Tackling the Opioids Overdose Epidemic: Methods of Detection of Fentanyl, its Analogues, and Metabolites

by

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With an average of 195 daily deaths due to synthetic opioids overdose in 2021,[1] the US have been facing an unprecedented opioids crisis.[2] Fentanyl and its analogues have been a major source of concern,[3,4] due to their high levels of addiction, fast-acting mechanisms, and detection challenges.[5-7] Fast, effective, and accurate identification and quantification of fentanyl, its analogues, and metabolites (hereinafter collectively abbreviated as FAMs) in blood and urine are essential[6,8] to help prevent overdose-related incidents and to enable agile medical response.[9-11] Nevertheless, further understanding of analytical techniques used for separation and detection of fentanyl and its analogues is crucial, as it would allow for the development of more sophisticated and portable devices.[12-14] Additionally, with the emergence of new analogues as fentanyl "designer" drugs (FDDs), the demand for novel detection methods is pressing. This thesis seeks to provide a comprehensive review of the US opioids crisis, with a focus on the different analytical techniques used for the separation and detection of FAMs. While traditional, well-established techniques, such as gas and liquid chromatography,[13,15-20] are extremely relevant to understand and explore, this review also seeks to bring attention to novel techniques that rely on electrochemical-based detection.[21-23] The recent emergence of electrochemical biosensors for drug detection applications[24,25] could help establish new paradigms in terms of public health policy response to the US opioids crisis, as such devices could have major impacts in curbing and preventing the rise of fentanyl and analogues-related overdoses.[26] This review explores electrochemical sensing as a viable detection method.[27] Relying on recent discoveries, it shows

how cyclic voltammetry,[23] differential pulse voltammetry,[28] chronoamperometry,[29] or field-effect transistors[21] could be used to detect fentanyl and its analogues. The challenges concerning sensitivity and selectivity are explored by understanding how carbon nanotubes (CNTs) could be used to selectively enhance electrochemical responses.[21,22] Although there are some challenges with collecting, using, and interpreting electrochemical data from biological samples,[29,30] recent advancements in the fields of statistical process control,[31,32] machine learning,[4,33,34] and predictive analytics[33-35] could help pave the way towards rapid large-scale development of reliable, accurate, and fast electrochemical sensors capable of identifying and determining concentrations of FAMs.

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Preface

This thesis represents the materialization of my personal interests at the intersection of electrochemistry, analytical chemistry, contemporary social issues, and statistics. At the forefront of challenges facing the world, the opioids crisis is particularly troubling due to the public health impacts within communities in the United States and abroad. Novel approaches to handle this crisis and its consequences shall combine a holistic, interdisciplinary approach, with an emphasis on the development of new electrochemical sensing technologies.

I became particularly interested in electroanalytical chemistry after being introduced to Prof. Alexander Star's research. I recall reading, for the first time, his paper on a tetrahydrocannabinol (THC) breathalyzer and feeling curious about how sensing occurs, what factors influence signal responses, and whether it would be possible to sense nature's many substances through electrochemical sensors. This interest was furthered by Prof. Shigeru Amemiya's course on electroanalytical chemistry. The countless evenings spent on assignments and the insightful after-class discussions with Prof. Amemiya triggered my interest in the mathematical and theoretical aspects of electrochemistry.

To consolidate my work as a graduate student, I wrote this thesis with a focus on methods of detection of fentanyl, its analogues, and metabolites (FAMs, see Appendix A), as I believe understanding those can help pave the way towards tackling the opioids crisis. To do so, I engaged with up-to-date research publications that help reflect on the current state of electrochemical sensing in the context of FAMs. Evaluating different sources and understanding the different methods of detection were challenging aspects of this work, along with comparing and contrasting sensing performances across different studies. Nonetheless, doing so has helped me to better understand how electrochemical sensors represent a promising, often overlooked opportunity for tackling the opioids crisis.

As you engage with this work, I encourage you to consider and appreciate both the scientific and technical aspects in addition to the broader societal implications. I hope this thesis encourages further research and innovation, shifting paradigms towards more effective and reliable ways to detect opioids and other substances in general, with a focus on public health and societal well-being.

Acknowledgements

First and foremost, I would like to thank my mother, Helianildes, for encouraging me to have the courage and perseverance to finish what I started, despite many challenges. I would also like to thank my father Sérgio, sister Andréa, niece Alice, grandmother Damiana, cousins, aunts, and uncles, most of which are in Brazil, feeling very proud of me. We are a family of hard-working first-generation doctors, healthcare workers, lawyers, engineers, and scientists who are proud to follow the footsteps of my grandfather Manoel da Silva (*In Memoriam*), who worked since he was 8 years old and passed away at 88 in the beginning of this year (2024). I wholeheartedly dedicate this thesis to him.

This work would not have been possible without the support of many mentors and colleagues in the Department of Chemistry and in the Star Research Group. First, I would like to thank Dr. Alexander Star for his patience, support, and encouragement towards both personal and professional goals. I couldn't have asked for a better advisor. Moreover, I would like to thank Mrs. Christie Hay for being a supportive administrator, Prof. Shigeru Amemiya for his exciting electrochemistry course and exchange of ideas, and Prof. Haitao Liu for his thought-provoking questions earlier in the program. I would also like to thank colleagues in the research group, including Faezeh Askari, Dr. Ganesh Kesavan, Jieyu Wang, Dr. Long Bian, Raihan Ahamed, Samia Afrin, Dr. Wenting Shao, Dr. Xiaoyun He, Zhengru Liu, and Zidao Zeng. I had amazing learning experiences with all of them, and I am very thankful for all the positive interactions we had in the lab.

I would also like to thank everyone who has been part of my personal, academic, and professional development. This would include very special people I have met all over Pennsylvania in the past few years as well as members of the Military School of Salvador (CMS), Primeira Chance Foundation, the Eastern Mediterranean International School (EMIS), New York University Abu Dhabi (NYUAD), University of Pittsburgh, and two private companies I have worked for. I am thankful to all of you.

1.0 An Overview of the US Opioids Crisis

Drugs have been part of human history, being used for both medical and recreational purposes across centuries.[36] Recently, drug abuse and overdose cases have been sources of major concern in the United States and worldwide,[1,11,19,36-39] due to increasing numbers of hospitalizations and deaths.[40] Additional concerns over the potential uses of drugs as chemical warfare agents[41-43] also contribute to the need for better detection methods of such substances. From an analytical chemistry perspective, drug detection has become even more relevant, with the emergence of new drugs[44-46] with distinct effects on the human body, depending on their classifications, sources, and acting mechanisms.[46]

Although cannabinoids are often reported as the most consumed drug worldwide, (see Figure 16, Appendix B),[45,47] their recreational or medical uses do not often lead to overdoses.[48] On the other hand, from the beginning of the 21st century, opioids have been a major public health concern,[1,3] especially in the United States, due to overwhelming numbers of intoxicated patients and overdose-related deaths.[1] With an average of 195 daily deaths due to opioid overdose in 2021,[1] the first wave of the US opioids crisis started in the end of the 20th century, with the rise of overdose-related deaths due to commonly prescribed opioids (*e.g.*, natural and semi-synthetic opioids).[46] The second wave started in around 2010, with a sharp rise in heroin consumption all over the country, leading to many overdose cases.[49,50] Finally, the third wave started around 2013, with the rise in the consumption of synthetic opioids, mainly fentanyl (see Figure 1).[51,52]

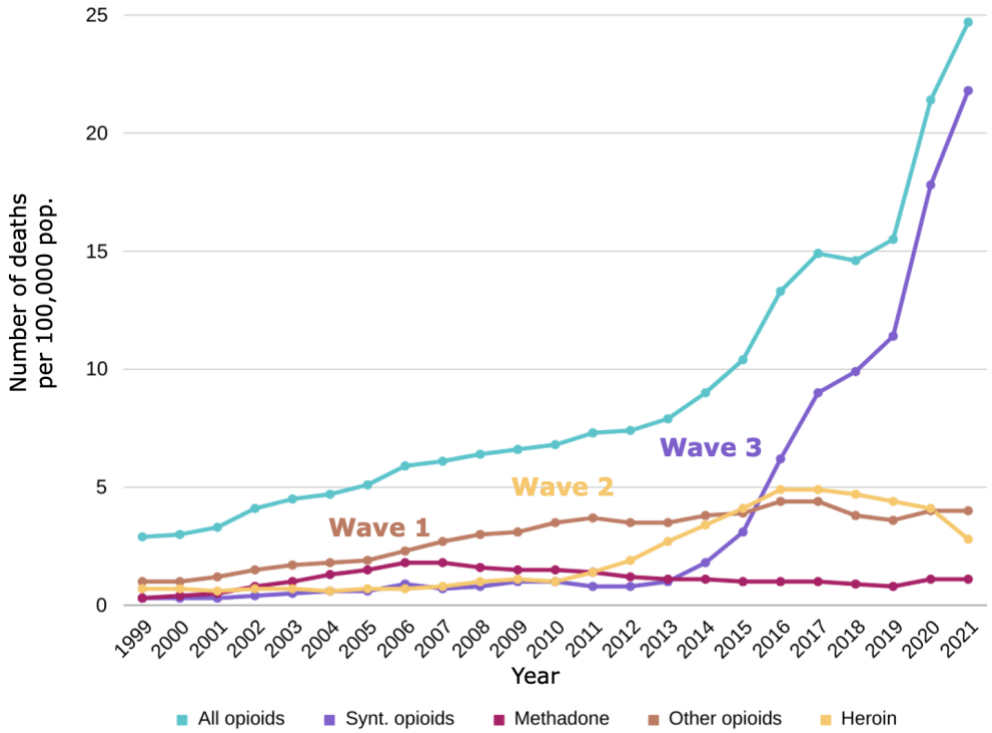

Figure 1. Death rates due to opioids overdose in the US increased substantially between 1999-2021. All opioids include synthetic opioids (mainly fentanyl), methadone, and other opioids (*i.e.*, natural and semisynthetic opioids). Data retrieved from CDC WONDER's database (available to the public).[1,39,53]

Although 2022 and 2023's datasets are still being aggregated and processed, recently released provisional death counts due to fentanyl overdoses are consistent with the increasing trends.[37,50] Thus, tackling the opioids overdose epidemic is an urgent public need, and that can only be fulfilled by enhancing detection methods of fentanyl and its analogues.

2.0 Fentanyl, its Analogues, and Metabolites (FAMs)

Herein, the terms above are briefly explained, followed by an in-depth exploration of such concepts. Emphasis is put on the chemical and structural properties of fentanyl and its analogues as well as their effects in the human body, which gives rise to the metabolites. Then, the importance of detecting such substances across different fields is explored.

Fentanyl is a powerful synthetic opioid that has caused elevated mortality rates due to overdoses.[1,52] Fentanyl presents the most pressing concern to the public health community, especially due to its widespread medical (*e.g.,* for severe pain management) and recreational uses.[3,54,55] Additionally, the drug is considered 20-50 and 50-100 times more potent than heroin and morphine, respectively.[56,57] Despite several concerns, fentanyl overdoses have skyrocketed in the past few years, especially during the peak of the Covid-19 pandemic and its aftermath.[38] Although fentanyl is a prescription drug, recent cases have shown that illegally manufactured fentanyl and its analogues have been mixed with a variety of recreational drugs, such as heroin,[58] cocaine,[51] and methamphetamine,[59] which increases challenges for detection as well as the risk of fatal overdose incidents (see Figure 17, Appendix B).[60-62] Even more concerning, considering the rise of vaping among teenagers and young adults, the most recent trend in the illegal market entails injecting fentanyl, its analogues, and/or other illegal substances into e-cigarette refill solutions, leading to fentanyl-laced vapes, which has caused overdose deaths among high school students in the US.[63]

Fentanyl analogues are a group of synthetic opioids that are chemically and structurally similar to fentanyl.[4,8,9] Although analogues' pharmacological effects are similar to fentanyl's (*e.g.*, pain relief, sedation),[64] their effects as well as toxicity and potency levels may vary

drastically.[16,65] With many of the analogues being produced illegally,[62] their uses in unregulated settings may lead to higher risks of overdose incidents, constituting a significant public health concern.[26] Additional concerns have emerged due to the rise of analogues known as fentanyl "designer" drugs (FDDs) which are produced illegally to mimic the pharmacological effects of fentanyl while circumventing regulations and detection methods.[44,45] Given the evolving landscape of fentanyl-related substances, there is an overwhelming demand for advanced, adaptative, and versatile detection methods.

Fentanyl metabolites are chemical compounds formed due to the breaking down of fentanyl in the body, through metabolic pathways.[66,67] These metabolites and their respective concentrations after consumption of the drug are key to understanding the drug's impact on the human body, specifically the relevant interactions with organ, molecular, and cellular systems.[11] Additionally, their concentrations and chemical activity can provide invaluable insights into the duration and intensity of the parent drugs' effects as well as on the pharmacokinetics of fentanyl and its analogues.[67,68] From an emergency medicine standpoint, comprehending the metabolomics of fentanyl and its analogues is crucial for ensuring patients' safety and for tackling drug abuse and overdose incidents with appropriate medical interventions.[11] Furthermore, from an analytical chemistry perspective, understanding the degradation of fentanyl and its analogues in the body can help identify what types of chemical interactions take place, and how those can guide the search for effective, molecular-based detection mechanisms (*e.g.*, CNT-based electrochemical sensors[21,22,69-71]).

2.1 Chemical and Structural Properties of Fentanyl and its Analogues

As previously mentioned, an additional challenge to the detection of fentanyl-like synthetic opioids lies on the analogues, defined as substances that are structurally similar to fentanyl.[8,9,14,27,71,72] To better understand the complexity of this challenge, it is relevant to understand how the chemical structure of fentanyl can be tweaked to obtain similar, potentially dangerous substances.[73,74] With a molecular weight of 336.47 g/mol,[75] fentanyl is composed of: (i) piperidine ring in the center; (ii) N-alkyl chain; (iii) amide group; (iv) aniline ring (see Figure 2).[76,77]

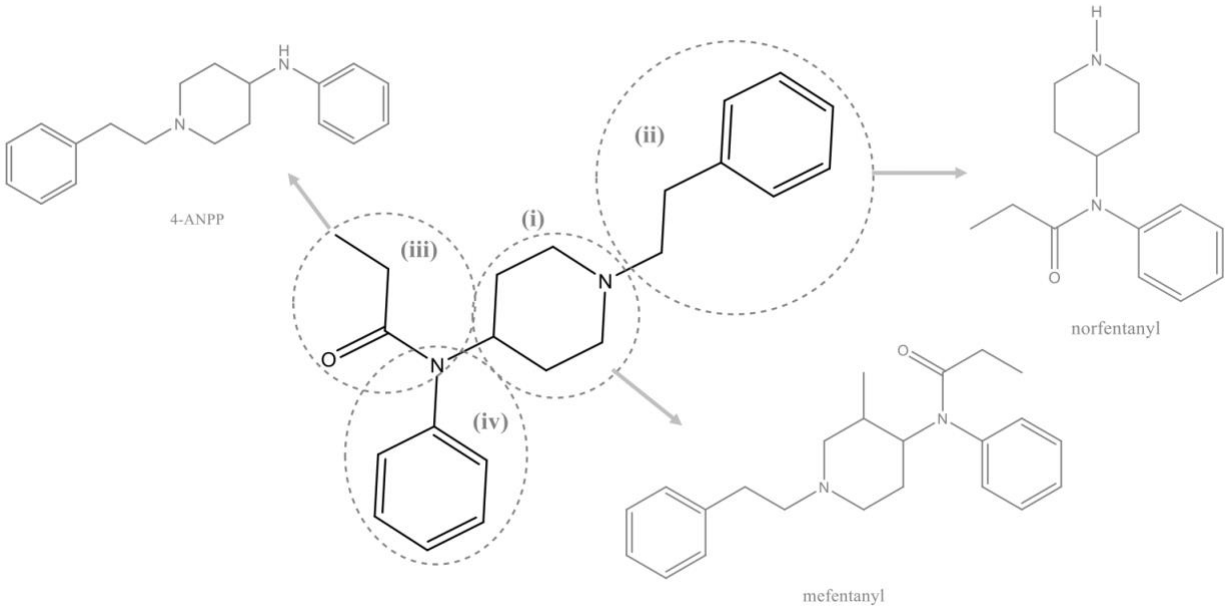

Figure 2. Fentanyl structure and its different functional groups that can be changed, giving rise to infinite possibilities of fentanyl-like structures (*i.e.*, analogues).[73-77]

Each of these functional groups shown in Figure 2 can be changed through group functionalization, leading to fentanyl-like molecules known as analogues.[73,74] For instance, by replacing the N-alkyl chain with a hydrogen, one can obtain norfentanyl, which is not harmful.[78] On the other hand, if instead of the amide group, a hydrogen atom is present (bound to the nitrogen), one has 4-anilino-N-phenethylpiperidine, which is toxic and often used as a precursor

for fentanyl production.[9,71-73] Likewise, the presence of a methyl group in the 3-piperidinyl position of the piperidine ring, the analogue would be mefentanyl (*i.e.*, 3-Methylfentanyl), which is extremely toxic.[9] As the possibilities of analogues are countless, these three examples are just few compared to the total number of analogues.[4]

Hence, taking into consideration the innumerous possibilities for changes, it is possible to obtain a great variety of fentanyl analogues.[78-80] Every year, new analogues are created, manufactured, identified, and seized, representing a growing concern over the current lack of reliable and accurate detection methods. Nevertheless, developing such methods is indeed challenging, due to the likelihood of false positives. Also, a method that may be efficient at detecting a single analogue (*e.g.*, fentanyl) might not be efficient at detecting other toxic, harmful fentanyl analogues (*e.g.*, carfentanil).[74,81]

The diversity of fentanyl analogues, their respective uses, toxicity levels, and applications can be exemplified in Table 1. The chemical structures can be found in Figure 18, Appendix C.

Table 1. Fentanyl and its analogues, along with their respective uses and toxicity levels. Analogues are often used in both medical and non-medical settings and their toxicity levels vary, with some being undefined.

Analogue	Uses	Toxicity
Fentanyl[49,80]	Severe pain treatment	50-100 times more potent than morphine. Lethal at 2 mg.
Sufentanil[81-83]	Anesthesia, pain relief in surgeries	5-10 times more potent than fentanyl. Lower lethal dose.
Alfentanil[83,84]	Anesthetic agent in surgeries	Less potent than fentanyl, more potent than morphine. Lethal dose not well-defined.
Carfentanil[43]	Tranquilizing large animals; not for human use	10,000 times more potent than morphine. Lethal at 20 µg.
Remifentanil[67,68]	Used in anesthesia, quick onset, and short duration in surgery	Rapid onset, short duration. Potent; specific lethal dose not well-defined.
Acetylfentanyl[85]	Not medically approved; illicit FDD	Like fentanyl but less potent. Lethal doses not well-defined.
Butyrylfentanyl[65,66]	Not medically used; illicit synthetic opioid	Less potent than fentanyl but more potent than heroin. Lethal dose not precisely defined.
3-Methylfentanyl[64]	Not used medically; illicit, highly potent opioid	Extremely potent, lethal doses in microgram range.
Lofentanil[86]	Experimental; not typically used in clinical settings	Extremely potent, more so than fentanyl. Exact lethal dose not clearly defined.
Ohmefentanyl[87]	Research chemical, not used medically	High potency. Specific lethal dose not well established.
Para-fluorofentanyl[88]	Illicit use; not approved for medical purposes	Like fentanyl. Lethal dose not specifically defined.
Fentanyl "designer" drugs (FDDs)[45,46]	Illicit use; not approved for medical purposes.	Varies, depending on the FDD produced; can be significantly more or less potent than fentanyl.

The list in Table 1 sheds light into the diversity of fentanyl analogues and their effects. While some are used in medical settings,[7] such as during surgeries and for pain management,[38] many of them have been used for illicit, recreational purposes.[11] It is also worrying that this list is not exhaustive, since there are many other fentanyl analogues that have been discovered and used in different settings as well as emerging, new analogues.[4,9] The variety of fentanyl analogues displayed in Table 1 helps one understand the importance of novel, accurate, and fast detection methods.

The emergence of new fentanyl analogues is particularly concerning, especially with the growing popularization of FDDs.[44] For this reason, the addition of a last row to the table highlights the importance of considering FDDs when developing detection methods for fentanyl

and its analogues. Among the analogues, there are examples that are way more potent than fentanyl itself, such as 3-Methylfentanyl and carfentanil.[8,9] The first has been used as a chemical warfare agent in the past,[44] whereas the second has raised concerns over its potential to be used as such, due to its commercial availability and high levels of toxicity to humans.[9] Nonetheless, the list also emphasizes how the toxicity levels vary widely, with many of the analogues not even having well-defined toxicity levels.[11] When dealing this diverse group of drugs, focusing on their detection becomes pressing and crucial, because some of them are intentionally designed to mimic the pharmacological effects of fentanyl, while avoiding being classified as illegal or detected by standard detection methods.[45]

Despite the importance of targeting such analogues, advancements in illegal drug manufacturing create the demand for detection methods that can be conceptualized, designed, created, and manufactured more quickly, as a means of quickly responding to the emergence of new analogues and FDDs.[43,44,46] Taking this into consideration, the emergence of fentanyl analogues becomes one of the most pressing, time-sensitive public health and safety challenges, as new FDDs are ready to flood within communities[37] and across borders,[89] relying on the absence of robust regulations and of fast, reliable, accurate, and versatile detection methods.

2.2 Fentanyl in the Human Body and its Metabolites

Current research approaches need to target not only the desired drugs but also their metabolites, since fentanyl, for example, gets metabolized to other analogues (see example of oxidative N-dealkylation of fentanyl in Figure 3).[82]

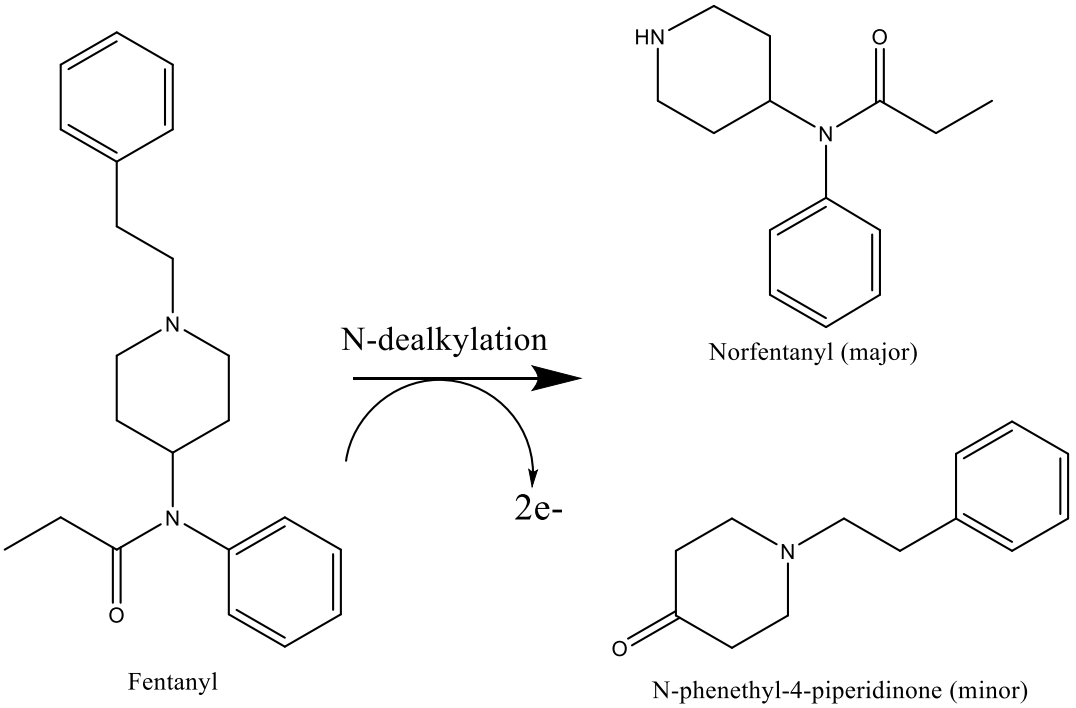

Figure 3. Fentanyl gets metabolized to norfentanyl (major product) and to 4-ANPP (minor product) via oxidative N-dealkylation.[82]

Norfentanyl can also be further oxidized, yielding other metabolites. Furthermore, there are other biotransformation pathways that fentanyl and their analogues can go through,[74,75,82] leading to further challenges for the detection of fentanyl-like drugs.[12,27,57,90]

Taking into account the aforementioned considerations and the state of the opioids crisis in North America, developing cheap, efficient, portable, and reliable detection methods for FAMs is extremely relevant and urgent.[12,27,57,89,90] To do so, one approach is to consider the potential of biomimetics in corroborating towards the development of innovative sensing technologies.[91,92] Understanding how fentanyl initially binds and eventually dissociates from the μ-opioid receptor (μOR) can provide relevant insights on the opioid-receptor interaction.[93] These insights can be particularly helpful in developing new therapeutic strategies and diagnostic tools focused on treatment and emergency response of overdoses related to fentanyl and its analogues.[91]

Furthermore, such insights can help guide the search and pave the way for molecular-based sensing mechanisms of FAMs.

From a biochemistry standpoint, the fentanyl molecule interacts with the μ-opioid receptor (μOR), a G protein-coupled receptor (GPCR).[93] When activated by ligand substances like opioids, μORs can prevent pain-associated signaling by translocating to the membrane, by blocking ionic channels, hence preventing neurons from transmitting pain signals.[93,94] In general, fentanyl binds to the μOR via multiple binding modes that are dependent upon the protonation states of specific aminoacids present in the structure of the receptor,[93] as shown in Figure 4a, where fentanyl molecules' carbon atoms are displayed in orange and the receptor's overall structure is shown in green and purple.

