless steel (a) and vinyl tile (b) samples. On-diagonal intensity reflects probability of correct classification. Off-diagonal intensities indicate that there is non-zero likelihood of that sample to be misclassified.

PLS-DA demonstrated that the CAS profile necessary for accurate classification persisted relatively well on stainless steel, a relatively inert surface. In contrast, we also investigated the ability of vinyl tile, a complex polymeric matrix, to retain the signatures needed for the statistical analysis. Like the stainless steel data, striking visual similarities between the test and calibration data sets can be seen in the scores plot given in Figure S-3 in Section 8 of the Supporting Information. The remarkable similarity between calibration and test scores may be surprising considering the large number of new

compounds introduced through exposure to the organic surface. New signatures were automatically ignored, however, since the variable set was pre-determined by the calibration data. Ultimately, signatures relevant to fentanyl synthesis appear persistent against even complex matrices like vinyl tile.

Examination of the class prediction plot given in Figure 4b shows that, again, the model has a tendency to confuse samples from Methods 3 and 5. Probabilities of misclassification of samples from Method 3 as Method 5 exceed 0.85 for all three samples. Again, the similar, trace CAS profiles of these routes explain the tendency for misclassification. Correct classification generally dominates any chance of the model's incorrectly assigning samples, however. Again, there was only a single erroneous PLS-DA assignment – Method 3 sample M3-VT1 was classified as a Method 5 sample.

PLS-DA of Individual Data Sets

As a final, though important, consideration, we investigated the degree to which individual techniques were able to discern differences amongst the six synthesis methods. PLS-DA analyses were performed GC, LC, and GC+LC data sets. The motivation behind this was to show the level of classification that could be performed in a laboratory with limited equipment and to highlight the benefits of using multiple sources of mass spectral data.

For the GC data alone, nine components were necessary to surpass the 90% cumulative variance threshold. This may indicate that differences among samples may not be sufficient to provide a robust predictive model. Analysis of LC data, however, needed only five components to exceed the variance threshold. The same is true for the

combined LC and GC data. Figure S-7 in Supporting Information shows scores plots from these PLS-DA analyses. Scores plots show that for the GC data, relatively poor separation was achieved by the first three, dominant components. LC data, however, was able to separate well many of the samples into their respective methods, though, again, Methods 3 and 5 and Methods 4 and 6 were difficult to resolve.

The overall ability of the data to statistically resolve the methods was gauged by the number of both incorrect and/or multiple (i.e. ambiguous) class assignments made by the model. The results from considering GC and LC scores data alone were mirrored during application of the predictive model, which often failed to unambiguously or correctly assign samples to the respective synthesis methods. In fact, of the 36 surface samples, six stainless steel and six vinyl tile samples were misclassified or multiply classified when using just GC data. LC data only provided a modest improvement - four stainless steel and six vinyl tiles samples were not classified properly. Combining the GC and LC data resulted in better discriminatory ability among the six routes. Three samples from each surface set were incorrectly assigned a route. The full, unabridged data set combing GC, LC, and ICP-MS results resulted in one stainless steel and three vinyl tile classification errors, which, again, includes both *wrong* and *multiple* assignments. These improvements in discriminatory ability highlight the power of using data from multiple orthogonal techniques for unambiguous classification of unknown samples.

Conclusions

A statistical analysis of chemical attribution signatures associated with the synthesis of fentanyl was presented. The results demonstrated that the combination of orthogonal techniques provided better discriminatory ability than that from individual analyses alone. Though we recognize that many more aspects of this method need to be addressed to assess its ultimately utility (synthetic variability, reaction conditions, CAS surface persistence, matrix effects, environmental conditions, etc.) this work provides a solid proof-of-concept work that aims to identify fentanyl CAS and to subsequently link them synthetic source. Through the application of the PLS-DA model, classification of "unknown" surface-exposed samples was possible even for synthetic strategies that are relatively similar or even share common synthetic steps. Also important to stress is that many important CAS are present at trace levels, further highlighting the power of statistical techniques for forensic attribution studies. Furthermore, purely objectively determined CAS were discussed in terms of their potential to be rationalized in terms of the specifics of the synthesis procedures. The authors feel it is critical to contextualize the "black box" nature often associated with statistical techniques with chemical knowledge to truly gain a deeper understanding of the origins of chemical attribution signatures. Again, we are working towards complete CAS identification and, also, this method's application towards other potential toxic materials. Ultimately, however, we believe this work further reinforces the beneficial synergy between multivariate statistical analysis and contextualizing results within a synthetic chemical framework while providing the most comprehensive attribution study of fentanyl to date.

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