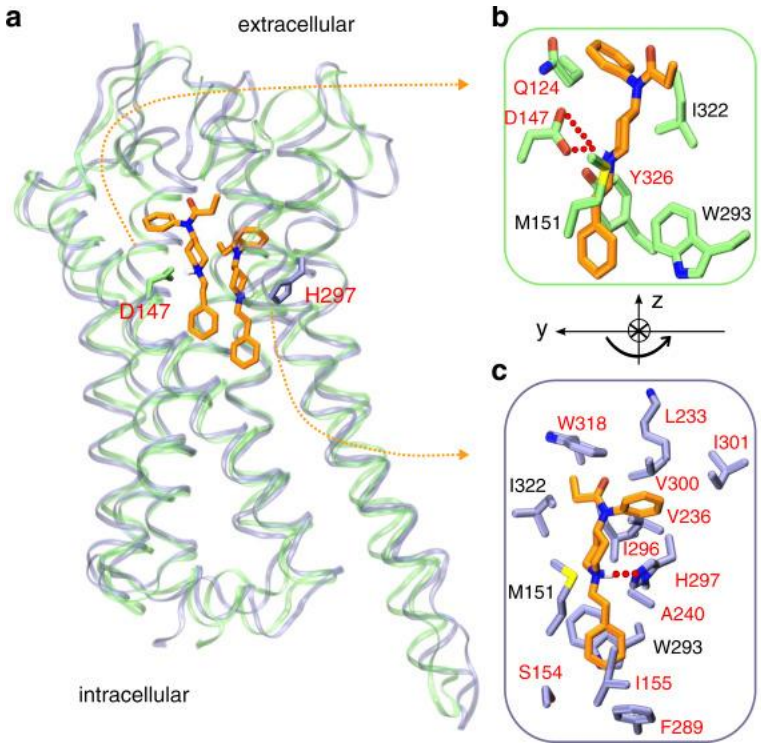

Figure 4. Binding of fentanyl to μ-opioid receptor.[93] Reprinted with modification (only panels a-c are shown, excluding panels d and e), from *Nat. Commun.* **2021**, 12 (1), 984, licensed under CC BY 4.0 DEED (https://creativecommons.org/licenses/by/4.0/).

The fentanyl-μOR interaction begins with the formation of a salt bridge between the former's positively charged piperidine amine group and the latter's negatively charged carboxylate group in Asp147 (D147),[93] as highlighted in Figure 1b. This electrostatic interaction facilitates the initial docking of fentanyl, orienting the molecule towards the μOR's binding pocket and facilitating stronger, subsequent binding interactions in the receptor's active site.[93] Nevertheless, it is worth mentioning that the strength of such interaction is highly influenced by the biochemical environment (*e.g.*, pH),[4,20,60,95] as it can affect the carboxylate's protonation and charge distribution. This dynamic nature of the interaction could create challenges in the context of using biomimetics as a means of developing CNT-based electrochemical sensors, since fentanyl can be present in a variety of biological matrices, all of which have different pH levels and conditions.[16,31,62,78]

Nevertheless, when interacting with the receptor, there are other underlying factors that can help facilitate the binding mechanism. For instance, the fentanyl-μOR is stabilized via hydrogen bonding between the former's piperidine amine and Hid297 (His297's Nδ-protonated tautomer),[93] as shown in Figure 4c. This interaction, also dependent on pH and other conditions, can enhance the positioning of the fentanyl molecule into the receptor's binding domain.[93,96] The roles of Asp147 (D147) in the initial binding and of Hid297's tautomerism in the stabilization of the fentanyl-μOR complex was only understood due to studies focusing on the unbinding of fentanyl, its kinetics, and mechanisms,[96] as summarized in Figure 5.

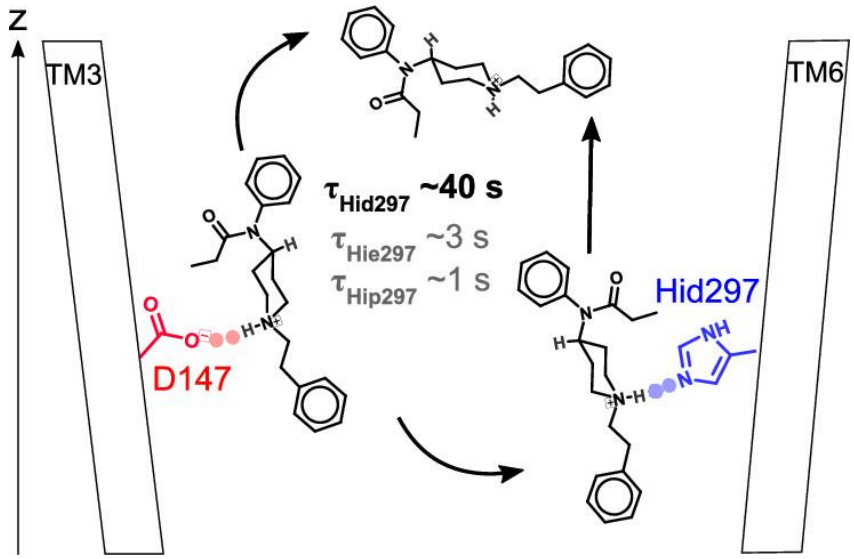

Figure 5. Unbinding of fentanyl molecule to D147 and Hid297.[96] Reprinted from *JACS Au* **2021**, 1 (12), 2208-2215, with no changes, licensed under CC BY-NC-ND 4.0 DEED (https://creativecommons.org/licenses/by-nc-nd/4.0/).

Factors that may enhance or dimmish the unbinding of D147 and of Hid297 to fentanyl molecules include the protonation states of aminoacids, the presence and competitive binding of other ligands (*e.g.*, from other drugs or antidotes, such as naloxone), and μOR's natural conformational changes.[96]

Additional binding affinity can be achieved due to hydrophobic interactions of fentanyl's phenethyl and aromatic groups with hydrophobic residues present in μOR' transmembrane helices, mainly Transmembrane Helix 3 and 6 (TM3 and TM6),[93,96] which is represented in Figures 4a and 5. Finally, Van der Waals forces and π-π stacking interactions between fentanyl and μOR's various hydrophobic residues and aromatic aminoacids, such as Phe221 and Phe289 (Figure 4c), take place as well, ensuring specificity and stability of the complex.[93,96]

Table 2 summarizes the aforementioned interactions and can provide an important framework for the development of CNT-based electrochemical sensors targeting fentanyl and its analogues.

Table 2. Chemical interactions between fentanyl and μOR, highlighting mechanisms and importance.[93,96]

Interaction	Fentanyl's Group	μOR's Group	Mechanism	Importance
Salt bridge	Piperidine amine	Asp147 (D147)	Positively charged amine group forms salt bridge with negatively charged D147's carboxylate.	Essential electrostatic interaction for μOR's activation/recognition of opioids.
Hydrogen Bonding	Piperidine amine	His297 (H297); Hid tautomer (Nδ-protonated)	Hydrogen atom from amine forms hydrogen bonding with Hid's nitrogen atom.	Secondary interaction, occurring when His297 is in the Hid state.
Hydrophobic	Phenethyl and aromatic groups	Transmembrane helix 6 (TM6)	Hydrophobic interactions occur between the phenethyl and aromatic groups and the transmembrane helices of μOR.	Enhanced by the hydrogen bond with H297, resulting in deeper insertion of fentanyl into the μOR water-filled intracellular cavity.
Van der Waals Forces	Alkyl and cycloalkyl groups	Several hydrophobic residues	Temporary polarization of electron clouds in the alkyl and cycloalkyl groups of fentanyl yield weak, non-specific forces, which interact with hydrophobic residues of μOR.	Although weaker, such interactions help position fentanyl within the binding site.
π-π stacking	Aromatic ring	Phe221 (F221), Phe289 (F289)	The π-electron clouds of fentanyl's aromatic rings and of μOR's phenylalanine residues overlap, stabilizing the interaction.	Stacking contributes to the specificity and stability of the binding, enhancing the overall interaction between fentanyl and μOR.

Understanding the fentanyl-μOR interactions can help enhancing the design of electrochemical sensors based on functionalized CNTs. Mimicking and replicating μOR's binding pocket via CNT-functionalization could help achieve this goal. For instance, salt bridges and hydrogen bonds suggest that charged and polar functional groups, respectively, could be incorporated on the surfaces of CNTs in order to mimic electrostatic and hydrogen-bonding interactions.

Furthermore, hydrophobic and π-π stacking interactions shown in Table 2 demonstrate the importance of including hydrophobic and aromatic functional groups in CNT-based electrochemical sensors.[71] Doing so would create complementary interactions between the sensing molecules and fentanyl's phenethyl and aromatic groups. Such interactions would

enhance specify and sensitivity towards fentanyl and its analogues, which is crucial, considering the variety of complex biological matrices[45,46,61,62] as well as contaminants (*e.g.*, other drugs[16]) that could be in the samples. Although these insights are further explored in *Section 6*, the aforementioned interactions and rationale can help pave the way towards strategic, interactions-oriented design of CNT-based electrochemical sensors.

2.3 Importance of Detection of Fentanyl, its Analogues, and Metabolites (FAMs)

The detection of fentanyl, along with its analogues and metabolites, is extremely important for addressing the ongoing opioids crisis. From a public health and safety standpoint, it is concerning that fentanyl is often mixed with other drugs,[61,62] leading to higher overdose risks.[37-39] Hence, accurate detection of fentanyl and its analogues in drugs can help the public by identifying its presence and concentrations.[1] Doing so could potentially save lives by helping users and healthcare providers to prevent overdose incidents.[91,92]

As such incidents become increasingly common in medical settings,[38] detecting fentanyl and its metabolites is of crucial importance for patient care.[11] Fentanyl overdoses require immediate and specific treatments,[19] hence rapidly identifying the presence and concentrations of the drug and its metabolites in the patient's body can be life-saving.[11]

Additionally, detection of fentanyl and its analogues are essential in the contexts of law enforcement[26] and forensic analysis,[96-98] aiding investigations of drug trafficking and distribution networks.[26] As drug abuse is a concerning public health and policy problem, effectively detecting and monitoring the flow of such substances across different communities can help researchers and practitioners to identify trends in drug abuse and develop robust harm reduction

and addiction prevention policies.[11] Finally, as fentanyl analogues are often manufactured and used as precursors in illegal fentanyl manufacturing process,[44] it is crucial to focus on detecting them as well.

3.0 Traditional Fentanyl Detection Techniques

In this section, traditional fentanyl detection techniques are presented in detail. Understanding how these methods work can help pave the way towards the development of more modern detection techniques.

3.1 Decentralized Methods

Decentralized methods of detection are highly versatile and mobile methods that can be used on the field. They are lightweight, small, and yield rapid results, though accuracy, specificity, and reliability are often compromised.

3.1.1 Colorimetric Tests to Perform Preliminary Screening

Colorimetric tests are rapid, simple tools that have been used for rapid screening of drugs by detecting the presence of a target analyte (*e.g.,* fentanyl) due to a color change upon the addition of reagent(s) to the sample.[97-99] In general, the mechanism of color change is a chemical reaction that is often caused by a change in oxidation state, complex formation, or other specific reaction between the reagent(s) and the functional group(s) of the target analyte.[97,98] Table 3 provides a summary of different examples of colorimetric tests used for detection of fentanyl, along with their specific reagents, colors, and mechanisms.

Table 3. Examples of colorimetric tests, with reagents, colors, and mechsnisms.[97-101]

Test	Reagents	Color	Mechanism
Froehde	Molybdic acid or molybdate salt and sulfuric acid	Greenish-brown or olive	Reduction of molybdate, reacts with various functional groups
Liebermann	Nitrite and sulfuric acid	Brownish-red or yellow	Nitrosation reaction with amine group
Mandelin	Ammonium vanadate and sulfuric acid (conc.)	Green or brown	Change in oxidation state of vanadium, reacts with alkaloids
Marquis	Formaldehyde and sulfuric acid (conc.)	Orange or brown	Reaction with amine group, with sulfuric acid as a dehydrating agent
Mecke	Selenious acid and sulfuric acid (conc.)	Green or blue-green	Complexation and oxidation reaction with alkaloids
Simon's	Sodium nitroprusside in acetaldehyde, water (step 1) and sodium carbonate in water (step 2)	Blue first, with no change in 2nd step	Reaction with primary amine group, and no reaction with secondary amine in second step

Some of these colorimetric methods shown in Table 3 are still commonly used nowadays by law enforcement and public safety entities, constituting a problem, since these colorimetric tests yield very questionable results.[95,98,99] Hence, these tests should only be used as preliminary screening and do not yield conclusive results. Based on the prevalence of false positives, some would argue that these methods should not even be used as preliminary screening.[95,99] The two main reasons why these tests fail are due to their lack of sensitivity and selectivity.[90,95] In the context of FAMs sensing, sensitivity is important due to the need to detect them at low concentrations or even at trace levels.[101,102] Selectivity is equally important,[103,104] because many samples containing fentanyl and its analogues are mixed with other substances and/or present in complex biological matrices that could interfere detection,[45,46,61,62,78] potentially leading to false positives/negatives.

3.1.2 Fentanyl Test Strips and its Widespread Use as Public Health Policy

Fentanyl Tests Strips have been widely used as a harm reduction strategy and public health policy.[11,26] The test strips are small, light-weight, and easy to use.[62] They provide fast results for

identifying the presence of FAMs. Fentanyl test strips work based on the components outlined in Figure 6. The liquid sample suspected of containing FAM is deposited onto the *sample pad*, which is designed to hold the sample and facilitate the capillary flow along the strip.[105,106] As the analyte flows towards the *conjugate pad*, it encounters immobilized antibodies (IAs) labeled with gold nanoparticles,[105,106] labeled in Figure 6 as IA-C and IA-T. The first, IA-C, is capable of binding to the binding partner (BP) and to the gold nanoparticles (Label), and of traveling across the strip until it binds to CA-C (capture antibody) in the *Control Line*, hence always yielding a red line.[105,106] The second, IA-T, which is initially bound to the gold nanoparticles, can bind to fentanyl, leaving the gold nanoparticles free to travel across the strip until it reaches the control line.[106] Then, the fentanyl-IA-T complex will selectively bind to CA-T (capture antibody) in the *Test Line*, yielding no red line.[106] Therefore, in the presence of fentanyl, only one red line can be observed, as demonstrated in Figure 6.

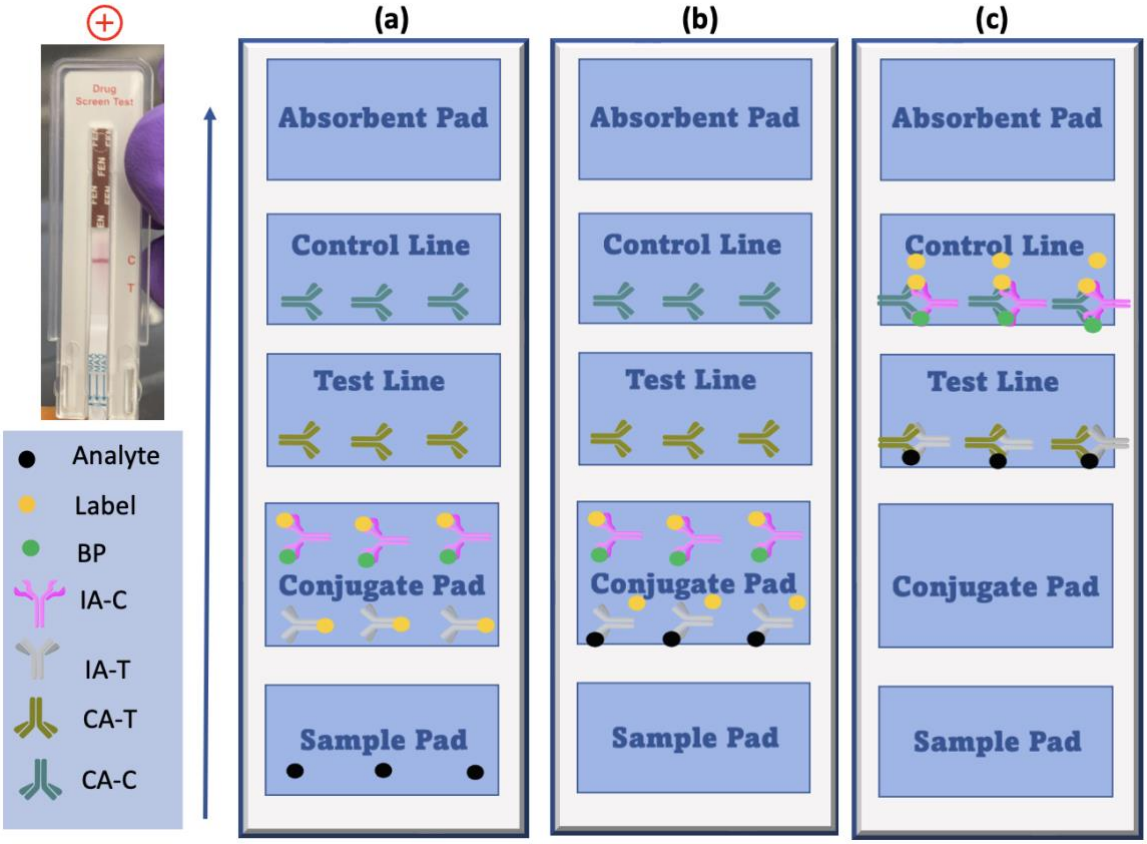

Figure 6. Schematics of a fentanyl test strip, showcasing the different areas and substances present in a positive test outcome. Left displays a fentanyl strip yielding a positive result for the present of norfentanyl and the legend. Pane (a) shows the initial state of the test strip in the presence of analyte, pane (b) the intermediary state, and pane (c) the final state. Analyte is fentanyl, Label is composed of gold nanoparticles, BP is the binding partner, IA-C and CA-C are immobilized and capture antibodies which selectively bind to each other in the control line, and IA-T and CA-T are immobilized and capture antibodies which also selectively bind to each other, but in the test line.[105,106]

In the absence of fentanyl, the Test Line would only have IA-T and CA-T bound to the gold nanoparticles, yielding a red color.[106] Likewise, the control line would also yield a red color due to the presence of IA-C and CA-C bound to the gold nanoparticles.[106] Hence, a negative result would yield two red lines. The *absorbent pad* in the end is important to draw the sample through the strip via capillary action and also to hold excess sample.[105,106] Capillary action is further corroborated by the material of the test strip, made of nitrocellulose membrane.[105,106] A fentanyl test strip is an example of a competitive (inhibition) immunoassay, since either the gold nanoparticles or the fentanyl molecules can bind to IA-T, eventually determining whether a red line will appear in the test strip.[26]

Understanding the basics of binding of fentanyl molecules to the IA-T antibody as a means of determining their presence through this test is extremely relevant, due to the possibility of the development of CNT-based electrochemical sensors functionalized with target antibodies that could help detect FAMs. Examples of current research in this direction include the development of an ultrasensitive CNT-based field-effect transistor used to detect norfentanyl in urine samples.[21] Although further review of this recent development will be explored in *Section 7*, it is worth mentioning that utilizing the high level of specificity between norfentanyl and its antibody can be an effective method towards enhancing the detection of FAMs,[21] along with CNT-based functionalization.[22] Doing so demonstrates the importance of reviewing a variety of detection methods of FAMs in order to guide future developments of effective, fast, and accurate electrochemical sensors.

3.1.3 Advantages and Shortcomings of Traditional Decentralized Methods

Traditional decentralized methods have advantages and shortcomings that are worth mentioning. Advantages include accessibility, cost-effectiveness, speed of results, and portability. Colorimetric methods[98,99] and fentanyl test strips[62] are relatively easy to use and widely available to the public, enabling accessible, on-site screening without requiring specific, expensive equipment or specialized training. Regarding cost-effectiveness, these methods are inexpensive, allowing for mass distribution, a relatively successful strategy adopted by many harm reduction programs.[11] The results from these techniques are fast and the devices required to perform testing are portable, being convenient for a wide range of circumstances, from personal use to law enforcement.[77,78]

Nevertheless, there are some limitations concerning the traditional decentralized methods. First, the high rates of false positives and negatives due to low sensitivity and specificity is extremely concerning.[26,27,95] For instance, a false positive result in a critical situation, such as a law enforcement setting, can lead to unnecessary, unjust detentions. Additionally, due to FAMs often being in low concentrations, mixed with other substances and/or present in complex matrices,[62] false negatives could be common,[26] leading to overdose incidents.[39,40] Given these shortcomings, while traditional decentralized methods are still widely used for pre-screening of samples, it is important to focus on the development of modern, decentralized methods that could offer the benefits of accessibility, cost-effectiveness, speed of results, and portability without compromising sensitivity and specificity.[90] By doing so, these two important factors are not restricted to traditional, centralized methods.[78]

3.2 Centralized Methods

A thorough understanding of the current state-of-the-art techniques used to separate fentanyl, its analogues and metabolites, and the different matrices they can be present in (e.g., saliva,[106,107] urine,[108-110] blood[9,16,78,79]) is paramount and is further explored herein. Although these centralized methods are not versatile and fast, they provide accurate and reliable results, often being used for detection and separation of fentanyl and its analogues after preliminary screening.[90]

3.2.1 Gas Chromatography - Mass Spectrometry (GC-MS)

3.2.1.1 Theoretical Overview

Often referred as the "gold standard" for drug testing, GC-MS can detect a wide variety of drugs across different matrices, including bodily fluids.[110] The instrumentation is composed of a gas chromatograph and a mass spectrometer.[111] Gas chromatography occurs in the following steps. First, the gas sample is collected (*e.g.*, vapors from the liquid sample) and then injected into a stream of inert gas, such as He, N_2, or H_2.[112] The injection of the sample can take place using a syringe or a sample loop attached to a valve, hence enabling automation and precise gas flow rate. After injected, the carrier gas and the gas sample travel through the column, which is placed under highly specific conditions.[110,111] The gas chromatograph takes advantage of a packed or open tubular column and its properties (*i.e.*, diameter, length, thickness of the film) in order to separate the analyte.[113,114] Several substances can be present in the analyte, and they travel through the mobile phase.[114] Substances with higher affinity to the stationary phase will elute last (higher retention times), whereas substances with lower affinity will do so first (lower retention times), hence allowing for separation to take place.[114] As the substances elute at different times, the mass spectrometer ionizes the molecules into fragments and then accelerates, deflects, and ultimately detects each ionized molecule separately, based on the mass-to-charge (m/z) ratios.[114] Based on the sizes of the peaks and their integrations, it is possible to determine the concentration of the different substances present in the analyte mixture.[114] Using the two techniques decreases the likelihood of false positives, as it is almost impossible for two substances to behave identically under both GC and MS. Additionally, under the same conditions, each substance is expected to elute at the same exact time, hence allowing for high reproducibility and accurate detection.[110,111,114,115]

3.2.1.2 Past Performances and Outcomes

In terms of toxicity, it is noteworthy that fentanyl, sufentanil, and alfentanil are extremely harmful to humans and other animals,[80,81] whereas norfentanyl is not considered toxic. Nevertheless, as the major metabolite of fentanyl, detecting norfentanyl can provide valuable information on fentanyl exposure.[109,116] These four aforementioned analogues were separated from urine samples using GC-MS and their limits of detection were also reported (see Table 4).[13]

Table 4. Fentanyl analogues and their respective retention times, molecular ions, and limits of detection (LOD) obtained via GC-MS.[13,78,108]

Analogue	Retention time (min)	Molecular ions (m/z)	LOD (ng/mL)
Fentanyl	4.800	148, 188, 202, 245	5.0
Fentanyl-D5	4.789	194, 250	5.0
Norfentanyl-TMS	3.230	154, 155, 247, 289, 304	5.0
Sufentanil	4.927	140, 187, 238, 289	5.0
Alfentanil	5.185	222, 268, 289, 359	2.0

It is worth mentioning that the GC column commonly used for the separation of the analogues is composed of 5% phenyl methylpolysiloxane (nonpolar GC column).[78] As a result, the most polar compounds are expected to elute the fastest, whereas the least polar ones are expected to yield higher retention times.[13,78] Table 4 shows that the most polar compound (*i.e.*, Norfentanyl-Trimethylsilyl) yields the lowest retention time, due to the higher polarity as a result of the absence of an N-alkyl chain linked to the piperidine ring.[78] Instead, a hydrogen is connected to the nitrogen, hence why this compound presents a higher polarity, eluting the fastest through the column. On the other hand, the other four analogues yield higher retention times, since they are less polar, due to the presence of the N-alkyl chains in their structures.[78] Depending on the types of structures present in their respective N-alkyl chains, their compounds may present different polarity indexes, hence explaining the differences between elution times.

Regarding the limits of detection, it is worth investigating whether such limits are enough, considering that fentanyl, for instance, is lethal at blood concentrations above 60 nM.[57,115]

Taking fentanyl as a reference, the limit of detection is 5.0 ng/mL, which is equivalent to 15 nM. This means that the limit of detection achieved by GC-MS is four times lower than the lethal dose of fentanyl in the blood. Hence, such limit of detection is considered ideal to prevent overdose-related deaths. Nonetheless, GC-MS is not considered a very practical technique to be used in emergency settings.[28] When medical staff receives a patient, they have a few hours or even minutes to decide whether to administer naloxone, which can have severe side effects.[116] Hence, it would be desirable to develop faster, more practical methods to determine fentanyl concentration in the blood. Additionally, it is worth mentioning that fentanyl gets metabolized to norfentanyl, hence why the concentrations of fentanyl in urine would correspond to only about 10% of the actual blood concentration. Bearing this in mind, it would be more appropriate to have a limit of detection of about 6 nM, instead of 15 nM, since GC-MS analyses are often performed in urine, a medium that provides faster, easier separation.[13,41,108]

Although separation of common fentanyl analogues from urine via GC-MS has been reported, correlation between the specific structures and the respective characteristic molecular ions has not been established yet. To better understand the results, the different fragments of molecular ions that result from the ionization step are hereby proposed. It is worth mentioning that the molecular ions herein reported are only the characteristic ones *i.e.,* the ones that are used to confirm the identity of the specific analogues (see Figures 7, 8, and 9).

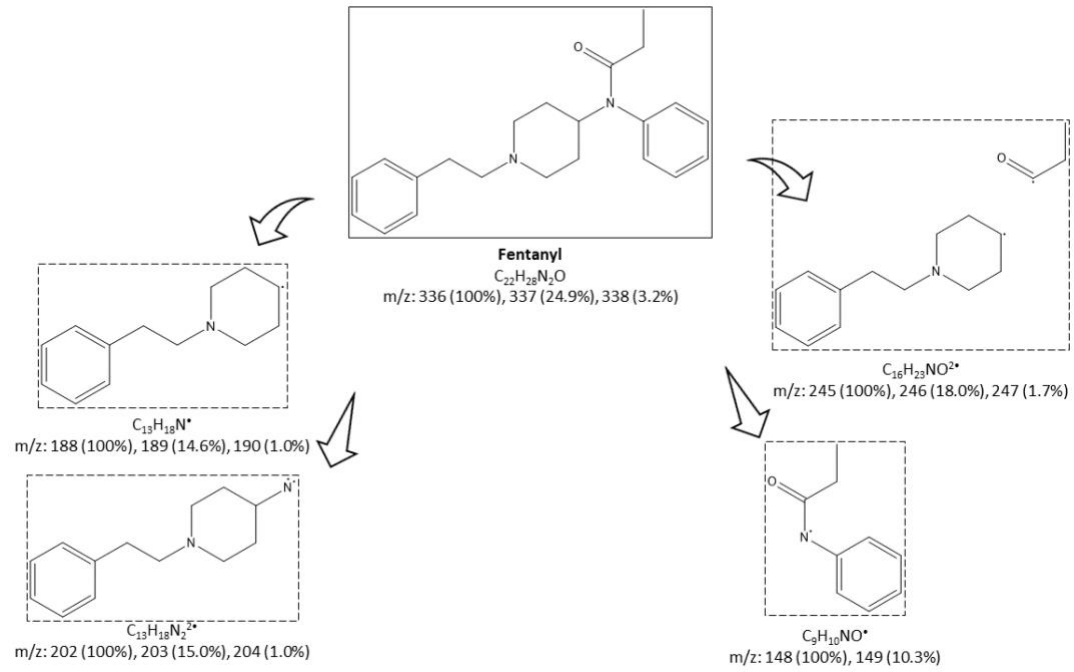

Figure 7. Fentanyl and its proposed characteristic molecular ions, based on fragmentation patterns and m/z calculations.

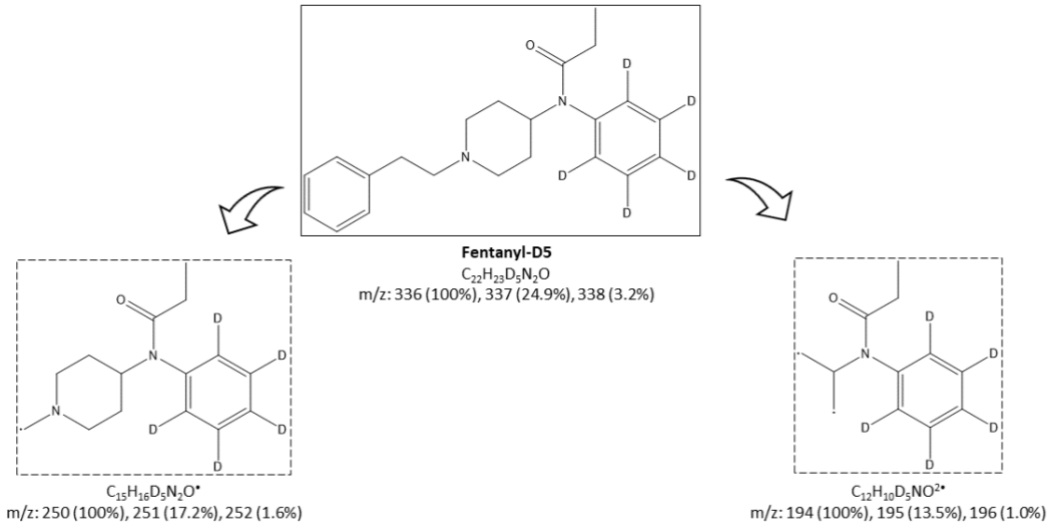

Figure 8. Fentanyl-D5 and its proposed characteristic molecular ions, based on fragmentation patterns and m/z calculations.

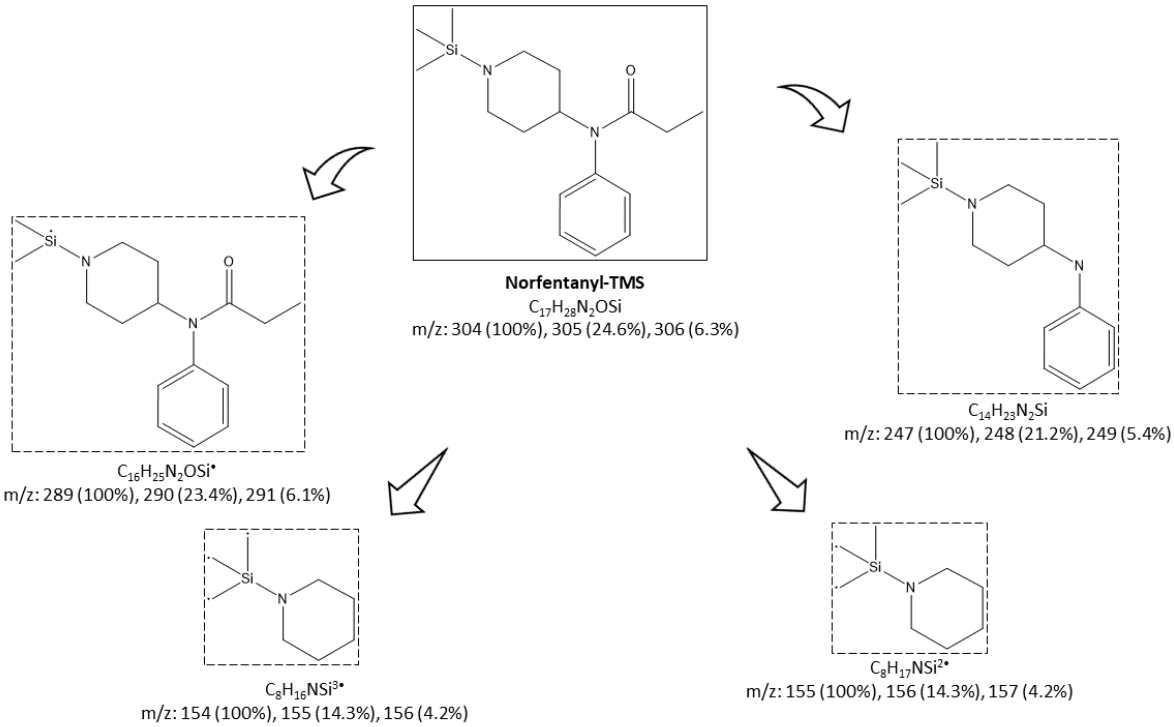

Figure 9. Norfentanyl-TMS and its proposed characteristic molecular ions, based on fragmentation patterns and m/z calculations.

Although not previously reported, proposing the structures of characteristic molecular ions (Figures 7, 8, and 9) is extremely important, given the variety of biotransformation pathways that fentanyl and their analogues can undergo,[75,81,82] as previously exemplified in Figure 3. By doing so, it is possible to predict possible transformations that can take place prior or after separations experiments. Moreover, in samples containing mixtures of an analogue and their respective metabolite(s), distinguishing between those can only be possible thanks to GC-MS or similar, advanced separation techniques.

An important distinction worth noting is the difference between non-targeted and targeted analysis.[80,117] The aforementioned limits of detection were reported for non-targeted analyses of fentanyl and their analogues.[78] In such scenarios, it is not known whether the analyte contains the specific analogues.[80] Nonetheless, in targeted analyses, it is assumed that the specific analogue is present,[79] allowing for even lower limits of detection, ranging between 0.1 and 5

ng/mL.[13,78,107,108] With these limits, it is then feasible to detect fentanyl at typical concentrations found in blood as well as its common metabolite, norfentanyl.[82] Targeted GC-MS analyses of fentanyl, norfentanyl, and other metabolites are particularly relevant for determining causes of death of intoxicated patients.[118-120]

The fentanyl/norfentanyl (metabolic) ratio is extremely important for identifying whether a patient died due to fentanyl overdose or due to other reasons. If this ratio is low, it is then known that most of the fentanyl has already been metabolized in the body, hence potentially ruling out overdose as a cause of death.[80,82] A more practical example of this approach can be seen in George Floyd's autopsy report,[121] which revealed a fentanyl/norfentanyl ratio of approximately 2.0, which is below the typical ratio for fentanyl overdose deaths (see Table 5).[80-82]

Table 5. Fentanyl and metabolites, their respective concentrations, and limits of detection (LOD) as reported in George Floyd's autopsy. Tests performed under targeted analysis in a blood sample using GC-MS.[121]

Substance	Concentration (ng/mL)	Concentration (nM)	LOD (ng/mL)
Fentanyl	11	33	0.10
Norfentanyl	5.6	17	0.20
4-ANPP	0.65	2	0.10

Hence, the report indicated that a significant amount of fentanyl found in the blood had already been metabolized. Additionally, the concentration of fentanyl in his blood was only 33 nM, which is almost half the concentration considered lethal.[57,90,115] The limits of detection are the lowest possible, taking into consideration that the analysis was targeted for the specific opioid (fentanyl) and its most common metabolites (norfentanyl and 4-ANPP). Data is consistent with previous reports that indicate that fentanyl gets mostly metabolized to norfentanyl and, in small amounts, to 4-ANPP as well.[80-82]

3.2.2 Liquid Chromatography (LC)

Liquid chromatography is also considered a suitable technique for separation of drugs from their matrices.[6,9,18,122-124] Like GC, the separation takes place depending on the interactions between the analyte's substances and the stationary and mobile phases.[123] There is a wide variety of materials that can compose the mobile and stationary phases, giving rise to many types of liquid chromatography.[123] For drug analysis applications, the mobile phase is often a liquid/mixture that carries the analytes through the stationary phase, composed of a column that has its inside region bonded with silica or a silica-based compound.[78] The instrumentation consists of solvent reservoirs which aid the flow of the mobile phase through the column.[77] The samples are then added through a multichannel valve, and travel through the column, together with the mobile phase.[78] Substances that have higher affinity to the mobile phase will elute first, whereas substances with higher affinity to the stationary phase will elute last.[123] LC enables a very efficient separation, due to the variety of mobile and stationary phases one can deploy.[123] When combined with mass spectrometry, each substance will be eventually ionized, and its molecular ions will be accelerated, deflected, and ultimately detected separately, according to their mass-to-charge (m/z) ratios.[122,123]

The diversity of mobile and stationary phase also allows for a wide variety of substances to be separated through this technique.[123] To improve the separation, the length of the column and the number of theoretical plates are key factors.[114,123,124] According to the Theoretical Plate Model, $HETP = {}^{L}/_{N}$, where $HETP$ is the number of Height Equivalent Theoretical Plates, L is the length of the column, and N is the number of theoretical plates.[125] This equation is based on the number of equilibrium states between the stationary and the mobile phases.[125] To optimize a separation, it is needed to increase the number of ideal equilibrium stages that would allow for an

efficient separation. To do so, increasing the number of theoretical plates (N) would improve the separation. This can be achieved by increasing the length of the column (L), but that would lead to a longer waiting time, hence why it is relevant to consider not only the efficiency of the separation but also how much time is needed for each separation.[123,126]

LC coupled with tandem or high-resolution mass spectrometry apparatus has allowed for even lower limits of detection.[122-126] While LC-MS/MS is usually preferred for targeted analysis only, LC-HRMS can also be used for non-targeted analysis.[78] These advancements have been extremely crucial for forensic and drug analysis applications, especially because of the various possibilities for fentanyl analogues.[73,78-81,117,124] With the latest emergency of FDDs,[127] it is often impossible to perform targeted analysis on the samples, which is a major challenge in clinical toxicology.[81] The development of FDDs is usually geared towards recreational purposes. New substances can yield new effects and users are willing to pay more for such drugs.[45] Additionally, due to difficulties in detection, FDDs can be transported more easily and with lesser logistical obstacles since authorities and law enforcement agencies are less likely to identify such substances.[81] Although naloxone (opioid antidote) may still be effective at neutralizing some of the effects of FDDs, the concentrations needed may be much higher, which can potentially lead to disastrous side effects.[116] Bearing this in mind, identifying and determining the concentrations of FDDs are essential steps towards preventing more overdose-related deaths.[81,127] Hence, targeted and non-targeted drug analyses using LC-MS/MS and LC-HRMS, respectively, become essential.[78-80]

3.2.2.1 Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS)

3.2.2.2 Theoretical Overview

LC-MS/MS is considered a very useful analytical technique, especially for drug detection, because it takes advantage of the extremely powerful separation ability of LC as well as of the sensitivity, selectivity, accuracy, and precision of the triple quadrupole mass spectrometry.[16,17,66,121-125] The LC-MS/MS has a powerful ionization source that readily nebulizes, desolvates, and ionizes the substances that come out of the LC column, hence giving rise to charged particles.[123] Then, under high vacuum, electromagnetic fields are applied, prompting the particles to travel through a series of quadrupole mass analyzers.[123] Each quadrupole enables further selectivity and precision.[123] For instance, the first quadrupole is designed to selectively accept only a specific molecular ion (based on its m/z), whilst rejection other particles with different m/z values.[123] Then, the molecular ions that pass through the first quadrupole collide with an inert gas, hence leading to further fragmentation.[123] Then, the third quadrupole is more useful to detect molecular ion fragments, which can be quantified through an electron multiplier.[123] Overall, molecules are split into molecular ions and further fragmented into molecular ion fragments. As such, every substance will have its fragmentation patterns, resulting in high specificity and selectivity.[124]

3.2.2.3 Past Performance and Outcomes

For LC-MS/MS analyses of fentanyl and its analogues, C18 columns (non-polar) are widely deployed, along with mobile phases constituted of formic acid in water or formic acid in acetonitrile (both polar).[14,78,79] Therefore, polar substances present in the analytes will elute first,

whereas non-polar substances will elute last. Table 6 below shows the different substances, along with their parent and product ions, and retention times.

Table 6. Substances identified and quantified via LC-MS/MS targeted analysis, along with their parent ions, product ions, and respective retention times. Product ions used for quantification of the substance are labeled with [q] were used for quantification of the substances. Cone volts ranged between 25-40V and collision energies ranged between 15-35eV.[14,78,79]

Substance	Parent ion (m/z)	Product ions (m/z)	Retention time (min)
Fentanyl	337.2	188.5[q], 105.6	4.87
Fentanyl-D5	342.2	188.5[q], 105.6	4.84
Norfentanyl	233	84.0[q], 176	3.61
Norfentanyl-D5	238	84.0[q], 181.9	3.59
Sufentanil	387.6	238.2[q], 355.7	6.72
Alfentanil	417.3	268.6[q], 197.7	4.93
3-Methylfentanyl	351.5	202.4[q], 105.6	5.70
Remifentanil	377.1	317.0[q], 345	4.05

Table 6 shows an example of how LC-MS/MS can be extremely useful for not only identifying the substances but also quantifying the analytes. Retention times are consistent with expectations, since the most polar substances (*e.g.*, norfentanyl, remifentanil) eluted first, whereas the least polar ones (*e.g.*, sufentanil, 3-methylfentanyl) eluted last. Nevertheless, it is noteworthy that the analytes were targeted, meaning that it was known these substances were present in the sample, hence allowing for the low limits of detection.

3.2.3 Liquid Chromatography with High Resolution Mass Spectrometry (LC-HRMS)

3.2.3.1 Theoretical Overview

LC-HRMS offers even more specificity and selectivity by measuring the exact m/z values instead of the nominal ones, hence allowing for differentiation between molecules with equal nominal masses. Advantages of LC-HRMS include the possibility of non-targeted analysis with a wide variety of substances present as well as the very small volume of analyte needed for a separation experiment. High resolution can be obtained via Quadrupole Time-of-Flight Mass

Spectrometry (Q-TOF-MS) or Orbitrap-MS.[117] The first is composed of four parallel rods (quadrupole), a collision cell (as described in LC-MS/MS above), and, most importantly, a time of flight (TOF) tube that allows for spectra to be generated.[117] As expected, lighter molecular ions travel faster through the TOF tube to the MS detect, hence enabling m/z values determination.[128,129] Orbitrap-MS consists of two electrodes that promote orbital travel of the molecular ions, hence trapping them between the electrodes.[117] Based on changes in current, a mass spectrum can be generated, allowing for highly precise m/z determination also.[129-131]

3.2.3.2 Past Performance and Outcomes

LC-HRMS non-targeted analysis of an analyte containing 44 opioid-related substances was performed and reported in literature,[14] showing how effective LC-HRMS can be at detecting a variety of fentanyl analogues as well as other opioid-related substances. C18 columns (non-polar) were deployed as the stationary phase, whereas the mobile phases were composed of ammonium formate, formic acid, and acetonitrile in water (polar).[14] The limits of detection for all substances ranged between 0.1 and 5 ng/mL.[14,78] Such limits of detection are considered very low, hence allowing for determining which specific substances were responsible for a potential overdose event as well as how much of the substance was present in the blood sample (see Table 7).

Table 7. Precursor ions targeted in $MS^{[2]}$ and $MS^{[3]}$ analyses of 44 opioid-related substances. For the purposes of this work, only 11 substances are herein reported, as those show the variety of opioid-related compounds that were separated using LC-HRMS in a blood sample.[14,78]

Substance	Precursor ion ($MS^{[2]}$)	Precursor ion ($MS^{[3]}$)
Fentanyl	337.16	-
Norfentanyl	233.07	-
Sufentanil	387.1	-
Alfentanil	417.15	-
3-Methylfentanyl	351.19	-
Heroin	370.14	-
Tramadol	264	246
Morphine	286	-
Ibuprofen	206.95	207
Naproxen	230.89	184.8
Naloxone	328.1	310

Precursor ions targeted for $MS^{[2]}$ and $MS^{[3]}$ analyses are shown in Table 7, along with the substances (note that brackets have been added in $MS^{[i]}$ to differentiate superscripts from citations). $MS^{[3]}$ (three-stage mass spectrometry, *i.e.,* Triple Quadrupole Mass Spectrometry) enables further fragmentation of precursor ions obtained by $MS^{[2]}$ (two-stage mass spectrometry, *i.e.,* Tandem Mass Spectrometry), hence prompting even further precision and selectivity.[14,78] Data above indicates that $MS^{[2]}$ is, most of the times, enough for the analysis of most opioid-related substances, and that using $MS^{[3]}$ is often unnecessary.[14,78] Nonetheless, when substances have equal nominal masses (which might occur, given the variety of fentanyl analogues), performing $MS^{[3]}$may be relevant.[78,79,107,132]

Herein, it is worth noting that fentanyl, norfentanyl, sufentanil, alfentanil, and 3-methylfentanyl are all toxic fentanyl-like substances, and that the precursor ions targeted were all different, despite the similar structures of these substances. This is a clear advantage of LC-HRMS, because it allows for separation of a variety of substances, even when they have similar molecular weights for the precursor ions.[14,79,124] In addition to these fentanyl analogues, other opioids were present in the blood sample, such as heroin, tramadol, and morphine.[14,78] Separation between fentanyl and other drugs is extremely relevant, considering that mixing drugs has been a

common practice among recreational users.[57,90,115] Common non-opioid drugs used for pain management, such as ibuprofen and naproxen (Non-Steroidal Anti-Inflammatory Drugs – NSAIDs[133]), were also successfully separated. Lastly, naloxone, a common opioid antidote, was also separated successfully. Analyzing the concentration of naloxone in blood is particularly relevant to ensure that the appropriate concentration has been given to a patient that may be undergoing fentanyl overdose, because naloxone has potent side effects that can indeed lead to death by overdose as well.[116]

3.2.4 Advantages and Shortcomings of Traditional Centralized Methods

The main advantage of traditional centralized methods is the high sensitivity and specificity, which is extremely desirable for sensing of FAMs, given their often-low concentrations, mixing with other substances and/or presence in complex biological matrices.[14,78] Furthermore, with the advent of data analytics and machine learning,[134] the quantitative and comprehensive data provided by these methods can be extremely helpful in toxicological analyses,[19] autopsies,[121] and early detection of new fentanyl analogues.[4] Nonetheless, shortcomings include the lack of accessibility, portability, cost, and resources, especially in areas with limited access to resources.[78,90] Given these limitations, it becomes crucial to develop more accessible, portable, and cost-effective sensors that carry the advantages of traditional centralized methods.[90] As further explored in the upcoming section, electrochemical sensors can be extremely promising in delivering such outcomes.

4.0 Electrochemical Sensing as a Viable Detection Method

Electrochemical sensing is a well-established analytical chemistry technique, often used for fast and accurate detection and quantification of a variety of chemicals.[24,134] Electrochemical sensing presents a viable and innovative detection method for fentanyl and its analogues due to a few relevant factors.[90] First, electrochemical sensors can be very sensitive[21,23] and selective.[22,135] Sensitivity is crucial for the detection of these drugs, as even trace amounts can be lethal.[103] Likewise, selectivity is relevant, because samples containing these drugs are often complex and contaminated with other complex substances, such as heroin[50,53] and cocaine.[13,95]

Furthermore, electrochemical sensing can yield fast responses that allow for real-time analysis of samples, which is necessary for medical emergencies and, consequently, effective overdose treatments.[26] With growing concerns over fentanyl analogues, such as carfentanil, being used as chemical warfare agents,[44] portability is also extremely relevant.[10] Electrochemical sensors are often miniaturized, and doing so would enable first respondents to perform on-site detection of substances which exposure could be dangerous or even deadly.[10,90]

From a feasibility standpoint, electrochemical sensors for the detection of fentanyl and its analogues would be more cost-effective than traditional, centralized methods, making them an invaluable alternative for communities with limited resources and growing concerns over the opioids crisis.[26,38] Additionally, the low power demands of electrochemical sensing technologies would enable fentanyl detection to occur through portable, small, and battery-operated devices, useful especially in remote locations.[26,136] Finally, modifications to the electrodes' surfaces and recent advancements in nanotechnology can make electrochemical sensors extremely versatile,[21] which could enable targeted detection of a variety of fentanyl analogues.[22]

4.1 Theoretical Overview

Herein, a theoretical overview on electrochemical sensing is presented. After introducing some classic examples of electrochemical sensing, the relevant background in the field of electrochemistry is explored. Such background includes the basics of migration and diffusion-oriented mass transfer, followed by an overview of electrochemical techniques and its respective models. the processes mediating electron transfer at the vicinity of the electrodes as well as the double-layer structure and its models. Then, important concepts for measuring efficiency of sensors are introduced, such as limit of detection and range. Understanding the aforementioned concepts is relevant to comprehend how electrochemical sensing can be an effective method for detecting FAMs.

4.2 Classic Examples of Electrochemical Sensing

Classic examples of applications of electrochemical sensing include sensors to measure pH,[136] oxygen,[137] and glucose[138]. Table 8 below presents some examples of sensors, along with their respective techniques, methods/concepts, limits of detection, and accuracy.

Table 8. Classic examples of electrochemical sensors, demonstrating how a variety of techniques based on different methods/concepts can provide different limits of detection and accuracy levels.

Sensor	**Technique**	**Method/concept**	**Limits of Detection**	**Accuracy**
pH Meter[137]	Potentiometric	Measures hydrogen ion concentration in a solution using a special electrode to detect potential difference.	0 to 14 pH	±0.01 to ±0.1 pH
Oxygen Sensor (Clark Electrode)[138]	Amperometric	Measures oxygen concentration using a cathode to reduce oxygen and produce a measurable current.	Trace amounts to 100% concentration	±0.1% to 1%
Glucose Sensor[139]	Amperometric/Biosensing	Detects glucose concentration in blood using enzyme-based electrode reacting with glucose.	As low as 0.1 mM	±15% of actual level
Biosensor[140]	Biosensing	Combines biological component with electronic component to detect specific molecules.	Picomolar to millimolar	Varies widely
Conductivity Meter[141]	Conductometric	Measures electrical conductivity of a solution, indicating ionic compound concentration.	Varies widely	High precision within range
Ion-Selective Electrodes[135]	Potentiometric	Measures specific ions in a solution using membrane-like electrodes selective to certain ions.	Nanomolar to millimolar	Typically within 1-2%
Potentiometric Sensor[142]	Potentiometric	Measures voltage changes due to ion concentration in a solution.	Micromolar to millimolar	Typically within a few percent
Amperometric Sensor[143]	Amperometric	Detects current produced by redox reaction of the analyte at an electrode surface.	Sub-ppm or lower	Generally within 5-10%

Table 8 is particularly relevant, because it shows how electrochemical sensing can be used for detecting a variety of chemicals in different types of samples and through distinct electrochemical techniques. The methods/concepts presented help highlight the versatility and selectivity of electrochemical sensing, as evidenced by how different electrochemical

measurements can help identify and quantify diverse analytes. The low limits of detection and high levels of accuracy could also corroborate to highlight the sensitivity and selectivity of such sensors. All these aspects further the idea the electrochemical sensors could be an extremely viable, robust method for sensing FAMs.

4.3 Migration and Diffusion-Oriented Mass Transfer, and Other Relevant Properties

Migration and diffusion are the two most relevant processes in electrochemical sensing, because they influence the rate at which analytes can reach the sensing electrode, hence affecting accuracy, sensitivity, and selectivity.[144] Migration is the movement of ions due to an electric field.[144,145] In an electrochemical system, upon application of an electric field, cations (positively charged) move towards the cathode (negative electrode), and anions (negatively charged) move towards the anode (positive electrode).[144-146] The electric field (E) represents the potential difference (*i.e.,* voltage) applied across the cathode and anode, such that the force applied on the cations and anions due to the electric field, as defined by Coulomb's law.[144-146] On the other hand, diffusion occurs due to the random thermal motion of ions or molecules in solution.[147] When a concentration gradient exists, ions or molecules will naturally move from high to low concentration areas.[146,147] Therefore, while migration is influenced mainly by the application of electric field,[144] diffusion is driven by the concentration gradient.[147]

Besides migration and diffusion, there are some important properties to be introduced. Although they are connected to and/or dependent on mass transfer in general, it is worth to explore them in order to comprehend their roles in sensing, the affected aspects, and the overall impact on sensing. Table 9 introduces such properties, along with relevant considerations.

Table 9. Properties related to migration and diffusion, along with their roles in sensing, affected aspects, and overall impact on electrochemical sensing.[143-148]

Concept	Role in sensing	Affected aspects	Impact on sensing
Electric field	Drives ion migration in electrolyte. Influences redox reaction rate at electrode.	Response time, Sensitivity	Strength of the field affects ion migration rate and can influence side reactions.
Velocity of ions	Determines ion transport speed to electrode. Affects electrochemical reaction kinetics.	Reaction kinetics, Efficiency of reaction	Higher velocity can improve reaction rate but may lead to non-uniform distribution of reactants.
Ionic mobility	Influences ion movement rate under electric field. Impacts electrochemical environment stability.	Sensor response, Accuracy in complex matrices	Higher mobility aids in faster establishment of equilibrium and better sensor response.
Ionic current	Proportional to fentanyl redox reaction. Used for quantifying fentanyl concentration.	Sensitivity, Signal-to-noise ratio	The magnitude of the current is directly related to the electrochemical reaction of interest.
Conductivity	Ensures efficient charge transfer. Reduces noise, enhances signal. Crucial for sensor environment.	Accuracy, Sensitivity, and Specificity	High conductivity is necessary for effective electron transfer during sensing.
Redox potential	Determines the potential at which fentanyl undergoes redox reactions. Essential for setting sensor operating conditions.	Selectivity, Operating voltage	Specific to fentanyl's electrochemical behavior; sets the necessary conditions for accurate detection.
Reaction kinetics	Affects the rate of the electrochemical reaction involving fentanyl. Influences the time to reach steady-state current.	Response time, Sensor stability over time	Faster kinetics lead to quicker sensor responses and more stable readings over time.

First, Table 9 introduces electric field, which drives ion migration, affecting the velocity of ions and ionic mobility. A higher electric field could lead to better sensitivity and response times, although could also yield higher noise signals, which can be challenging to be processed and analyzed.[144,148]

It is worth noting that ionic velocity and ionic mobility refer to how fast, and the ability of ions to move through the medium, respectively.[144] The first can deeply impact the reaction kinetics, which could also lead to the presence of improved signal responses.[144] The second can impact sensor response and accuracy in complex matrices, due to faster establishment of reaction equilibrium.[144] Conductivity is also another important parameter that can aid accuracy, sensitivity, and selectivity, and can be enhanced by appropriate electrolyte solution (medium)

and sensor design.[24,148] In addition, determining the correct redox potential can be extremely relevant, especially in techniques which sensing relies on reduction and/or oxidation peaks.[90,149] Lastly, understanding the reaction kinetics is crucial for deciding the correct approach to collect, process, analyze, and interpret the data.[144,148]

All the aforementioned parameters, including migration and diffusion, affect the ion flux, which is the net rate of movement of a species per units of area and time.[150] The ion flux is defined by the derivation of the Nernst-Planck equation, such that ion flux $J = -D\nabla C + Cv + \frac{Dze}{k_B T} CE$,[150] where D is the diffusion coefficient, ∇C the ionic concentration gradient, v the ionic velocity, z the valence of ionic species, e the elementary charge k_B the Boltzmann constant, T the absolute temperature, and E the electric field.[144] It is worth mentioning that the negative sign in front of D only indicate the direction of ion flux, consistent with Fick's first law of diffusion.[147] In electrochemical sensing, the ion flux plays a crucial role in signal generation, sensitivity, selectivity, response times, stability, and reproducibility.[144,148]

In the context of electrochemical sensing, current responses are usually more useful due to the influence of the surface area of the electrode on the signal response. It is defined that the current can be obtained by $I = JA$, such that A is the electrode's surface area and I is the electric current, defined as the rate of flow of electric charge through a conductor.[149-151] Hence, based on the relevant current equations for amperometry (Amper.), cyclic voltammetry (CV), differential pulse voltammetry (DPV), and square-wave voltammetry (SWV), $I_{Amper.}(t) = \frac{nFACD^{1/2}}{(\pi t)^{1/2}}$, $I_{CV} = 0.4463nFAC\left(\frac{nFDv}{RT}\right)^{1/2}$, $I_{DPV} = \frac{nFAD^{2/3}v^{1/2}C\Delta E_p{}^{1/2}}{RT}$, and $I_{SWV} = \frac{2n^2F^2A\omega DC}{RT}$, where n is the number of electrons, F is the Faraday constant, and t is the time. Notice that, in the four techniques, $I_\forall \propto D^\alpha, n^\beta, C, A$, such that $\alpha_{Amper.,CV,DPV,SWV} = {}^1/_2, {}^1/_2, {}^2/_3, 1$ and $\beta_{Amper.,CV,DPV,SWV} =$

$1, {}^{3}/_{2}, 1, 2$ (see Table 10 for the description of these techniques, along with models involved and the aforementioned equations as well as Appendix D for a description of variables and constants used in these equations).[144]

Bearing the current equations in mind, it is relevant to demonstrate how the four variables above can impact current responses. For simplification purposes, only the equation for amperometry is herein considered. However, since $I_{\forall} \propto D^{\alpha}, n^{\beta}, C, A$, the conclusions drawn hereinafter can be extended to CV, DPV, and SWV, within different orders of magnitude due to the different proportionalities and bearing in mind the currents expressed in the latter techniques correspond to the peak current. Figure 10 shows how Log(Current) vs. Log(Time) are affected by increasing D, n, C, and A values (see Appendix E for Python script that created the plots based on the current equation for amperometry).

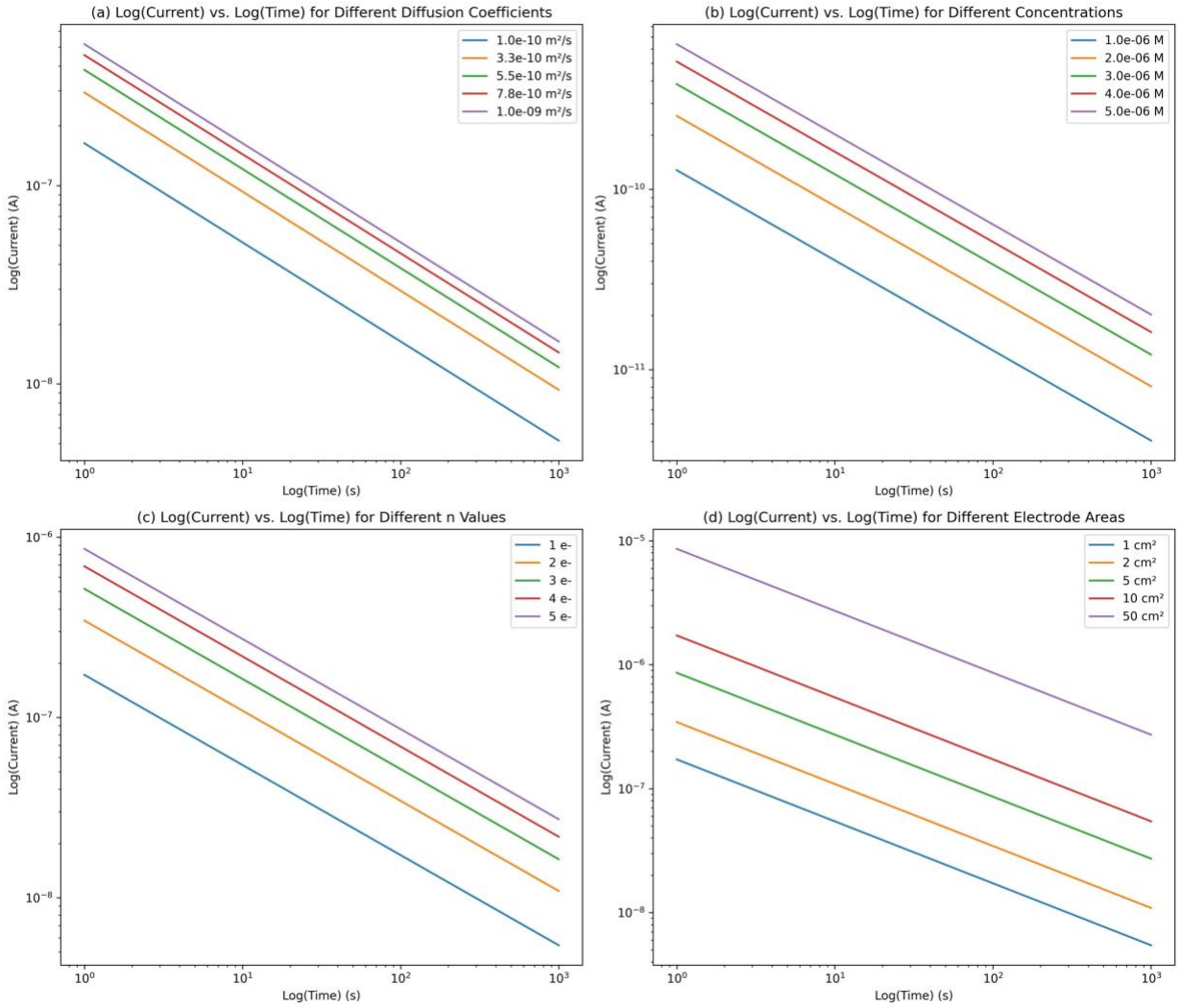

Figure 10. Four plots displaying Log(Current) vs. Log(Time) demonstrate how different diffusion coefficients (a), concentrations (b), number of electrons (c), and electrode areas (d) affect ion flux.

Since $I_\forall \propto D^\alpha, n^\beta, C, A$, plots in Figure 10 successfully demonstrate the impact of these parameters on the current responses of amperometry, CV, DPV, and SWV. In the case above involving amperometry, with increasing diffusion coefficients, concentrations, number of electrons, and electrode surface areas, higher current responses can be clearly observed across different times. The Log scale in the base 10 was used to facilitate the comparison between current responses across different times.

In the context of electrochemical sensing of real samples of FAMs, changes to the aforementioned parameters could potentially aid their detections. For diffusion coefficients, slightly increasing the temperature of the sensor system could increase the diffusion coefficient of the analytes, as the latter is directly proportional to temperature.[144] Furthermore, selecting a solvent that enhances mobility of FAMs molecules could also increase the diffusion coefficient, hence increasing the current, leading to better signals.[148] Although these solutions may not have a significant impact, understanding their effects could be helpful. On the other hand, changing the concentrations of analytes is not practical since the goal is to detect and quantify them.

Concerning the number of electrons, those are usually dependent on the electrochemical (redox) reaction that takes place between the FAM substance and the electrode's surface. For instance, in the electrooxidation of fentanyl, $n = 2e^-$, since two electrons are lost in the reaction.[149] However, these is some potential for this value to be increased by using side reactions that can take place in the presence of the desired analyte that involve a higher number of electrons. Lastly, increasing the electrode's area is feasible. To do so, electrode's surfaces can be modified by using high surface area-to-volume and/or porous nanomaterials, such as carbon nanotubes, polymers, nanocomposites, and nanoparticles.[23,75,90,152-154] Dropcasting such materials on the electrode can be an effective way to optimize the area by increasing the number of active sites available for the electrochemical reaction,[144,148] facilitating migration-driven mass transfer of the analyte's molecules from solution to the vicinity of the electrode, hence leading to higher currents and, consequently, enhanced signaling responses.

4.4 Overview of Electrochemical Techniques and its Models

In the previous subsection, amperometry, CV, DPV, and SWV were briefly mentioned in order to explain how different electrochemical properties can impact current responses,[18,23,90] which are crucial for electrochemical sensing of substances. Herein, these four techniques are explored in further detail, along with EIS and FETs. Table 10 shows the six main relevant methods for electrochemical sensing, along with additional details (see Appendix D for the descriptions of variables and constants present in the relevant equations).

Table 10. Main methods relevant for electrochemical sensing, along with their inputs, outputs, sensitivity levels, relevant models, and respective relevant equations related to the method.[25,144,146,148]

Method	Input	Output	Sensitivity	Relevant Models	Relevant Equation
Amperometry	Fixed potential	Current over time ($I(t)$)	High	Cottrell, Steady-State, Convection-Diffusion	$I(t) = \frac{nFACD^{1/2}}{(\pi t)^{1/2}}$
Cyclic Voltammetry (CV)	Cycled potential sweep	Current response (I_p)	Moderate	Butler-Volmer, Randles-Ševčík, Nicholson-Shain	$I_p = 0.4463nFAC\left(\frac{nFDv}{RT}\right)^{1/2}$
Differential Pulse Voltammetry (DPV)	Potential pulses on a linear sweep	Current at pulse intervals (I_p)	High	Laviron's equations, modified Randles-Ševčík for pulse techniques	$I_p = \frac{nFAD^{2/3}v^{1/2}C\Delta {E_p}^{1/2}}{RT}$
Square-Wave Voltammetry (SWV)	Square-wave potential pulses	Current at each pulse (I_p)	Very high	Osteryoung equation, non-equilibrium models	$I_p = \frac{2n^2F^2A\omega DC}{RT}$
Electrochemical Impedance Spectroscopy (EIS)	AC potential of varying frequency	Impedance (Z) over frequency range	Moderate to high	Equivalent Circuit, Randall's Circuit, CNLS fitting	$Z = Z' + iZ''$
Field-Effect Transistors (FETs)	Voltage-controlled electric field across conductive channel	Drain current (I_D)	Extremely high	Shockley for FETs, Charge Transport, BioFET, ChemFET	$I_D = \frac{1}{2}\mu C_{ox}\frac{W}{L}(V_{GS} - V_{th})^2$

Based on the information presented in Table 10, a few observations are worth mentioning. First, amperometry can be useful in the detection of FAMs due to models like the Cottrell Model and its related equation, which relates time-dependent current with the concentration of electroactive

species.[143,144] Therefore, amperometry can be well suited for real-time monitoring of FAMs as it can detect concentration changes with high sensitivity and provide instant responses, which could be especially promising in the context of continuous monitoring of important metabolites, such as norfentanyl, in blood, for example.[23]

For CV, Butler-Volmer and Randles-Ševčík models and the derived equation are extremely relevant, as they help explain the relationship between current and potential curves.[144,148] The peak currents may be correlated with concentrations of electroactive species,[148] which could corroborate to the detection and quantification of FAMs. In addition, these models can aid in enhancing the understanding of electrochemical properties and redox reactions involving FAMs, which could eventually help identify specific FAMs based on their unique electrochemical signatures. In the context of low-detection concentrations of FAMs, DPV can be a relevant, highly sensitive method.[21,23,90] The modified Randles-Ševčík model and its pulse-modified equation can further assist in determining the peak currents corresponding to the oxidation and reduction reactions of FAMs, hence enabling quantification of their concentrations even within complex matrices. In some contexts, SWV could potentially detect even lower concentrations of FAMs, by quickly and efficiently screening samples, since the method relies on quickly scanning wide potential ranges and measuring the current outputs.[144,153] This method could be useful for analyzing FAMs and determining their reduction and oxidation potentials as well.

Moreover, Table 10 highlights EIS and FETs, which could also be helpful for the detection and quantification of FAMs. The first utilizes circuit models (*e.g.,* equivalent and Randall's circuits) in order to interpret the spectrum of impedance, obtained as a result of applied alternating current (AC) potentials over varying frequencies.[92,144] EIS circuit models are used in

breaking down the impedance spectrum profile into elements (*e.g.*, resistive, capacitive, and inductive) that provide relevant information about electrochemical processing occurring at the interface between the electrode and the electrolyte solution.[70,113] By doing so, enhanced sensitivity towards interfacial changes, hence enabling deeper studies into the kinetics and mechanism between FAMs and the sensor surface. This understanding can help establish how to approach the detection and quantification of FAMs, hence why this method is often used as a characterization technique before selecting the appropriate detection method.[144,148]

Lastly, FETs can be used as biosensors or chemically sensitive FETs (*i.e.*, BioFETs or ChemFETs) that operate on the Shockley model for FETs and other charge transport theories relating gate voltage to drain voltage.[21,22,92] Binding of FAMs to receptors and recognition elements on the FET gate can modulate channel conductance,[148] hence altering the drain current which can be measured during detection of FAMs. This transduction mechanism, along with the very high sensitivity of surface-modified FETs,[21,92,104] could make this method highly suitable for quantification of FAMs at very low concentrations, even when mixed with complex biological matrices or other substances. The main challenge lies on identifying what types of receptors and recognition elements that target FAMs can be used to help modify, along with nanomaterials, the FET gate electrode.[21,104,155] Biomimetic approaches have been explored recently,[90] often inspired by the biochemical interactions described previously in *Subsection 2.2*. Further exploration into the applicability of modified FETs into sensing of fentanyl's metabolite norfentanyl is provided in *Subsection 6.6.3*.[21-23,104]

4.5 Sensitivity, Limit of Detection, Linear Range, and Other Concepts

Herein, a few important concepts are introduced here in order to facilitate the understanding of subsequent sections.

In the context of electroanalytical chemistry, sensitivity is the ability of an analytical technique to detect changes in the analyte concentration.[151] Usually, this ability is quantified by the slope of the calibration curve, which represents the signal change or related response per unit of concentration.[151,156] Developing electrochemical sensors with high sensitivity is crucial for detecting species at low concentrations or trace levels.[24,148] Regarding FAMs, high sensitivity is paramount, since substances like fentanyl, carfentanil, among others, can be extremely toxic and even deadly at very low concentrations.[62]

Limit of Detection (LOD) is the lowest concentration of a target analyte that can be detected, but not necessarily quantified, whereas Limit of Quantitation (LOQ) is the lowest concentration that can be quantified with appropriate accuracy and precision.[157] Mathematically, $LOD = k \times \frac{\sigma}{S}$ and $LOD = k' \times \frac{\sigma}{S}$, where σ is the standard deviation of the blank sample (to account for noise), S is the slope of the calibration curve, and k, k' are factors that represent the desired confidence level for detecting the target analyte above the background noise and are often set to ≈3 (3σ rule) and ≈10 (10σ rule), respectively.[151,156,157]

Besides LOD and LOQ, the linear range is also an important concept, as it represents the concentration span over which the analytical method yields a linear response proportional to the target analyte's concentration.[151,157] Since concentration can be quantitatively determined with high confidence at the LOQ, the linear range usually starts slightly above the LOQ and extends to the point at which the calibration curve is no longer linear.[151,156,157]

Lastly, in certain scenarios, the dynamic range can be also relevant, since it represents the concentration span over which the analytical method can provide relevant, reproducible measurements.[156] It entails both the linear and non-linear parts of the response curve, from the LOD until the saturation point or the point at which the target analyte gets depleted.[151,156] While the linear range only considers the direct, proportional relationship between response and the target analyte's concentration, the dynamic range can be relevant, especially when determining whether a detection method is useful, reliable, accurate, and quantitative enough.

In many recent studies on the electrochemical detection of FAMs,[21,23,90,104,153,157-160] the LOD and the linear range are mostly used due to easier comparisons as well as due to the fact the lower limit of the linear range is often only slightly above the LOQ. Nonetheless, LOQ reporting could be useful for facilitating accurate comparisons between electrochemical sensors with traditional, centralized analytical techniques.

5.0 Challenges of Electrochemical Sensing of FAMs

Although electrochemical sensing offers relevant and promising solutions for the detection of FAMs, there are several interdisciplinary challenges that impact the production, effectiveness, and widespread adoption of electrochemical sensors. These challenges are herein explored and include inherent limitations of electrochemical sensors that engender sensitivity concerns, selectivity challenges of FAMs with mixed-substance samples and in complex biological matrices, and technological and methodological constraints.

5.1 Inherent Electrochemical Sensing Limitations

There are some inherent limitations to electrochemical sensing as a method for detecting and quantifying FAMs. These limitations stem from the fundamental nature of electrochemical processes and the interactions between the sensor's components and the analyte, its biological matrices (*e.g.*, blood,[7] saliva,[106] sweat[28]), and other substances present (*e.g.*, heroin, cocaine,[95] other drugs[62-64]).

5.1.1 Detection Limits and Sensitivity Concerns

Main limitations in electrochemical sensing lie on attaining accurate detection of FAMs at the required sensitivity and detection limits. Due to the intrinsic potency and lethality of fentanyl and many of its analogues, trace amounts – often in picogram to nanogram levels – need to be detected, especially for public safety reasons.[104] Challenges for achieving high sensitivity levels

through electrochemical sensors concern electrode materials,[23] surface functionalization,[21] interference rejection,[18] miniaturization and integration,[90] and signal amplification.[28]

First, the development of advanced and integrated electrode materials with high electroactivity and surface area is challenging.[161,162] Although materials like nanocomposites,[23] conductive polymers,[163] metal-organic frameworks,[22] and CNTs[22] are promising candidates, integrating them into robust, sensitive, and reproducible sensing devices remains difficult. Reasons include lack of materials' stability over time, reproducibility, cost, and scalability.[140]

Nevertheless, surface functionalization of the aforementioned materials could represent a relevant opportunity. By modifying electrode surfaces with specific recognition molecules, such as antibodies,[21] aptamers,[158] or molecularly imprinted nanoparticles,[163] selectivity and sensitivity could potentially improve, resulting in detection at lower detection limits. However, challenges to this approach include the role of environmental factors, such as pH and ionic strength, in binding efficiency, the non-specific binding of surface's materials to other substances, which could lead to false positives, and the lack of stability of recognition molecules, especially antibodies and aptamers.[164]

Therefore, interference rejection is also an important goal when developing electrochemical sensors for detecting FAMs. Since these substances are often found in samples with diverse compositions and within complex biological matrices,[78] other interfering substances present could help mask or distort signals, leading to false negatives.[46] The varying concentrations of these interfering substances further complicates the challenge, prompting the need for increased sensitivity and selectivity towards FAMs.[164]

Moreover, another factor that could compromise sensitivity is the pressing demand for miniaturization and integration of the sensors, given portability and on-site detection needs.[57,90]

While advances in microfabrication and nanotechnology could help attain smaller sensors with integrated signal processing capabilities,[31] challenges regarding fabrication precision, power consumption, and data processing and connectivity still need to be overcome.[46] Implementation of statistical process control methods can be an effective way to enhance fabrication precision at the nanoscale.[165]

Lastly, signal amplification[152] and filtering[166-168] strategies need to be further developed in order to amplify FAMs signals while mitigating the signals of other interfering substances. With innovations in data analysis and machine learning,[33,73,90] further development of these strategies could aid the extraction of valuable information from sensors' signals, facilitating their processing, analysis, and interpretation.[148,149] When addressing the detection limits and sensitivity challenges, a deeper understanding of the role of interference from biological matrices and environmental conditions is crucial, as those can greatly impact sensor performance, reliability, and data quality acquisition.[31,46,71]

5.1.2 Interference from Biological Matrices and Environmental Conditions

The presence of complex biological matrices and varying environmental conditions imposes difficulties for the detection of FAMs, as these factors pose serious problems to the accuracy and reliability of sensors' readings.[16,31,71] Electrochemical sensors may be severely affected by the complexity of biological matrices (*e.g.,* blood, saliva, urine) due to the presence of various interfering substances, such as proteins, salts, and other metabolites.[16,46,78] These substances can bind to the sensor's surface, resulting in non-specific adsorption that diminishes or even inhibits the sensor's ability to selectively detect FAMs.[16,46,78,158] This interference may

result in decreased sensitivity and specificity, raising the likelihood of false positives or negatives.

Environmental conditions, including temperature, humidity, and pH, may also influence sensor performance.[4,71,145,168-170] Temperature changes can affect the kinetics of electrochemical reactions and stability of sensors containing biological recognition molecules, such as aptamers and antibodies.[23,167-171] Likewise, changes in humidity can alter the hydration layer of the sensor's surface, creating a barrier for signal response.[71,172] Lastly, pH changes can influence the charge and conformation of both the biological recognition molecules and the FAMs, potentially affecting the binding efficiency and, consequently, the sensor's sensitivity.[71,160,164,171]

To overcome these challenges, incorporation of specific binding sites and utilization of blocking agents could potentially prevent non-specific interactions. With this concern in mind, recent research approaches have included aptamer-[158] and antibody-functionalization[21,139] of CNTs, along with other functional groups that could help prevent unwanted interactions as well as protect sensor's functionalized CNTs from degradation.[71] In addition to physical modifications to sensor's surfaces, further data analysis and processing techniques could be implemented in order to differentiate between electrochemical responses of FAMs and their complex biological matrices.[4] To prevent interference from environmental conditions, enhanced calibration methods and data processing techniques that account for their influences could be adopted as well.[30]

Overall, developing robust and adaptable sensors that could selectively detect FAMs while also enduring different environmental conditions could be a solution to some of these challenges. To do so, innovative approaches to materials science,[4] sensor design,[91] and data processing[152,166-

[168] would be critical to overcome the aforementioned difficulties, with the goal of achieving higher levels of sensitivity and specificity.

5.1.3 Stability and Reproducibility Concerns

To reliably detect FAMs, electrochemical sensors need to show high levels of stability and results' reproducibility. To do so, materials (*e.g.*, functionalized CNTs) should not degrade, sensor fabrication should be consistent, and sensors should consistently detect substances in different biological matrices under different environmental conditions.[71,171] Besides measures that were previously mentioned to prevent materials degradation and inconsistencies in detection, the manufacturing process of these sensors should adhere to strict quality controls. This could be achieved by implementing statistical process control methods to ensure sensing of substances is consistently reliable.

5.2 Selectivity Challenges with Mixed-Substance Samples

5.2.1 Discrimination of Fentanyl from Analogues, Other Opioids, and Substances

Distinguishing between fentanyl, its various analogues, other opioids, and other often mixed substances has been a challenge for sensor-based detection of FAMs. This challenge emerges due to structural similarities between these substances, as previously explained in *Section 2.* Although centralized traditional methods of detection are capable of separating and identifying these substances, as demonstrated extensively in *Subsection 3.2*, further efforts need to be applied in order to develop electrochemical sensors. These efforts to prevent interference from other substances within a sample should focus not only on the nanofabrication of materials

(*e.g.*, functionalized CNTs), but also on advanced data processing techniques, capable of handling between mixed-substance signals effectively. This could potentially be achieved via signal amplification[152] and filtering[166-168] techniques.

5.2.2 Cross-Reactivity and False Positives/Negatives

Despite promising advancements and future directions of electrochemical sensors, cross-reactivity and false positives/negatives is still a limitation. To minimize these factors, reliable confirmatory tests via traditional centralized methods will likely remain extremely relevant.[26] Comparisons and statistical analyses between quantitative results of electrochemical sensors and such methods, together with advancements in machine learning,[4] could help pave the way towards more sensitive, selective, and reliable sensors.

5.3 Technological and Methodological Constraints

5.3.1 Choice of Electrochemical Techniques and Their Limitations

Table 11 below summarizes the main factors concerning the choice of electrochemical techniques towards the detection of FAMs.

Table 11. Different techniques, along with their purposes in FAMs detection, advantages, and limitations.[24,25,144,146,148,151,161]

Technique	Purpose in FAMs detection	Advantages	Limitations
Amperometry	Continuous monitoring of FAMs concentrations	Direct correlation with concentration enables real-time monitoring	Potential must be constant
Cyclic Voltammetry (CV)	Differentiate FAMs by CV spectra	High sensitivity and can reveal redox mechanisms	Overlapping peaks in complex samples
Differential Pulse Voltammetry (DPV)	Detect low concentrations of FAMs	Improved resolution and suitability for low concentrations	Interfering substances can affect signals
Square-wave voltammetry (SWV)	Trace level detection in complex samples	High sensitivity and rapid analysis	Electrode fouling or varying concentrations required
Electrochemical Impedance Spectroscopy (EIS)	Insights on binding mechanisms	Non-destructive	Difficult data interpretation and requires surface characterization
Field-Effect Transistors (FETs)	Highly selection detection via changes in conductance upon binding	Highly sensitive and viable for miniaturization	Sensitive to interfering substances and environmental conditions. Difficult large-scale fabrication.

Among the techniques presented in Table 11, Amperometry and EIS are the least feasible. Amperometry can only be performed at a constant potential throughout the detection process, which is a limitation due to the lack of versatility and adaptability when detecting FAMs at varying concentrations, mixed with other substances, and within complex biological matrices. While EIS can be a useful technique for studying binding mechanisms between FAMs and nanomaterials, its feasibility is compromised mainly due to the need for surface characterization, posing challenges to miniaturization and versatility.[148,149]

CV, DPV, SWV, and FETs offer the most feasibility towards the development of electrochemical sensors to quantify and detect FAMs. CV is highly sensitive and, like EIS, can also aid the studies of mechanisms and redox behavior of FAMs and their interactions with the nanomaterials deposited on electrodes. In some circumstances, DPV can enhance resolution and sensitivity of signals by applying a controlled, defined series of voltage pules, making it useful for detection of FAMs in low concentrations. Although SWV can be useful for trace level

detection of FAMs in complex samples, the technique requires electrode fouling and varying concentrations of the target analyte as standards, leading to fabrication and sample availability constraints, respectively. If these concerns are addressed, SWV can be an extremely feasible technique. Lastly, FETs can enhance resolution and sensitivity in low concentration samples due to the fact that, even small changes in mass or charge at the sensor's surface can yield noticeable changes in conductance. FETs also present high input impedance and can be fabricated on a very small scale, hence preventing signal distortion, and enabling miniaturization, respectively.[148,149]

In general, collecting and processing electrochemical data from these three techniques could be useful for detecting FAMs with sensitivity and selectivity. However, electrode preparation and surface modifications remain an important obstacle to overcome.

5.3.2 Need for Advanced Nanomaterials and their Importance in Sensor Modifications

At the forefront of electrochemical sensing technologies, there has been a growing tendency of integrating advanced nanomaterials into devices as a means of enhancing sensor sensitivity, selectivity, and stability. In the context of FAMs detection and quantification, nanomaterials offer a critical opportunity, given the needs previously outlined throughout this section. Nonetheless, selecting the appropriate nanomaterials can be a challenge, considering their importance in the sensor modification and fabrication processes. To better understand some of the different nanomaterial candidates, Table 12 summarizes them, highlighting their potential purposes in FAMs detection, along with advantages and limitations.

Table 12. Different nanomaterial candidates for modification and fabrication of electrochemical sensors, along with their potential purposes in FAMs, advantanges, and limitations.

Nanomaterial	Purpose in FAMs detection	Advantages	Limitations
Carbon Nanotubes (CNTs)[71]	Enhance sensor sensitivity and selectivity	High electrical conductivity, mechanical strength, and functionalization	Fabrication and signal processing
Conductive Polymers[172]	Enhance sensor sensitivity and stability	High conductivity and functionalization	Degradation over time and environmental sensitivity
Metal Nanoparticles[163]	Amplify signals	Catalytic properties and large surface-to-volume ration (spherical)	Lack of stability and potential for aggregation
Graphene[166]	Rapid electron transfer can enhance signal responses	High surface area and electrical properties	High number of defects and cost-prohibitive
Molecularly Imprinted Polymers (MIPs)[173]	Enhance selectivity via selective binding sites	High selectivity due to specific recognition sites	Limited reutilization and may lack stability

Table 12 shows five relevant nanomaterials that could be potential candidates for modification and fabrication of electrochemical sensors targeting FAMs.

Carbon Nanotubes (CNTs) are often considered one of the best nanomaterials for sensor development due to their high electrical conductivity and mechanical strength,[71,174,175] which are crucial properties for enhancing sensor sensitivity and selectivity.[71] Furthermore, CNT functionalization creates new opportunities for the design of highly specific interaction sites[71] that can help target FAMs, which could make CNT-based sensors particularly effective in discriminating between target analytes, mixed substances, and complex biological matrices. When compared to other nanomaterials, CNTs can often be more easily integrated into a variety of sensor platforms that perform different electrochemical techniques, enabling versatility in the sensor design and aiding miniaturization.[71]

Nonetheless, CNTs integration into sensor platforms is challenged mainly by three factors. First, achieving uniform dispersion of CNTs onto the sensors' electrode(s) can be difficult, due to CNTs tendency towards aggregation/bundling due to van der Waals force.[71] These aggregates/bundles can lead to inconsistent electrochemical properties,[71] hence diminishing

sensor sensitivity, stability, and consistency of measurements. To prevent this from happening, innovative surface protection strategies that can help prevent aggregation could be adopted, such as terminally linking CNTs to DNA polymers[176] or other nanomaterials. Nonetheless, the second challenge lies on the functionalization of CNTs, which can be challenging. Besides the need for functional groups that can effectively target FAMs, it is also important to ensure that functionalization does not compromise the integrity of CNTs, which could lead to degradation of their properties. Lastly, the complex and intricate fabrication process of functionalized CNTs needs to be scalable and cost-effective. Yet high-quality CNT synthesis and integration into sensors can be costly and difficult, hence limiting large-scale fabrication.[71] However, with the emergence and implementation of new nanofabrication techniques, this challenge could be resolved. Overall, CNTs remain a good candidate of nanomaterial for modifying electrochemical sensors that target FAMs, as further explored in *Section 6*.

Among other nanomaterials presented in Table 12, graphene might be considered the least ideal one. Despite its promising high surface area and electrical properties, graphene presents a variety of defects (*e.g.,* Stone-Wales, single vacancy, multiple vacancy, line, and carbon adatoms)[177] that can limit their accessibility and versatility for widespread sensor fabrication. Additionally, these defects can have substantial impacts on the electrical, chemical, and mechanical properties of this nanomaterial. Nonetheless, graphene defects can be dealt with by many material science strategies, including improved controlled synthesis,[71] post-synthesis treatment,[71] the use of graphene derivatives (*e.g.,* graphene oxide, reduced graphene oxide),[153] and combination with other materials (*e.g.,* nanoparticles,[163] polymers[23]).[155] All these strategies can help mitigate the impact of defects on graphene's properties,[155] hence facilitating sensor development and fabrication.[69] Despite the high cost of such strategies and challenges for large-

scale fabrication, graphene's propensity to defects could even be beneficial in the context of electrochemical sensing,[69] as the rational introduction of specific defects could help enhancing sensing of target analytes,[155] such as FAMs. Although this approach to graphene defect management has emerged recently,[178] rational defect engineering, aided by advancements in machine learning, could provide great opportunities in the field of electrochemical sensing technologies.

Besides CNTs and graphene, conductive polymers, metal nanoparticles, and MIPs can be excellent complementary materials, by both complementing one another and/or aiding CNT- and graphene-based sensor design.[71,173,176] Conductive polymers can provide a conductive and stable matrix in which metal nanoparticles can be embed, combining the polymers' stability and functionalization capabilities[172] with the catalytic properties and signal amplification potential of metal nanoparticles.[163] This combination may result in sensors with higher sensitivity and winder dynamic range, suitable for detecting FAMs. Likewise, MIPs can be combined to nanomaterials like CNTs or conductive polymers in order to enhance sensitivity and also improve selectivity by providing specific recognition sites. This way, it is encouraging that combinations of nanomaterials, together with strategic molecular targeting of FAMs, can pave the way towards electrochemical sensors that are sensitivity and selective, capable of detecting and quantifying FAMs, despite the challenges.

5.3.3 Data Acquisition, Analysis, and Interpretation

In addition to the previously explored challenges, data acquisition, analysis, and interpretation remain at the cornerstone of developing electrochemical sensors that target FAMs. There are two main data acquisition challenges to be addressed. First, there is lack of signal

stability and reproducibility when obtaining electrochemical signals, especially due to the presence of interfering substances and complex biological matrices.[21,22,160] Secondly, the demand for higher sensitivity and detection at lower concentrations is challenged due the need for trace detection of FAMs.[26,104,153] Lastly, as previously mentioned, interference from other substances may undermine sensor selectivity, which is crucial, especially with the emergence of new analogues.[4,9] CNT-based functionalization of electrochemical sensors could pave the way towards solving these problems, as they could provide stable, reproducible, sensitive, selective, and low-concentration data acquisition of electrochemical signals.[71]

Although recent advancements point towards improvements in data acquisition, analysis of electrochemical signals remains a challenge. Signal processing and noise reduction techniques need to be enhanced in order to filter out noise and interfering signals while retaining and potentially even amplifying target analyte(s') signals.[33,73] This could be achieved with data filtering and amplification techniques,[152] enhanced by current advancements at the forefront of data science, statistics, and machine learning.[4,33] These advancements give rise to other concerns related to reliability of algorithms and models focused on electrochemical signals. While overall advancements in the field could contribute to the analysis of electrochemical data, the principles of electrochemistry need to be further understood in order to apply and develop such algorithms and models. Inaccuracies in the process could lead to unreliable models that misinterpret the sensor's output, leading to false positives and negatives.

Developing data interpretation approaches to electrochemical signals of FAMs is also an important obstacle to be overcome.[4] The main challenge in this area lies on the lack of standardization and regulation concerning FAMs.[26] Since traditional centralized methods will likely remain the "gold standard" as confirmatory tests, it is crucial to develop databases

containing both electrochemical responses as well as data outputs from “gold standard” methods, as a means of potentially drawing upon correlations, conclusions, standards, and calibration approaches. This would allow more easily FAM-based detection to be integrated into current diagnostic frameworks for enhanced reliability and accuracy. Moreover, a robust database structure could provide clinicians and researchers with important tools to further develop machine learning algorithms and statistical techniques, aimed to identify and detect fentanyl, emerging analogues, and indirectly related biomarkers that could indicate exposure to FAMs. These advancements could lead to improved tackling of the fentanyl opioids crisis, with faster and more accurate detection.

6.0 Carbon Nanotubes (CNTs) to Selectively Enhance Electrochemical Responses

Herein, CNTs and their relevant properties are explored, from their basics to potential uses as a means of selectively enhancing electrochemical responses, especially in the context of detection and quantification of FAMs. Although there are other nanomaterials that could be suitable for these purposes, as explained in the previous section, CNTs are considered one of the most promising ones, hence the need to further understand their groundbreaking potential.

6.1 Definition and Types of Carbon Nanotubes (CNTs)

Late 20th century was marked by the advent of new paradigms concerning carbon-based materials, with the conceptualization, design, and synthesis of distinct allotropic forms of carbon, such as fullerenes and carbon nanotubes (CNTs).[174,175,179] Defined as helical or cylindrical rolled-up graphitic sheets,[175] CNTs can be single- or multi-walled, respectively abbreviated as SWCNTs and MWCNTs. The first type consists of a single layer of CNTs, whereas the second type consists of many interconnected nanotubes, enabling lengths of hundreds of nanometers.[71] Over the past three decades, CNTs have been widely researched and their physicochemical properties have been relevant for a variety of commercial applications in many areas, such as microelectronics,[139] medicine,[28] energy,[180] environmental remediation,[181] biosensing,[24,25,148,161] and chemical sensing.[25,31,71,90] Applications of CNTs take advantage of their remarkable physical, structural, electronic, and chemical properties, such as high electrical conductivity, thermal conductivity, tensile strength, and mechanical stability.[22,69,71,182]

6.2 Physicochemical Properties of CNTs and Suitability for Electrochemical Sensing

Physicochemical properties of CNTs make them extremely suitable for being used as nanomaterials for electrochemical sensors of FAMs. Table 13 summarizes the properties of CNTs, describes them, and briefly state the advantages of such properties in the context of electrochemical sensing of FAMs.

Table 13. Main properties of CNTs, with descriptions and advatanges for electrochemical sensing of FAMs.[71]

Properties	Description	Advantages for Electrochemical Sensing of FAMs
Electrical conductivity and high electron transfer rates	Metallic or semiconducting behavior due to arrangements of carbon atoms and delocalized electrons, facilitating electron movement.	Enhanced electron transfer yields improved signals, high sensitivity to changes in electron properties upon binding of FAMs, and rapid detection capabilities due to efficient conductivity.
Mechanical strength and durability	High tensile strength and elasticity due to strong covalent bonds and cylindrical structure.	Durable material for sensors, with long-lasting performance and stability in diverse environmental conditions, ensuring accuracy of responses.
Chemical stability and resistance	Chemically inert nature but has potential for functionalization to facilitate interactions.	Less degradation in complex biological matrices and less interference from other substances, enhancing selectivity. Functionalization can yield higher specificity to FAMs.
Tunability	Properties can be tuned via functionalization and structural modification.	Customizable selectivity and sensitivity towards FAMs in complex biological matrices, with adaptability to various sensors' types and techniques.
Modification and doping	Structural modifications and embedding of dopants can tailor electrical, physical, and chemical properties.	Fine-tuning of sensor's properties can enhance sensor performance. Sensors can be modified to possess better selectivity, sensitivity, stability, adaptability, and versatility.
Large surface area and electrocatalytic nature	Surface area provides more active sites for chemical interactions, while electrocatalytic nature can facilitate electrochemical reactions at the surface.	Sensitivity is enhanced by larger surface areas, as more binding sites are available. Signals can be amplified due to redox reactions involving FAMs being catalyzed by CNTs, potentially yielding better detection limits and sensor performance.

Bearing the aforementioned properties in mind, using CNTs to facilitate sensing of FAMs is shown to be a promising approach. First, electrical conductivity enables enhanced electron transfer, leading to better signal responses, which are further aided by the high sensitivity to changes in electron properties. This is important for the rapid and accurate detection of FAMs, because faster electron transfer can help ensure timely signal responses.

Furthermore, the inherent mechanical strength and durability of CNTs[71,149] can help make sensors reliable and stable under various conditions, which is suitable for long-term monitoring in diverse environments.[71,168,169,181] This robustness is relevant for long-term sensor performance and accurate detection. In addition, the potential for functionalization offers another crucial advantage. Strategic functionalization can provide resistance to degradation in complex biological matrices and aid selectivity to FAMs by choosing relevant functional groups and nanomaterials. Doing so could help reduce false positives by preventing interfering substances from triggering misleading signal responses.

Moreover, the tunability of CNT properties through functionalization and structural modification opens up the possibility of custom-designed sensors,[21,71,149,183] engineered towards adaptability, selectivity, and sensitivity. The latter can be further aided by modification of CNTs via dopants like nanoparticles, microfibers, and other nanomaterials.[153,155]

6.3 Sensing-Oriented Chemical Interactions Mediated by CNTs

The predominant chemical interactions mediated by CNTs, along with the countless possibilities for analyte-oriented functionalization, enable them to become excellent candidates for chemical sensing applications.[21] Herein, this subsection focuses on CNT-based sensing mechanisms that could be relevant for electrochemical sensing of FAMs. Such mechanisms are mediated by CNT-analyte interactions that may occur at the sidewalls of CNTs (*i.e.*, intra-CNT), at the spaces between CNTs (*i.e.*, inter-CNT), or between the CNTs and the metal electrodes (*i.e.*, Schottky barrier modulations), as shown in Figure 11.[71]

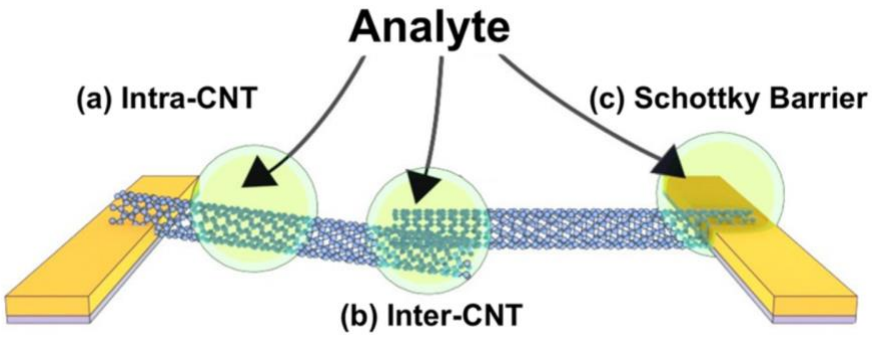

Figure 11. CNT-based sensing of analytes can occur via intra-CNT (a), inter-CNT (b), and Schottky barrier (c).[71] Reprinted (adapted) with permission from *Chem Rev*. **2019**, 119 (1), 599-663. Copyright 2019 American Chemical Society.

Figure 11 shows how a given analyte could be sensed by CNT-based sensors. It is important to note that, while it does not display functional groups attached to the CNTs, it is extremely relevant to understand that functionalization of CNTs can play a decisive role in sensing.[184] Bearing this in mind, there is actually a variety of sensing mechanisms that are not necessarily encompassed by the aforementioned figure. Such mechanisms could include interactions at the interface between CNTs and attached nanomaterials, for example. Nevertheless, for the purpose of introducing the general mechanisms, this work will focus hereinafter on the three main sensing mechanisms presented.

6.3.1 Intra-CNT Sensing Mechanisms

Intra-CNT sensing mechanisms are the ones that arise as a result of interactions between the analyte and the sidewalls of CNTs.[71] Predominant interactions involve π-π stacking, Van der Waals forces, covalent bonding, electrostatic interactions, and hydrogen bonding.[71] π-π stacking arises due to the staking of π-electron systems,[71] present in aromatic groups of fentanyl and its analogues, with the π-electron clouds of CNTs, which are prevalent especially in noncovalent

functionalization. As a result, these π-electron clouds facilitate the selective adsorption of aromatic compounds[71] (*e.g.,* fentanyl), hence enhancing sensor selectivity.

In addition to π-π stacking, the Van der Waals forces are weak intermolecular that arise due to induced electrical interactions between molecules and atoms of CNTs and of analytes.[71] In the context of sensing, Van der Waals forces contribute to physisorption phenomena, hence facilitating the adsorption of non-polar regions (or molecules) of FAMs, impacting sensor's baseline conductivity and response to target analytes.[70] Indirectly, covalent bonding can also contribute to the sensing of FAMs, due to the modifications to the CNT structure through strong chemical bonds between CNTs and the desirable functional group(s) that can help target specific analytes.[71] These functional groups can also help prevent sensor degradation by adding surface protection groups that can diminish the impact of complex biological matrices, for example.[71] Thus, with the introduction of functional group(s) mediated by covalent bonding, sensor specificity and stability can be significantly improved.

Electrostatic interactions can also play an important in CNT-based sensing of analytes.[71] These forces arise from attraction or repulsion between charged sites and/or polar groups present in the CNT's surface and in the analyte's molecules.[70,71] Depending on the overall structure of the CNTs, certain analytes may be selectively adsorbed by the CNTs, hence affecting the sensor's response characteristics.[71] Given the structure of fentanyl and many of its analogues, the presence of polar groups may help stabilize or disrupt the CNT-based sensor matrix, hence potentially giving rise or diminishing signal responses.

Lastly, hydrogen bonding between CNTs and analytes may also impact CNT-based sensing by aiding interactions with hydrogen bond donors or acceptors.[71] In the context of FAMs, hydrogen bonding with CNTs can play a crucial role in sensing. For example, due to the

presence of certain functional groups in the structure of fentanyl, it can act as both a hydrogen bond donor and acceptor. As a hydrogen bond donor, the nitrogen atom of the piperidine ring may donate a hydrogen atom to a hydrogen bond acceptor present in the CNT's sidewall structure. Alternatively, the amide group in fentanyl, which consists of a nitrogen atom connected to a carbonyl group, can act as a hydrogen bond acceptor. The nitrogen's lone electron pair may accept a hydrogen atom from a hydrogen bond donor present in the CNT's sidewall structure. Therefore, depending on the interaction and environment surrounding the analyte (*e.g.*, fentanyl or similar-structure analytes/metabolites), it can act as either a hydrogen bond donor or acceptor, contributing to the detection by CNT-based sensors.

6.3.2 Inter-CNT Sensing Mechanisms

Inter-CNT sensing mechanisms are the ones that arise as a result of interactions between the CNTs' interfaces and exposed molecules[71] (*i.e.*, target analytes, interfering substances). Predominant mechanisms are based on the modulation of intertube conduction and CNT network responses to analyte exposure.[71] The modulation of intertube conduction consists of physical changes in a CNT network due to the presence of an analyte.[71] Specific examples highlighted in literature include the swelling of polymeric matrices that encapsulate CNTs.[71] By correlating the swelling index with drops in conductance due to CNT's physical changes,[71] previous works have shown it is possible to distinguish between natural rubber composites[185] and, using a similar rationale, to detect porphyrins[186] and volatile organic compounds that were covalently[187,188] and noncovalently[189,190] attached to polymers. Alternatively, other works have shown that the disassembly of polymeric protective coatings around SWCNTs can be correlated with increase in conductivity, paving the way towards sensing based on polymer-based dewrapping around

SWCNTs.[71] By doing so, this approach has proven to be effective[71] in detecting diethyl chlorophosphite[182] and ionizing radiation.[191,192]

More recent works have further developed on these fundamental concepts of inter-CNT sensing mechanisms by combining SWCNTs with advanced materials, such as metal-organic frameworks (MOFs), to provide new sensing platforms.[22,23,31,184,186] For example, this recent work on SWCNT@MOF composites for norfentanyl detection[22] represents an important step in not only inter-CNT but also Schottky barrier modulation sensing mechanisms, paving the way towards MOF-modulated CNT-based sensors (see Figure 12 showcasing the graphical abstract).

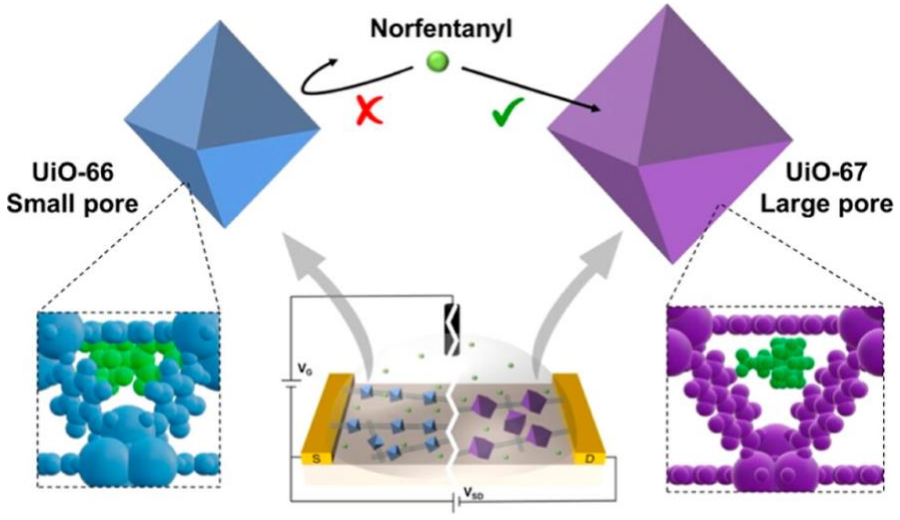

Figure 12. Graphical abstract of recent study showcasing how SWCNT@MOF composites can detect norfentanyl via size-matching and selective interaction, aided by FET sensing. Reprinted from *ACS Appl. Mater. Interfaces* **2024**, *16*, 1361-1369, with no changes, licensed under CC BY 4.0 DEED (https://creativecommons.org/licenses/by/4.0/).

The approach adopted in this work combined CNT's electrical properties with the selective filtering capability of MOFs as a means of potentially providing a sensitive and specific sensing technology.[22] Based on the interaction between the CNT networks and the MOFs, the core principle of this approach relies on MOFs modulating the electrical environment surrounding the CNTs, hence enabling the sensor to respond to target analytes by selective interaction and size-

matching, as shown in Figure 12. This process is aided by FET sensing, representing the integrated inter-CNT/Schottky barrier modulation aspects of the work.[22] Doing so leads to changes in conductance, leading to strong sensing responses towards norfentanyl when CNT was integrated with a specific composite (*i.e.*, SWCNT UiO-67).[22]

Although the achieved limit of detection (LOD) was of 48ng/mL, which is not very low when compared to other approaches,[22] it is important to highlight that this study successfully demonstrated the potential of MOFs to modulate CNT-based devices' conductance, by providing size-based detection of norfentanyl. Further improvements to the adopted strategy, such as further functionalization of CNTs[71] and tuning the composition and structure of the MOFs,[193] could help yield better results. Overall, this work represents a promising strategy to be considered when developing CNT-based sensors that rely on inter-CNT sensing mechanisms.

6.3.3 Schottky Barrier Modulation

Schottky barrier modulation relies on deliberate modifications to the height and width of the energy barrier formed at the junction of a metal and a semiconductor.[71,194] This energy (Schottky) barrier controls the movement of charge carriers (*e.g.*, electrons and holes) across the interface, hence affecting the electrical characteristics of the junction.[71,194]

The modulation of Schottky barriers, commonly triggered at the junctions between CNTs and metal electrodes, is an important approach to improve the performance of electrochemical sensors.[21,71] It consists of a combination of interactions, based upon electrodes' metal choice, CNT deposition methods, and the interactions of the sensor matrix with the target analytes.[184,194] All these factors can affect sensor responsiveness to specific analytes, hence showcasing the relevancy of Schottky barrier modulation in the context of sensor design and optimization.

The nature of interactions between substances and the Schottky barrier-modulated sensor matrix yields important implications.[21,23,92,194] By modulating the Schottky barrier, sensor responsiveness to analytes can be increased or decreased.[194] Doing so enables customization of sensor functionality, hence becoming an essential consideration in the context of designing CNT-based sensors for the detection of FAMs.[21] To detect substances and understand their interactions with CNT-based electrodes, different characterization techniques can be used, such as cyclic voltammetry (CV) and field-effect transistors (FETs) measurements, which allow for measuring current and conductance, respectively.[21,23,184] These approaches can enable the development of more selective and sensitive electrochemical sensors.[71]

Beyond the molecular interactions at the Schottky barrier, the overarching device architecture can be critical to sensor performance.[71] Sensor design should consider materials used for electrodes as well as CNT deposition techniques in order to maximize sensors' relevant signal responses.[71,153] The strategic optimization of device architecture can help isolate and amplify desired sensing signal responses from target analytes, hence increasing sensor selectivity and sensitivity.[71,153]

As an example of Schottky barrier modulation in sensor design, researchers have been able to develop an ultrasensitive norfentanyl sensor fabricated from CNT-based FET as a means of detecting fentanyl exposure in urine (see Figure 13).[21]

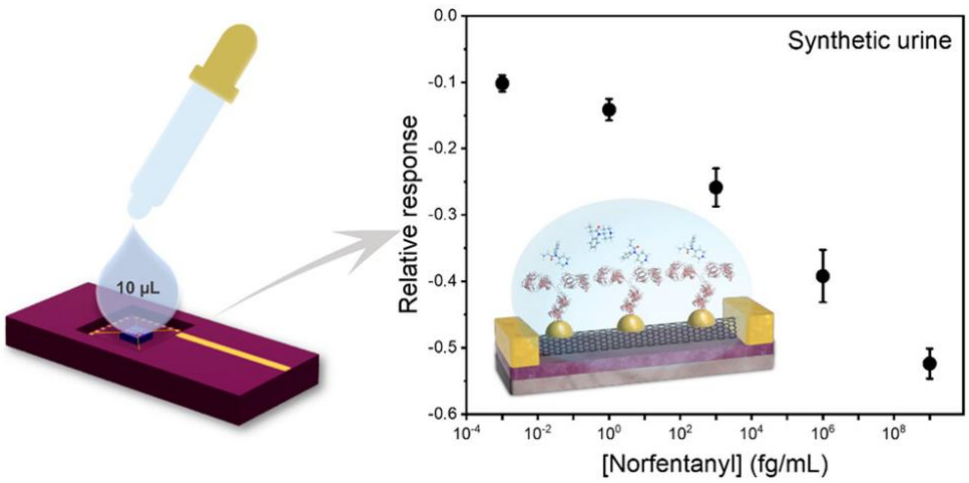

Figure 13. Graphical abstract of recent studying showcasing the development and application of semiconductor enriched SWCNT-based FET biosensor towards the detection of norfentanyl at extremely low concentrations (fg/mL scale). Retrieved from *ACS Appl. Mater. Interfaces* **2023**, *15*, 31, 37784-37793, with no changes, licensed under CC BY 4.0 DEED (https://creativecommons.org/licenses/by/4.0/).

The work shown in Figure 13 illustrates the intricate interplay between device architecture and interactions at junctions between CNTs and metal electrodes that significantly affect sensor performance.[21] To do so, the study presented a semiconductor-enriched SWCNT-based FET biosensor attached to norfentanyl antibodies for the detection of norfentanyl metabolite in urine samples.[21] Exploring different sensor configurations and an oriented immobilization strategy for the antibodies yielded an extremely low limit of detection (LOD) for norfentanyl, showcasing the sensor's ultrasensitivity and reliability.[21] The modulation of the Schottky barrier between the SWCNTs and metal electrodes played a key role in this remarkable achievement.

To attach the antibodies, two approaches were adopted, namely direct coupling and a gold nanoparticle (AuNP) method, and both affect the electrical characteristics of the sensor and the interactions with the target analyte.[21] These methods highlight the device architecture as a key element of sensor design, where the structure of the sensor matrix affected the sensor performance. Antibody functionalization substantially improved sensor performance, by altering conductance responses, shifting threshold voltages, and changing Schottky barrier's heights.[21]

Both approaches achieved outstanding LODs (*i.e.,* 2.0 fg/mL and 3.7 fg/mL, respectively),[21] with the AuNP approach providing a more robust platform for antibody functionalization, less sensitive to interfering substances, often present in real-world samples. In summary, outcomes and conclusions of this study should be thoroughly considered and investigated when designing CNT-FET-based sensors that rely on Schottky barrier modulation to detect FAMs.

6.4 Sensing-Oriented CNT Functionalization Strategies

Sensing-oriented functionalization strategies for CNTs have been developed to enable sensing of various target analytes, such as environmental pollutants,[145,181] gases,[186,188] biomolecules,[173,192] and various other substances.[71,184] These strategies aim to enhance the inherent properties of CNTs, such as high surface area, electrical conductivity, and mechanical strength, [71] in order to suit the desired sensing applications. The functionalization approaches can be broadly divided into noncovalent and covalent methods, which offer different advantages depending on the applications.[71,184] Tailored to target analytes in different contexts, noncovalent and covalent functionalization strategies leverage the unique properties of CNTs to fabricate sensitive, selective, and stable sensors.[71,184]

6.4.1 Noncovalent Functionalization

Noncovalent functionalization relies on the physical adsorption of molecules onto the CNT surface without the formation of covalent bonds, which can be useful for maintaining the integrity of CNTs, their structure, and desirable properties.[71,184] This can be achieved through previously mentioned interactions, such as $\pi - \pi$ stacking with aromatic molecules, van der

Waals forces, and electrostatic interactions.[71] This approach can be particularly relevant in the context of sensor fabrication, as noncovalent functionalization can help increase the solubility and dispersibility of CNTs in different media,[71,150] facilitating deposition onto electrodes while ensuring solution homogeneity.

Specifically, $\pi - \pi$ stacking and van der Waals are crucial to the absorption of aromatic molecules and surfactants onto the CNT surface,[193] which could help enhance selectivity and sensitivity of CNT-based sensors towards aromatic compounds (*e.g.,* fentanyl and some of its analytes) and non-polar molecules.[71] Furthermore, noncovalent functionalization through wrapping of CNTs with polymers (*e.g.,* conducting polymers,[173,182] DNA,[176] and other biopolymers[172,189]) can aid specific binding sites for target analytes. As previously discussed, these polymer-wrapped CNT-based sensors can boost enhanced selectivity via a variety of supramolecular interactions, such as hydrogen bonding, metal-ligand coordination, and host-guest chemistry.[71]

6.4.2 Covalent Functionalization

Alternatively, covalent functionalization relies on chemical bonds between functional groups and the CNT's surface. By covalently attaching different functional groups, biomolecules, and nanoparticles, electrochemical sensors with high specificity towards target analytes can be fabricated.[71] Adopted methods include modular functionalization, which enables the attachment of multiple functional groups, thus increasing specificity.[71] Another method relies on end-tip functionalization, which consists of modifying CNT's ends with specific groups or molecules.[71] This approach is particularly promising, as modifications can provide selective

binding sites for target analytes, while preserving the conductive properties of the CNT's sidewalls.[71]

Decoration of CNTs with metal nanoparticles, such as Pd, Au, or SnO_2,[163] have shown to improve their catalytic and electron transfer properties,[71] enabling sensing with high sensitivity of gases like H_2, CO, and NO_2.[178] Furthermore, hybrid materials composed of CNTs covalently bound to different chemicals, such as metal oxides or conducting polymers, have been proven to enhance detection capabilities for a variety of analytes.[23]

6.5 CNT-Based Materials and Electrochemical Sensing of FAMs

6.5.1 Sensing of Fentanyl

Herein, seven recent studies on CNT-based electrochemical sensing of fentanyl are explored, with the goal of comprehending how their approaches could help contribute towards the development of more rapid, sensitive, selective, reliable detection and quantification methods. All studies took advantage of the direct electrooxidation of fentanyl on the surface of modified electrodes in order to obtain current responses that correlate with concentrations of fentanyl. To better comprehend this process, Figure 14 shows the proposed mechanism for fentanyl oxidation, consistent with reaction mechanisms of the oxidation of drugs with similar structures, namely domperidone and itraconazole.[23,149,154]

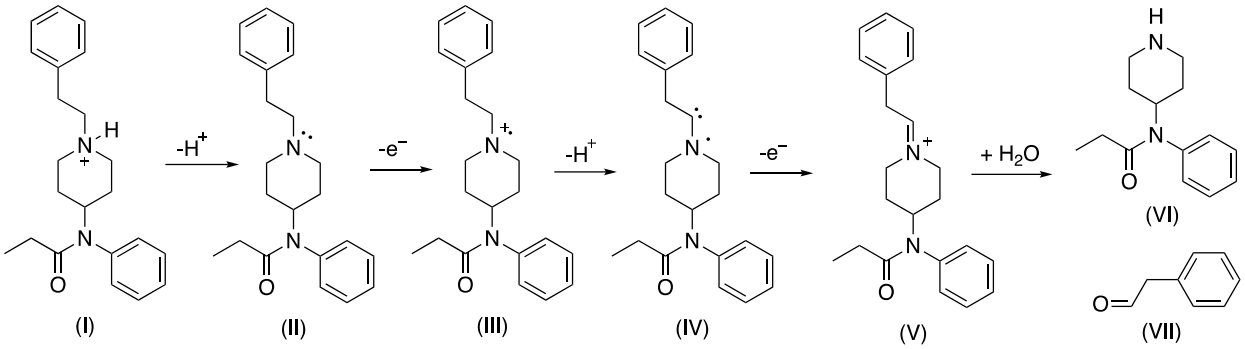

Figure 14. Proposed mechanism for fentanyl electrooxidation.

In Figure 14, fentanyl molecule in solution is represented by I and II, which are protonated and deprotonated, respectively.[23,149,154] Fentanyl, as shown in II, loses an electron from the piperidine ring's nitrogen in order to form an intermediary radical cation (III).[23,149,154] Then, III loses a proton, forming an unstable radical (IV), which, seeking stabilization, loses another electron, yielding a quaternary Schiff base (V) base.[23,149,154] This base undergoes hydrolysis, [23,149,154] forming norfentanyl (VI) and 4-anilino-N-phenethylpiperidine (VII, 4-ANPP), which are also the two major metabolites of fentanyl degradation in the human body.

It is worth mentioning that, although some studies have proposed mechanisms for the electrooxidation of fentanyl based on the oxidation of similar drugs and related electrochemical experiments,[23,149,154] there are many questions surrounding the mechanism. Some studies have raised concerns over norfentanyl molecules not being able to be reduced back to fentanyl.[153] However, this could potentially be explained by the hypothesis that the electrooxidation of fentanyl is irreversible, hence why no reduction peaks of norfentanyl can be observed.[149]

Table 14 showcases the seven different studies that rely on the electrooxidation of fentanyl and on the design and modification of electrodes as a means of detecting the substance.

Table 14. Summary of seven important studies that rely on the electrooxidation of fentanyl.

Studies (referred as 1-7, in order)	*Sens Actuators: B. Chem* **2019**, 296, 126422[90]	*J Am Chem Soc* **2020**, 75, 1209-1217[149]	*Mater Sci Eng C* **2020**, 110, 110684[154]	*Microchim Acta* **2023**, 190, 414[159]	*ACS Appl Mater Interfaces* **2023**, 16, 190−200[160]	*Alex Eng J* **2024**, 87, 515-523[23]	*Microchim Acta* **2024**, 191, 159[69]
Detection method(s)	SWV	DPV	CV	CV	CV	CV	DPV
Type of electrode	Carbon screen-printed electrodes (SPE)	Glassy Carbon Electrode (GCE)	GCE	Reduced graphene oxide GCE (rGO/GCE)	SPE	GCE	GCE
Materials deposited on electrode	MWCNTs, polyethylenimine (PEI), and ionic liquid mixture (IL)	MWCNTs	Carbon NanoOnions (CNOs)	Flower-like Covalent Organic Frameworks (TpTa-COFs)	Naloxone-AuNPs@ ZIF-8 nanocomposite	Graphitic carbon nitride (g-C_3N_4) and polyaniline (PANI)	Vacancy-rich r-$Fe_2(MoO_4)_3$:MWCNTs composite
Deposition method	Dropcasting of MWCNT-PEI-IL hydrogel composite nanomaterials at room temperature	Abrasion immobilization of MWCNTs onto preheated (for 5 min at 50°C) GCE	Dropcasting of CNOs/DMF dispersion onto GCE, then heating at 50°C	Dropcasting COFs/EtOH dispersion onto rGO/GCE, then drying with infrared lamp	Dropcasting of nanocomposite/N-methyl-2-pyrrolidone suspension onto SPE	Dropcasting of g-C_3N_4-PANI/HCl suspension onto GCE, then drying with infrared lamp	Dropcasting of r-$Fe_2(MoO_4)_3$:MWCNTs/water suspension onto GCE
Media and/or biological matrices	Phosphate buffer solution (PBS) (0.1 M, pH 7.4) and fentanyl powder	PBS (0.1 M, pH 7.4), human blood serum, and urine	Phosphate buffer (PB) (0.1 M, pH 7.0), human blood serum, and urine	Human blood serum, interfering substances in PBS (67 mM, pH 7.38)	PBS (unreported concentration, pH 7.2) and urine	PBS (0.1 M, pH 7.5) and urine	PBS (0.1 M, pH 7.0), human blood serum, and urine
Interfering substances	Acetaminophen (APAP), caffeine, glucose (Glu), theophylline	Uric acid (UA), ascorbic acid (AsA)	Glu, salts, H_2O_2, fructose, cysteine, sucrose, citric acid	AsA, Glu, UA, methamphetamine (mAMP), morphine	Heroin, morphine, cocaine, and sufentanil	AsA, Glu, UA, APAP, salts, mAMP, caffeic acid, cocaine, quinine	Urea, UA, Glu, AsA, citric acid, APAP, salts
LOD/µM, Linear ranges* /µM and respective R^2 values	10	0.1	0.3	0.033	29.72	0.006	0.006
	10-100	0.5-100	1-10; 10-60	0.1-0.99; 0.99-6.54*	297.2-2972	10-920	0.02-0.2; 0.2-10*
	0.988	0.9979	0.9893; 0.9925	0.9907; 0.9914	0.99	0.99977	0.9924; 0.9998

* Linear ranges herein reported were obtained by two different experiments with same controllable parameters and conditions, hence complementing each other.

The research documented in studies 1-7 shown in Table 14 represents important advancements in the design of fast, sensitive, and reliable detection system. At the forefront of these studies, the novel materials and electrochemical techniques employed contribute to understanding different approaches to high-performance electrochemical sensing of fentanyl.

Studies 6 and 7 achieved very low LODs (both 0.006 μM),[23,69] due to advanced electrode modification and material selection strategies. In study 6, the electrode is modified by graphitic carbon nitride (g-C_3N_4), which presents high surface area and conductivity, and polyaniline (PANI), a conductive polymer.[23] This hybrid structure facilitates electron transfer and increases the surface area available for analyte interaction,[23] hence yielding enhanced sensitivity, as evidenced by the low LOD. Similarly, study 7 used a vacancy-rich r-$Fe_2(MoO_4)_3$:MWCNTs composite, combining the catalytic properties of r-$Fe_2(MoO_4)_3$ with the high surface area and conductivity of MWCNTs.[69] This composite enabled a high sensitivity and selectivity towards fentanyl, enabling study 7's sensor to detect fentanyl in various media, including blood, with a LOD of 6 nM,[69] which is 10 times less than the reported fentanyl's lethal concentration in blood (60 nM).[56,114] Therefore, in both studies, strategic engineering of these nanocomposites and their deposition onto electrodes' surfaces demonstrates the critical role of sensor design in fentanyl sensing.

Overall, all seven studies emphasized the importance of thoughtful, strategic engineering of CNT-based sensors as a means of detecting fentanyl. For example, studies 1, 3, and 5 investigated the potential of MWCNTs, Carbon NanoOnions (CNOs), and Zeolitic Imidazolate Frameworks (ZIFs, a type of MOF) to improve fentanyl sensing sensitivity and selectivity.[90,154,160] The excellent electrical properties and large surface areas of these

nanomaterials were shown to offer good platforms for electrooxidation of fentanyl, hence enabling consistent and reliable signaling responses.

Studies 2 and 4 further emphasize how innovative material selections and strategic electrode modifications can significantly improve the performance of electrochemical sensors targeting fentanyl. To do so, study 2 showcases a GCE modified by MWCNTs, used for fentanyl detection via DPV.[149] The high electrical conductivity and the large surface area of the MWCNTs are critical for sensing, as they enhance the electroactive surface of the GCE.[149] This modification enables faster electron transfer and more active sites for interaction with fentanyl molecules, this improving sensitivity and selectivity.[149] The abrasion immobilization of MWCNTs onto the GCE aided sensor stability,[149] which is essential for reliability and reproducibility of sensor's measurements, especially when exposed to biological matrices like human blood serum and urine.

Alternatively, study 4 investigated the detection of fentanyl through CV, with reduced Graphene Oxide (rGO)/GCE electrode modified with flower-like Covalent Organic Frameworks (TpTa-COFs).[159] The integration of COFs onto the conductive rGO substrate is a promising approach that combines COF's high surface area and porosity with rGO's high conductivity.[159] By doing so, enhanced electrochemical performance can be achieved, as the COFs provide a structured, high-surface-area matrix, resulting in faster electron transfer and increased sensitivity.[159] Long-term stability and reliability of the sensor were achieved with dropcasting the COFs, followed by drying with an infrared lamp,[159] ensuring good adhesion of the nanomaterials to the modified electrode.

Deposition techniques like dropcasting were a key element for the application of these advanced nanomaterials. This method, illustrated in all studies expect Study 2, guarantees a

uniform and stable layer of sensing material on the electrode surface, crucial for consistent and reliable sensor performance. Later treatments, such as heating or drying, help improve the adhesion and stability of the material on the electrode and contribute to the reproducibility of signaling responses.

The reproducibility is complemented by the detection techniques selected, namely CV and DPV, further aid high sensitivity and specificity. By combining CNT/nanomaterials-based functionalization of CNTs, all studies have shown that CV and DPV are capable of yielding satisfactory signaling responses in the form of currents, which were correlated with concentrations of fentanyl in a variety of samples, even when mixed with several interfering substances and within different biological matrices.

In summary, studies 1-7 point towards a comprehensive approach towards the development of more sensitive and selective electrochemical sensors for the detection and quantification of fentanyl. The high LODs in studies 6 and 7 demonstrate the potential of using advanced nanomaterials to modify electrodes, followed by sensitive and selective signaling responses obtained by CV or DPV. Study 7 pivoted by detecting not only fentanyl but also other analogues, without interference, hence its performance becoming the focus of the subsequent subsection.

6.5.2 Sensing of Fentanyl Analogues

Study 7 is noteworthy due to the development of a sensor capable of detecting not only fentanyl but also three analogues with high levels of sensitivity and selectivity.[69] To do so, vacancy-rich r-$Fe_2(MoO_4)_3$ and MWCNTs were used to modify GCEs.[69] Sensor performances were outstanding, with remarkably small LODs and LOQs, as shown in Table 15.

Table 15. LODs, LOQs, Ranges, and R^2 values of fentanyl, sufentanil, alfentanil, and acetylfentanil in Study 7.[69]

Analytes	Fentanyl	Sufentanil	Alfentanil	Acetylfentanil
LOD/μM	0.006	0.007	0.018	0.024
LOQ/μM	0.02	0.023	0.06	0.08
Linear ranges*/ μM	0.02-0.2; 0.2-10	0.02-0.2; 0.2-10	0.04-10	0.1-10
R^2 values	0.9924; 0.9998	0.9973; 0.9991	0.9990	0.9996

* Linear ranges herein reported were obtained by two different experiments with same controllable parameters and conditions, hence complementing each other.

When compared to lethal concentrations of fentanyl, sufentanil, alfentanil, and acetylfentanil, these LODs are very small (*i.e., 6 nM* ≈ 10 times smaller than the reported 60 nM lethal concentration of fentanyl in blood[57,115]). Since sufentanil is 5-10 times more potent than fentanyl,[83] the LOD of 7 nM is considered satisfactory enough for preventing most overdose deaths. Although lethal doses for alfentanil and acetylfentanil are not well-defined, they are estimated to be less potent than fentanyl,[84,85] rendering the LODs of 18 nM and 24 nM potentially sufficient to prevent overdose deaths related to these substances as well.[69] Such low LODs reflect the high sensitivity of the developed electrochemical sensor, which is crucial for early detection in biological samples.

The LOQs for the aforementioned analytes are also small, especially for fentanyl and sufentanil, indicating the sensor's ability to quantify them at low concentrations. Given these two analytes are the most potent among the four, these LOQs can be considered satisfactory, although further improvements could be desirable, particularly for sufentanil, given its higher potency and lethal dose than fentanyl. Additionally, further improvements to LOQs could be extremely crucial for analysis of trace levels and to help achieve better understanding of the pharmacological effects at different consequences, below the lethal ones.

The linear ranges were determined to be 0.02-10 μM for fentanyl and sufentanil, 0.04-10 μM for alfentanil, and 0.1-10 μM for acetylfentanil,[69] reflecting the wide ranges of concentrations that the sensor is capable of detecting the presence of these substances. This is

important to enable sensing of different concentrations of fentanyl and its analogues present in different samples and their compositions. These ranges were all reported with R^2 values higher than 0.99, indicating good linearity of the sensor's response. This linearity can allow for accurate quantification over the ranges, potentially increasing the sensor reliability when exposed to real-world samples.

Overall, study 7 presents a prime example of how to take advantage of CNT-based electrochemical sensors, by taking advantage of the properties of specific nanomaterials and MWCNTs in order to enhance sensitivity and selectivity towards not only fentanyl but also three of its analytes.[69] By doing so, sensors like the one reported could provide an effective tool to help tackle the opioids overdose crises. In future studies, it could be relevant to attempt to develop sensors capable of detecting an even larger variety of analytes, while maintaining sensitivity, selectivity, reliability, and stability.

6.5.3 Sensing of Fentanyl Metabolites

Fentanyl degradation in the body results in two main metabolites, namely norfentanyl and 4-ANPP.[109,116] Since the first is the major metabolite, current approaches to detect fentanyl exposure have focused on the detection of norfentanyl, especially in urine[108,110] and interstitial fluid (ISF) samples.[57]

Table 16. Five important studies focused on norfentanyl detection through a variety of methods and aided by diverse modified electrodes.

Studies (referred as 8-12, in order)	*J Am Chem Soc* **2020**, 142, 5991-5995[57]	*ACS Nano* **2022**, 16, 3704-3714[104]	*Anal Chem* **2022**, 94, 12706-12714[153]	*ACS Appl Mater Interfaces* **2023**, 15, 37784-37793[21]	*ACS Appl Mater Interfaces* **2024**, 16, 1361-1369[22]
Detection method(s)	SWV	Aptamer-based Graphene Field-Effect Transistor (AptG-FET), yielding correlation between modulated voltage shift and analyte concentration	SWV	Semiconductor-enriched (*sc*-) SWCNT-based FET with norfentanyl antibodies attached via direct coupling or AuNP approach	SWCNT@MOFs-based FET, with focus on size-matching detection
Type of electrode	Hollow microneedle working electrodes filled with carbon paste	G-FETs with integrated side-gate Pt electrodes.	Reduced graphene oxide GCE (rGO/GCE)	*sc*-SWCNT-based FET, with interdigitated gold source and drain electrodes on a Si/SiO_2 chip	Interdigitated electrodes modified with SWCNT@MOF composites to create FETs
Materials deposited on electrode	MWCNTs and hybrid of AuNPs with a reduced graphene film	Aptamers specific to three opioid metabolites were functionalized on graphene surface of G-FETs.	GO was deposited onto the GCE and electrochemically reduced to form rGO	*sc*-SWCNTs were deposited to form conducting channels and provide a platform for antibody functionalization	Variations of SWCNT@UiO-MOF composites with different pore sizes
Deposition method	Filling of electrode microneedles with carbon paste	Functionalization with a 1-Pyrenebutanoic Acid Succinimidyl Ester (PBASE) linker, followed by incubation with aptamers	Electrophoretic deposition, followed by electrochemical reduction	Dielectrophoresis (DEP) for *sc*-SWCNTs between interdigitated electrodes directly or together with AuNPs, for the second approach	DEP of SWCNT@MOF composites on prefabricated interdigitated electrodes
Media and/or biological matrices	Skin-mimicking gel (agarose)	Wastewater samples	PBS	PBS and synthetic urine	PBS
Interfering substances	Nerve agents	Noroxycodone and EDDP (also target analytes; opioids metabolites)	Fentanyl, alfentanil, carfentanil, lorazepam, heroin, cocaine, caffeine, sucrose	Other opioids' metabolites, *i.e.*, normorphine (NM), norhydrocodone (NH), 6-acetylmorphine.	Other drugs' metabolites: NM, NH, and benzoylecgonine (BZ)
LOD/μM, ranges/μM and respective calibration sensitivities	Not reported	$1.83 \cdot 10^{-4}$	Detection not achieved due to lack of significant redox peak	Direct coupling and AuNP approaches	$2.092 \cdot 10^{-4}$
	40-400	Not reported.		$8.61 \cdot 10^{-9}$ and $1.59 \cdot 10^{-8}$	Not reported
	Not reported	Not reported; estimated to be ≈0.106		$4.30 \cdot 10^{-13}$ - $4.30 \cdot 10^{-10}$ and $4.30 \cdot 10^{-10}$ - $4.30 \cdot 10^{-7}$	Not reported
				0.069 and 0.021	

The recent developments in electrochemical sensors for norfentanyl detection illustrated in Table 16 are remarkable, as they highlight the use of different nanomaterials and sensing methods in order to increase sensitivity and selectivity.

It is worth mentioning that study 8 was focused on not only detecting norfentanyl but also nerve agents, given public safety concerns related to these substances.[57] An innovative approach to electrode design yielded hollow microneedle working electrodes filled with carbon paste, which were then used to deposit MWCNTs and a hybrid of AuNPs with a reduced graphene film.[57] The study claims to be able to successfully detect and differentiate between nerve agents and norfentanyl through induced electrooxidation of fentanyl (observed by a SWV peak), although LODs were only reported for the sensing of nerve agents. Additionally, given concerns over proposed mechanisms of the electrooxidation of fentanyl, it is concerning that the study relies on the electrooxidation of fentanyl to norfentanyl as a means of detection, especially without performing a thorough characterization of this redox reaction. This concern is further exacerbated by the fact that artificial ISF, used in the experiments,[57] may not fully encompass the complex composition of real ISF. Nonetheless, the study provides an interesting model for skin-based microneedle sensing that could reveal exposure to dangerous nerve agents and opioids.[57]

Also using SWV, study 10 focused on detection using a rGO/GCE-based sensor to target a variety of substances. By doing so, it achieved some remarkable achievements by successfully detecting fentanyl with a LOD of ≈6 nM, consistent with studies 6 and 7 presented in Table 14. The method was also sensitive to fentanyl analogues, namely carfentanil and alfentanil. Sensing was shown to be selective, with no interference due to the presence of substances like heroin, cocaine, caffeine, and sucrose. However, the study was unable to obtain redox peaks for norfentanyl, which prevented detection. Thus, further studies on the mechanisms of redox

reactions involving the fentanyl-norfentanyl pair could be critical to aid the development of reliable electrochemical sensors.

Studies 9 and 11 both relied of aptamer-based approaches combined with FETs to detect norfentanyl, in the presence of other opioids' metabolites, with no interference.[21,104] While study 9 relied on graphene-based FET,[104] study 11 took an innovative approach, taking advantage of the properties of *sc*-SWCNTs in order to produce the norfentanyl sensor with perhaps the lowest LOD reported in literature (of 2.0 fg/mL, for direct coupling approach).[21] Further details about this study have been explored in *Subsection 6.3.3*.

Lastly, study 12 achieved a LOD comparable with study 10, while relying on an intriguing, innovative approach to norfentanyl sensing, which consisted of using variations of SWCNT@UiO-MOF composites with different pore sizes in order to detect norfentanyl based on size, while filtering other interfering substances.[22] Although study 12 did not achieve a very low LOD, when compared to other sensors in literature,[22] it paved the way towards a creative approach to opioids sensing. Further studies focused on further functionalization of composites, combined with advanced statistical methods and machine learning algorithms, could help enable SWCNT@UiO-MOF-based electrochemical sensors to detect norfentanyl and other substances, potentially with improved sensitivity.[22] Further details on this study have been previously explored in *Subsection 6.3.2*.

Overall, the studies reported in Table 16 demonstrate how the use of nanomaterials, such as SWCNTs, MOFs, and graphene, could help improve the sensitivity and selectivity of norfentanyl electrochemical sensors. These advancements suggest an encouraging future for the electrochemical sensing of FAMs, which could help tackling the opioids crisis.

7.0 Current Approaches and Future Directions of Electrochemical Sensing

To summarize the challenges of electrochemical sensing of FAMs, and to provide insights concerning recent breakthroughs and a framework towards future directions, Figure 15 is presented below, followed by column-specific subsections.

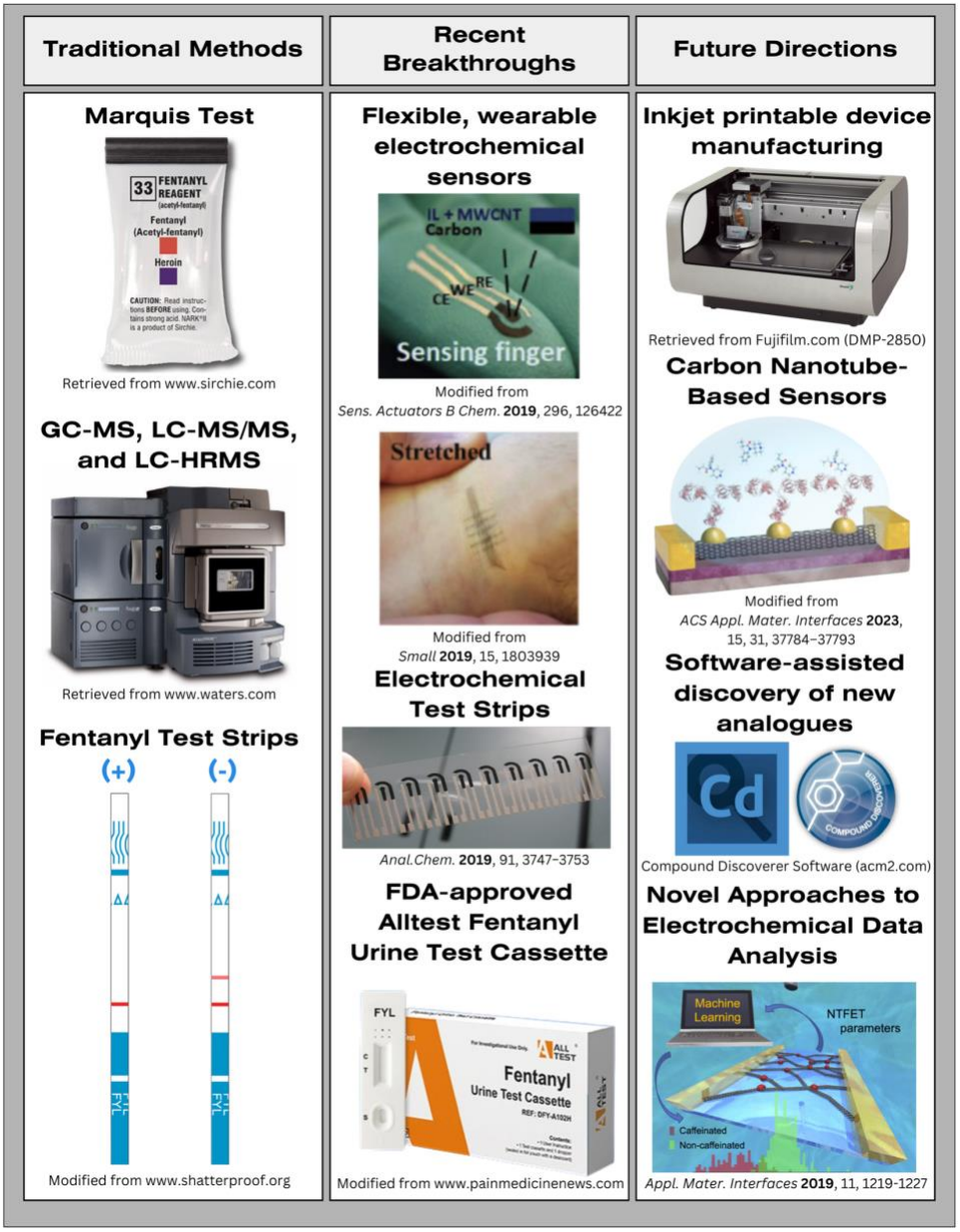

Figure 15. Traditional methods, recent breakthroughs, and future directions for the detection of FAMs. See Appendix F for Copyright Notes.

7.1 Traditional Methods: From Colorimetric Test Strips to Lab-Based Techniques

Traditional methods of detection of FAMs include previously mentioned traditional methods, divided into decentralized (*e.g.,* Marquis Test and fentanyl test strips) and centralized techniques (*e.g.,* GC-MS, LC-MS/MS, and LC-HRMS).[78] Although the decentralized techniques have several limitations, they are still useful to some extent in certain contexts and help establish a foundation for future research and developments. The Marquis Test has been widely used by law enforcement for prescreening, although this test has challenges, as false positive results may occur often due to the mixing of fentanyl with other drugs. Furthermore, the Marquis Test may not be able to detect fentanyl analogues, which may offer even more risk than fentanyl itself. Fentanyl test strips have been widely used by drug abuse and overdose prevention programs as a harm mitigation strategy, with strips being distributed to drug users so that they can test for the presence of fentanyl contamination in their drugs of choice, such as cocaine and heroin. These strips may yield false negative results (3.7%), putting users at risk, as well as false positive results (9.6%), which could lead to decrease in the public's trust over this harm prevention strategy.[170] Additionally, these strips may not be effective in detecting the fentanyl analogues, hence making users of other drugs vulnerable to potent and deadly FDDs.

7.2 Recent Breakthroughs: The Emergence of Versatile Sensors and The First FDA-Approved Fentanyl Urine Test Cassette

Recent breakthroughs include advancements in the conceptualization of flexible, wearable electrochemical sensors that could be printed onto different types of materials via lithography or inkjet printing. These provide flexibility and real-time collection and analysis of samples. Nonetheless, such sensors still present challenges concerning sensitivity, field reliability due to

environmental conditions, and large-scale manufacturing. Such sensors paved the way towards the emergence of electrochemical test strips, which have proven to be more reliable, sensitive, and selective than traditional fentanyl test strips. With the recent approval of the first FDA-approved over-the-counter test for the preliminary detection of "fentanyl" in urine, it could be relevant to investigate how sensing occurs, what the LOD, LOQ, and Ranges are, as well as whether the sensor is sensitive, selective, stable, and reliable. Further investigation on this sensor could help lay the foundation towards the development of accessible, easy-to-use, and inexpensive electrochemical sensors. By doing so, commercially available electrochemical sensors could help mitigate the number of overdose incidents related to fentanyl and its potent analogues.

7.3 Future Directions: From Advancements in Nanofabrication to Improved Data Approaches

Advancements in nanofabrication are relevant to ensure reliability of nanomaterial deposition and decoration of electrodes, hence consistency of measurements and high levels of sensitivity. Using inkjet printing technology for large-scale manufacturing of electrochemical sensors could be crucial in ensuring reproducibility of results, consistency of measures, sensitivity, selectivity, stability, and reliability. This could potentially be achieved by adopting statistical methods to ensure quality control standards for device manufacturing, such as statistical process control (*e.g.*, Laney P' Control Charts).

Additionally, further exploration into the use of CNTs and other nanomaterials to modify or decorate electrodes would be crucial. With the outstanding results reported in recent studies,[21]

understanding how to optimize sensors to become more sensitive and selective is extremely relevant. Bearing these advancements in mind, it is also relevant to consider how to translate these state-of-the-art sensing devices into feasible, inexpensive, and accessible devices. Improvements to nanofabrication as well as nanomaterials selection taking costs into consideration are paramount for ensuring future, large-scale manufacturing of these remarkable sensors.

Improved data analysis and processing approaches could become extremely relevant in the era of emerging fentanyl analogues. Recent studies have developed a technology capable of automatically detecting and identifying "unknown" fentanyl analogues, by combining HRMS analysis, followed by data processing and automated data interpretation with the Compound Discoverer software.[4] With the advent of machine learning, algorithms could be used to predict what future analogues could be like, enabling early detection and predictive analytics models to perform early risk analysis in order to protect the public.

Lastly, advancements to data analysis techniques in the field of electroanalytical chemistry could help amplify and filter electrochemical signals, ensuring sensitivity and reliability. Statistical approaches combined with computational methods could pave the way towards enhanced detection of a variety of substances (see Appendix G for example of application of Savitzky-Golay filter to filter noisy CV signals).

8.0 Conclusion

This thesis provided a comprehensive review of the US opioids crisis, with a focus on the different analytical techniques used for the detection of FAMs. While traditional, well-established techniques, such as gas and liquid chromatography are extremely relevant to understand and explore, this review emphasized the novel techniques that rely on electrochemical-based detection. Relying on recent discoveries, this work showed how cyclic voltammetry, differential pulse voltammetry, chronoamperometry, or field-effect transistors could be used to detect fentanyl and its analogues. The challenges concerning sensitivity and selectivity are explored by understanding how carbon nanotubes (CNTs) could be used to selectively enhance electrochemical responses. Although there are some challenges with collecting, using, and interpreting electrochemical data from biological samples, recent advancements in the fields of statistical process control, machine learning, predictive analytics, and data analysis could help pave the way towards rapid large-scale development of reliable, accurate, and fast electrochemical sensors capable of identifying and determining concentrations of FAMs. Ultimately, electrochemical methods explored throughout this work could help establish new paradigms in the detection of FAMs, which would hopefully lead to more effective ways to the tackle the ongoing opioids crisis, in the US and worldwide.

Appendix A List of Acronyms and Abbreviations

4-ANPP: N-phenethyl-4-piperidone
APAP: Acetaminophen
AsA: Ascorbic Acid
AuNPs: Gold Nanoparticles
BP: Binding Partner
BZ: Benzoylecgonine
CA (-C or -T): Capture Antibody (at the Control or Test Lines)
CDC: Centers for Disease Control and Prevention
CNT: Carbon Nanotubes
COF: Covalent Organic Framework
CV: Cyclic Voltammetry
DEP: Dielectrophoresis
DMF: Dimethylformamide
DPV: Differential Pulse Voltammetry
EDDP: 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EIS: Electrochemical Impedance Spectroscopy
EtOH: Ethanol
FAMs: Fentanyl, its Analogues, and Metabolites
FDA: Food and Drug Administration
FDDs: Fentanyl Designer Drugs
FET: Field-Effect Transistor
GC(-MS): Gas Chromatography (- Mass Spectrometry)
GCE: Glassy Carbon Electrode
Glu: Glucose
IA (-C or -T): Immobilized Antibody (at the Control or Test Lines)
IL: Ionic Liquid
ISF: Interstitial Fluid
LC: Liquid Chromatography
LC-HRMS: Liquid Chromatography with High Resolution Mass Spectrometry
LC-MS/MS: Liquid Chromatography with Tandem Mass Spectrometry
LOD: Limit of Detection
LOQ: Limit of Quantitation
MIP: Molecularly Imprinted Polymer
MOF: Metal-Organic Framework
$MS^{[2]}$: Tandem Mass Spectrometry
$MS^{[3]}$: Triple Quadruple Mass Spectrometry
MSLE: Mean Squared Logarithmic Error
MWCNT: Multi-Walled Carbon Nanotube
NH: Norhydrocodone
NM: Normorphine
PANI: Polyaniline
PB: Phosphate Buffer
PBASE: 1-Pyrenebutanoic Acid Succinimidyl Ester
PBS: Phosphate Buffer Solution
PEI: Polyethylenimine
SPE: Carbon Screen-Printed Electrode
SWCNT: Single-Walled Carbon Nanotube
SWV: Square-Wave Voltammetry
TMS: Trimethylsilyl
UA: Uric Acid
ZIF: Zeolitic Imidazolate Framework
g-C_3N_4: Graphitic Carbon Nitride
mAMP: Methamphetamine
rGO: Reduced Graphene Oxide
μOR: μ Opioid Receptor

Appendix B Worldwide Consumption of Drugs and The Rise of Fentanyl-Caused Cocaine and Heroin Overdoses

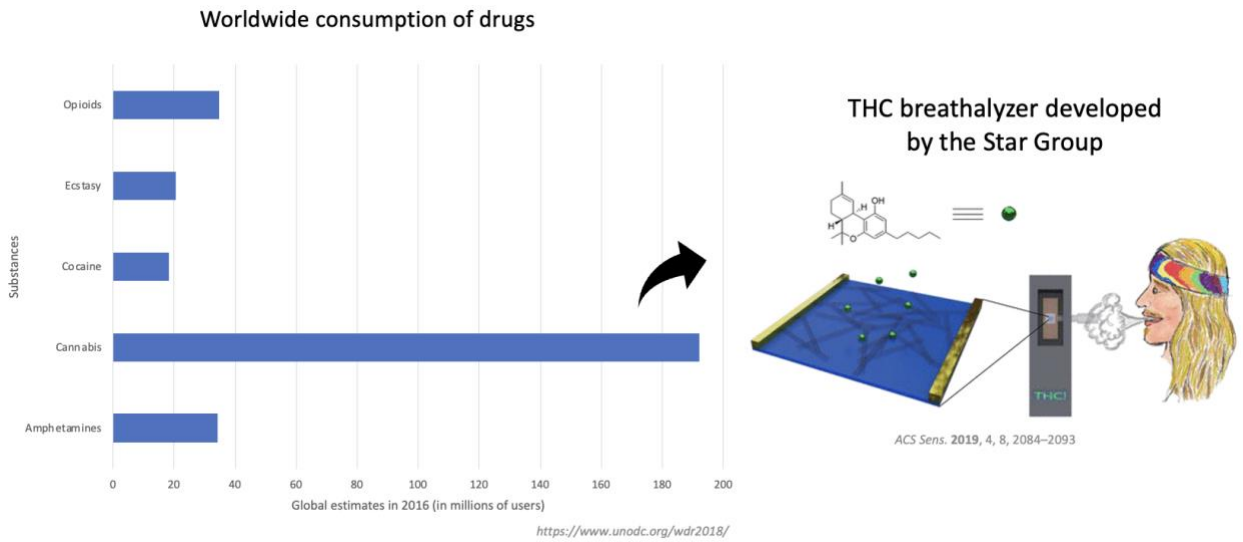

Figure 16. Cannabis is the most commonly consumed drug worldwide. Scientists from the Star Research Group have developed an electrochemical sensor to detect tetrahydrocannabinol (THC) vapors. Opioids are, together with amphetamines, the second most consumed drugs. Reprinted (no changes) with permission from *ACS Sens.* **2019**, 4, *8*, 2084-2093, Copyright 2019 American Chemical Society.

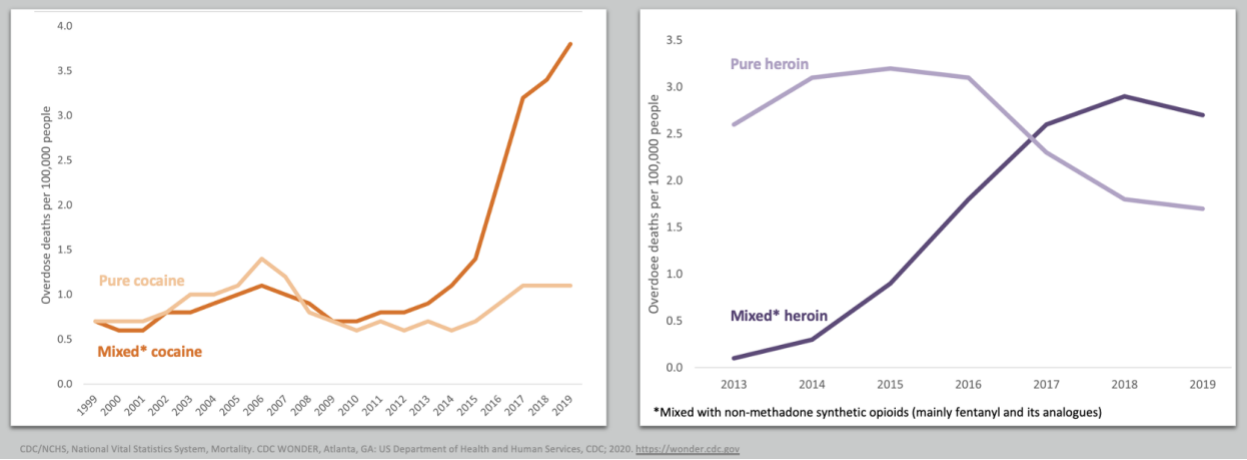

Figure 17. Overdose deaths involving consumption of pure cocaine has remained generally steady in the past two decades, whereas deaths due to pure heroin overdoses have decreased in the past few years. However, there has been a sharp increase in overdose deaths due to cocaine and heroin being mixed with fentanyl and its analogues.

Appendix C Chemical Structures of Fentanyl and some Analogues

The chemical structures below complement Table 1. The intent is to showcase the diversity of fentanyl analogues. Given the emergence of Fentanyl Designer Drugs (FDDs), there can be countless possible analogues, hence why the chemical structures presented below in no way exhaust the list of the growing number of fentanyl analogues.

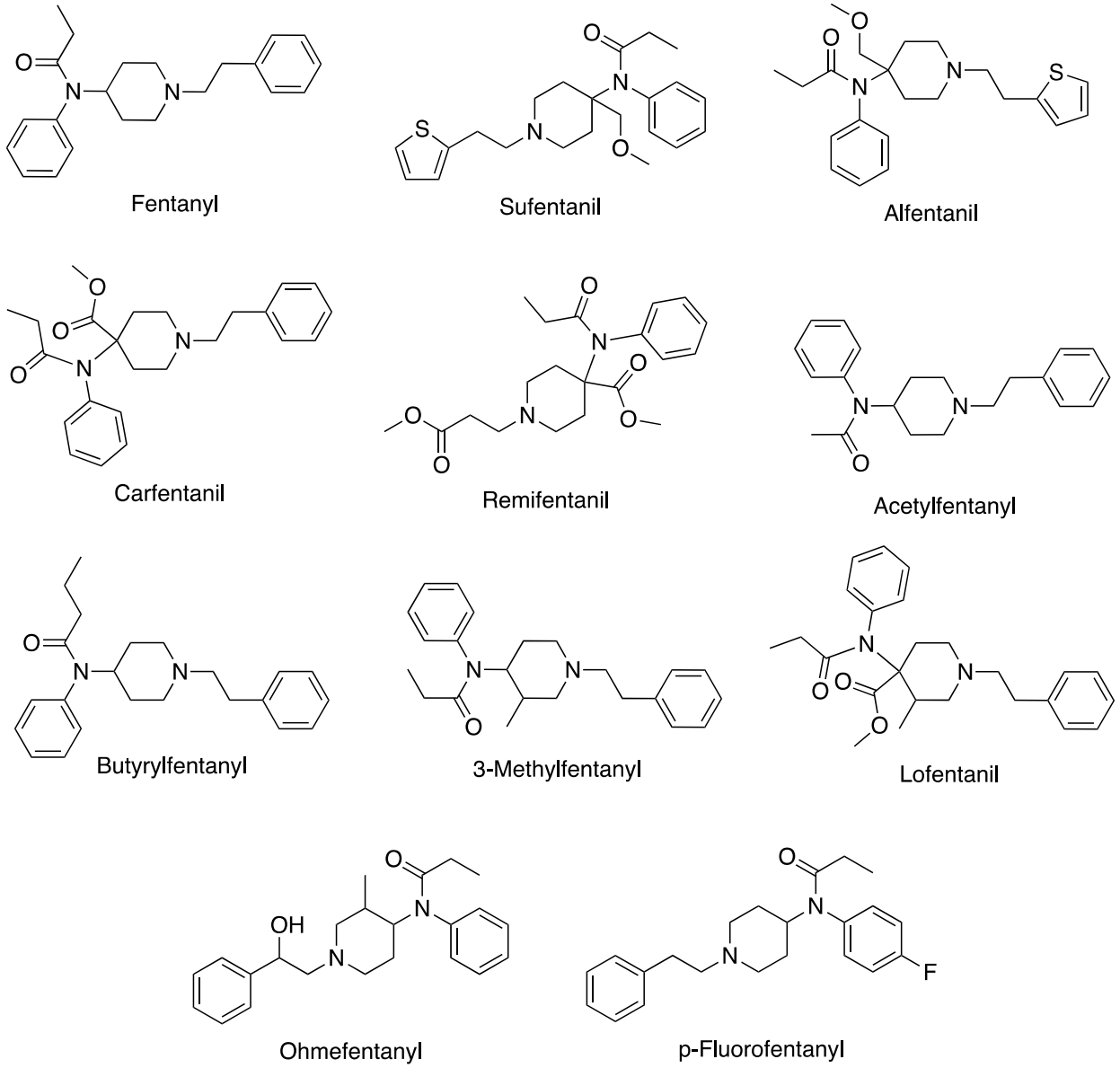

Figure 18. Chemical structures and common names of fentanyl and some of its known analogues.

Appendix D Description of Variables and Constants in Electrochemical Techniques useful for Sensing (shown in Table 10)

Amperometry

$I(t) = \frac{nFACD^{1/2}}{(\pi t)^{1/2}}$, such that:

- $I(t)$: current at time t after a step change in potential
- n: number of electrons transferred in the redox reaction per molecule of analyte
- F: Faraday's constant, ≈96485 C/mol
- A: electrode's surface area
- C: concentration of electroactive species involved in the redox reaction
- D: diffusion coefficient of the electroactive species
- t: time passed since the beginning of the electrochemical reaction (or reaction step).

Cyclic Voltammetry (CV)

$I_p = 0.4463nFAC\left(\frac{nFDv}{RT}\right)^{1/2}$, such that:

- I_p: peak current for CV experiment
- n, F, A, C, D: same as aforementioned
- v: scan rate of voltage
- R: universal gas constant
- T: temperature in Kelvin

Differential Pulse Voltammetry (DPV)

$I_p = \frac{nFAD^{2/3}v^{1/2}C\Delta {E_p}^{1/2}}{RT}$, such that:

- I_p: peak current for DPV experiment
- n, F, A, C, D, v, R, T: same as aforementioned
- ΔE_p: pulse amplitude in DPV

Square-Wave Voltammetry (SWV)

$I_p = \frac{2n^2F^2A\omega DC}{RT}$, such that:

- I_p: peak current for SWV experiment
- n, F, A, C, D, v, R, T: same as aforementioned
- ω: frequency of the square wave

Electrochemical Impedance Spectroscopy (EIS)

$Z = Z' + iZ''$, such that:

- Z: complex impedance
- Z': real part of the impedance (*i.e.*, resistive component)
- Z'': imaginary part of the impedance (*i.e.*, reactive component)

Field-Effect Transistors (FETs)

$I_D = \frac{1}{2}\mu C_{ox}\frac{W}{L}(V_{GS} - V_{th})^2$, such that:

- I_D: FET drain current, in the saturation region
- μ: mobility of the charge carriers in the semiconductor material
- C_{ox}: capacitance per unit area of the gate oxide
- W, L: width and length of semiconductor channel, respectively
- V_{GS}: voltage difference between the gate and source terminals
- V_{th}: FET threshold voltage

Appendix E Python Script for Log(Current) vs. Log(Time) Plots, Shown in Figure 10

Log(Current) vs. Log(Time) plots were created with varying parameters in order to demonstrate the effects of diffusion coefficient, concentration, number of electrodes, and electrode area onto current responses, in the context of amperometry. By changing the equations for current (*I*), it is possible to apply similar principles to other techniques as well.

```
"""
Author: Rodrigo Silva Ferreira
Project: MSc Thesis in Chemistry - University of Pittsburgh
Date: 04MAR2024
Purpose: Demonstrate how varying diffusion coefficients, concentrations, n values,
and electrode area
        affect Log(Current) vs. Log(Time) plots
"""
import numpy as np
import matplotlib.pyplot as plt

n = 1 # Fixed n (e-)
F = 96485.3329  # Faraday constant in C/mol
A = 1E-4  # Area of electrode in m^2 (= 1 cm²)
pi = np.pi # pi value
D_values = np.linspace(1E-10, 1E-9, 5)  # Varying diffusion coefficients in m^2/s
C_values = np.linspace(1E-3, 5E-3, 5)  # Varying concentrations in mol/m^3
D_fixed = 1e-9  # Fixed diffusion coefficient in m^2/s
C_fixed = 1e-3  # Fixed concentration in mol/m^3
n_values = [1, 2, 3, 4, 5] #Varying n values
A_values_cm2 = [1, 2, 5, 10, 50] #Varying Areas
A_values = [A * 1e-4 for A in A_values_cm2]  # Convert from cm² to m²
t = np.linspace(1, 1000, 1000)  # Time range in seconds

plt.figure(figsize=(14, 12)) # Create plot

# Plot 1: (a) Log(Ion Flux) vs. Log(Time) for Different Diffusion Coefficients
```

```
plt.subplot(2, 2, 1)
for D in D_values:
    I = (n * F * A * C_values[2] * np.sqrt(D)) / (np.sqrt(pi * t))
    plt.loglog(t, I, label=f'{D:.1e} m²/s')
plt.title('(a) Log(Current) vs. Log(Time) for Different Diffusion Coefficients')
plt.xlabel('Log(Time) (s)')
plt.ylabel('Log(Current) (A)')
plt.legend()

# Plot 2: (b) Log(Ion Flux) vs. Log(Time) for Different Concentrations
plt.subplot(2, 2, 2)
for C in C_values:
    I = (n * F * A * (C / 1000) * np.sqrt(D_values[2])) / (np.sqrt(pi * t))
# Convert C to mol/L
    plt.loglog(t, I, label=f'{C / 1000:.1e} M')  # Label in M
plt.title('(b) Log(Current) vs. Log(Time) for Different Concentrations')
plt.xlabel('Log(Time) (s)')
plt.ylabel('Log(Current) (A)')
plt.legend()

# Plot 3: (c) Log(Ion Flux) vs. Log(Time) for Different n Values
plt.subplot(2, 2, 3)
for n in n_values:
    I = (n * F * A_values[0] * C_fixed * np.sqrt(D_fixed)) / (np.sqrt(pi * t))
    plt.loglog(t, I, label=f'{n} e-')
plt.title('(c) Log(Current) vs. Log(Time) for Different n Values')
plt.xlabel('Log(Time) (s)')
plt.ylabel('Log(Current) (A)')
plt.legend()

# Plot 4: (d) Log(Ion Flux) vs. Log(Time) for Different Electrode Areas
plt.subplot(2, 2, 4)
for A, A_cm2 in zip(A_values, A_values_cm2):
    I = (n_values[0] * F * A * C_fixed * np.sqrt(D_fixed)) / (np.sqrt(pi * t))
    plt.loglog(t, I, label=f'{A_cm2} cm²')
plt.title('(d) Log(Current) vs. Log(Time) for Different Electrode Areas')
plt.xlabel('Log(Time) (s)')
```

```
plt.ylabel('Log(Current) (A)')
plt.legend()
plt.tight_layout()
plt.savefig('CurrentVSTime.jpeg', dpi=300) # Save as .JPEG
```

Appendix F Copyright Notes on Figure 15

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Appendix G Data Processing of Noisy Signals using Savitzky-Golay Filter, including Plots Generated, MSLE outputs, Resistance to Noise Analysis, and Python Scripts

Original CV data was obtained from publicly available dataset with CV results containing Fc^{+}/Fc redox peaks. Dataset was retrieved from Copley, G.; Gibson, E. Cyclic Voltammetry of a Cobaloxime Catalyst raw data. *Newcastle University*: **2019** (licensed under CC BY 4.0). Noisy CV data was obtained by applying pseudorandom noise to the original CV data, whereas the filtered CV data was obtained by applying the Savitzky-Golay filter to the noisy data, without knowledge or influence of the original data.

Four different models were applied, such that the first two had low window sizes (*i.e.*, 31) and low and high polynomial orders (*i.e.*, 1 and 5) and the last two had high window sizes (*i.e.*, 101) and low and high polynomial orders (*i.e.*, 1 and 5). In further analyses, it could be relevant to scan dozens or even hundreds of combinations of window sizes and polynomial orders, and to perform automated statistical analysis for each in order to determine which model (*i.e.*, window size, polynomial order) would yield best results.

The levels of noise applied, in order, were 0, 10, 30, 50, 70, 90, and 100%. Mean Squared Logarithmic Error (MSLE) were reported in order to determine the error due to deviation of the filtered data from the original CV data, in the peak region (*i.e.*, between -0.5 and 0.2 V). See Figures 19-25 below, displaying the original CV data, noisy data, and filtered data for each of the four different models, at different levels of noise. Besides the fit to the curves, notice that the MSLE values are reported.

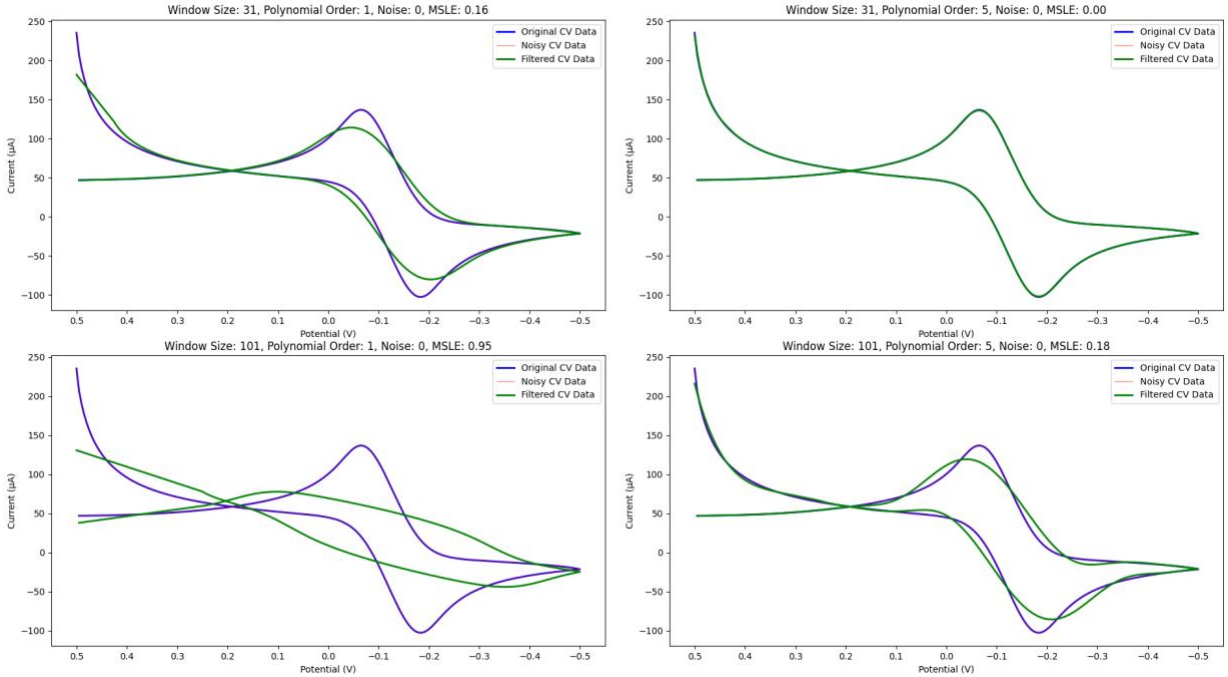

Figure 19. Application of Savitzky-Golay filter with no noise (control), with four different models (window size, polynomial order)

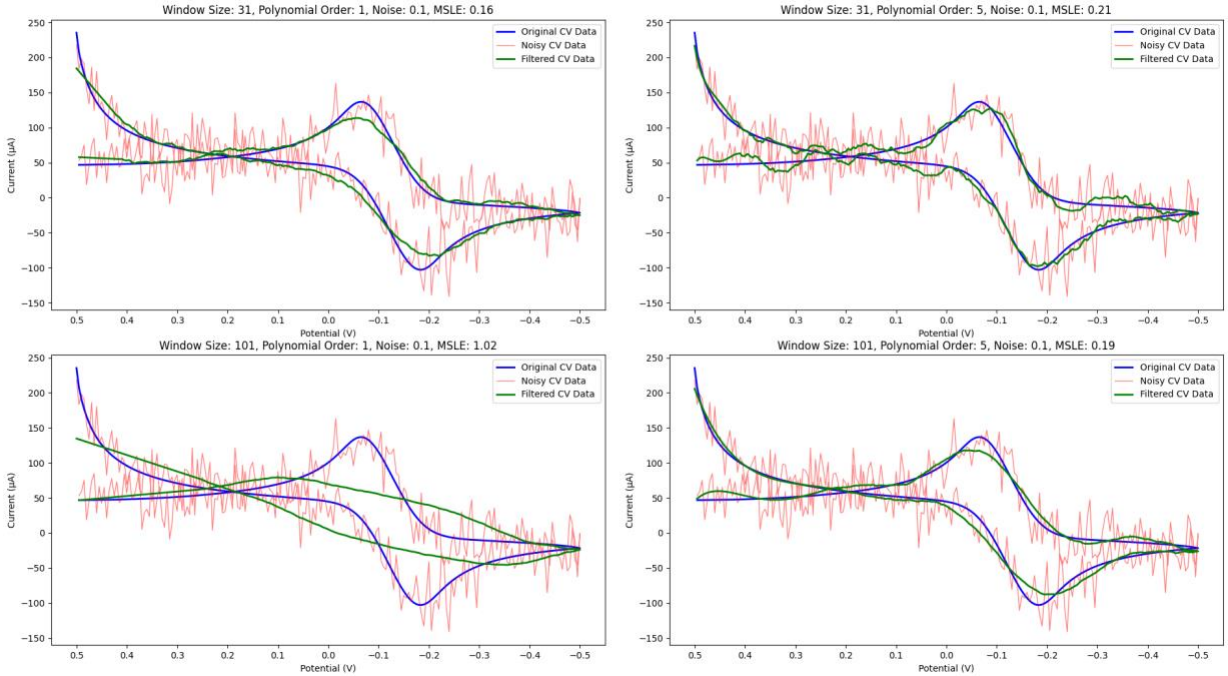

Figure 20. Application of Savitzky-Golay filter with 10% random noise, with four different models (window size, polynomial order)

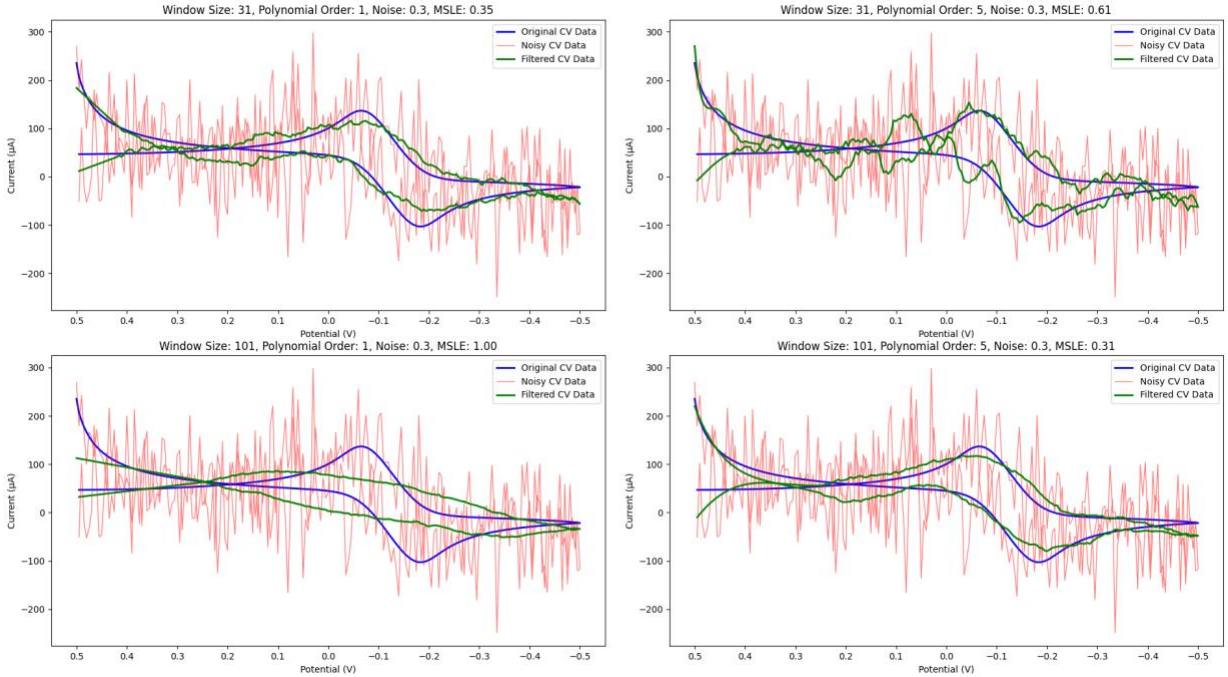

Figure 21. Application of Savitzky-Golay filter with 30% random noise, with four different models (window size, polynomial order).

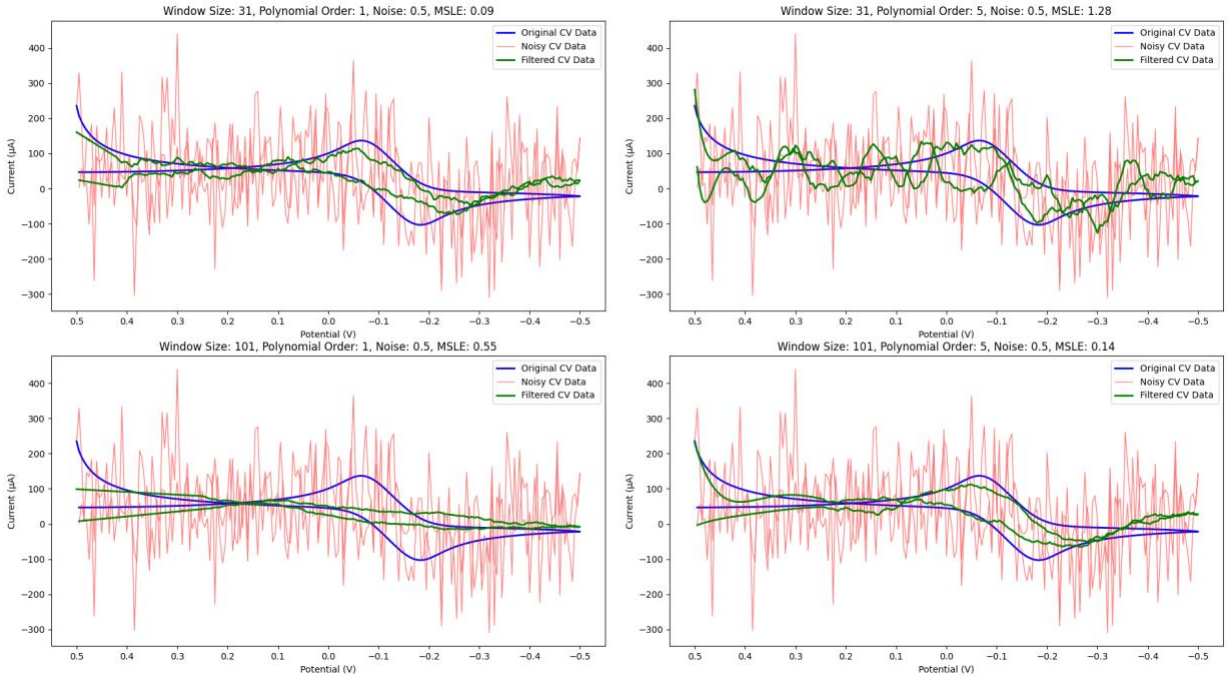

Figure 22. Application of Savitzky-Golay filter with 50% random noise, with four different models (window size, polynomial order).

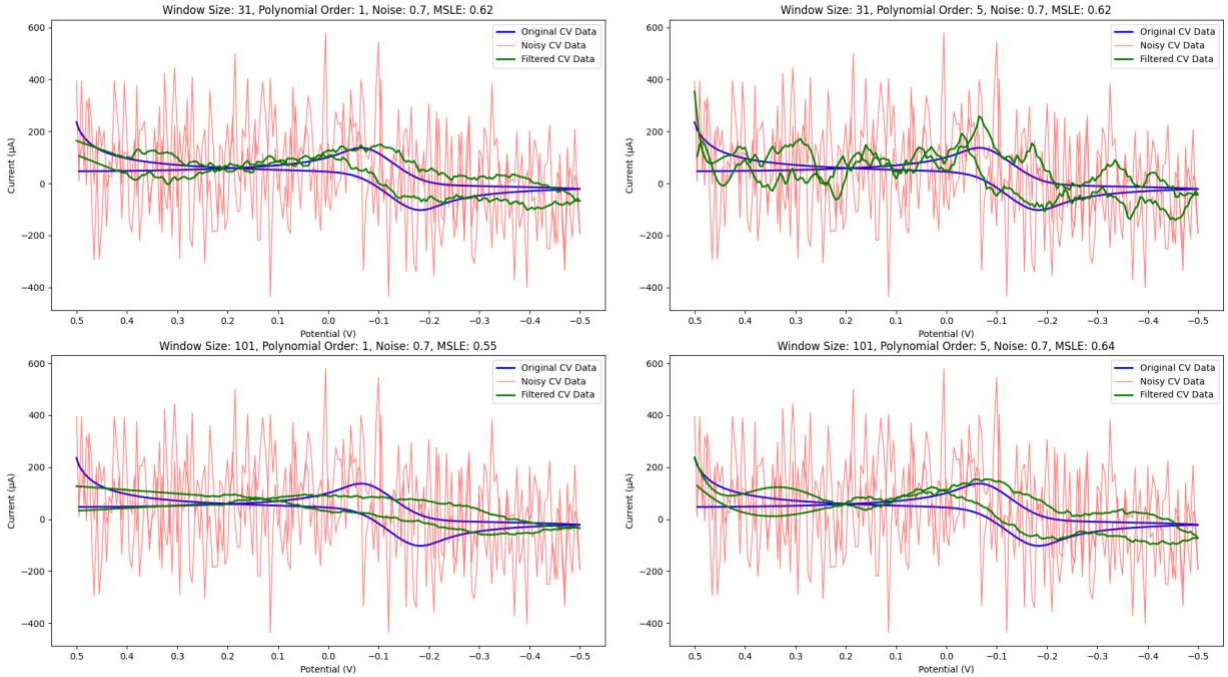

Figure 23. Application of Savitzky-Golay filter with 70% noise, with four different models (window size, polynomial order).

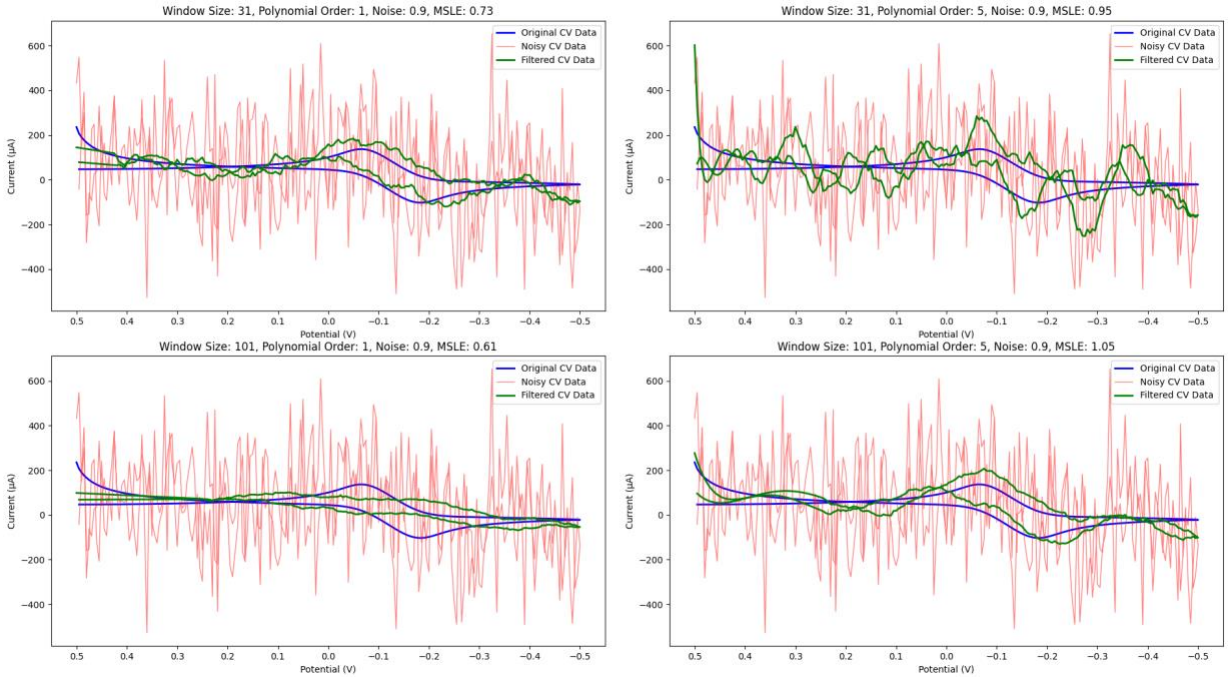

Figure 24. Application of Savitzky-Golay filter with 90% random noise, with four different models (window size, polynomial order).

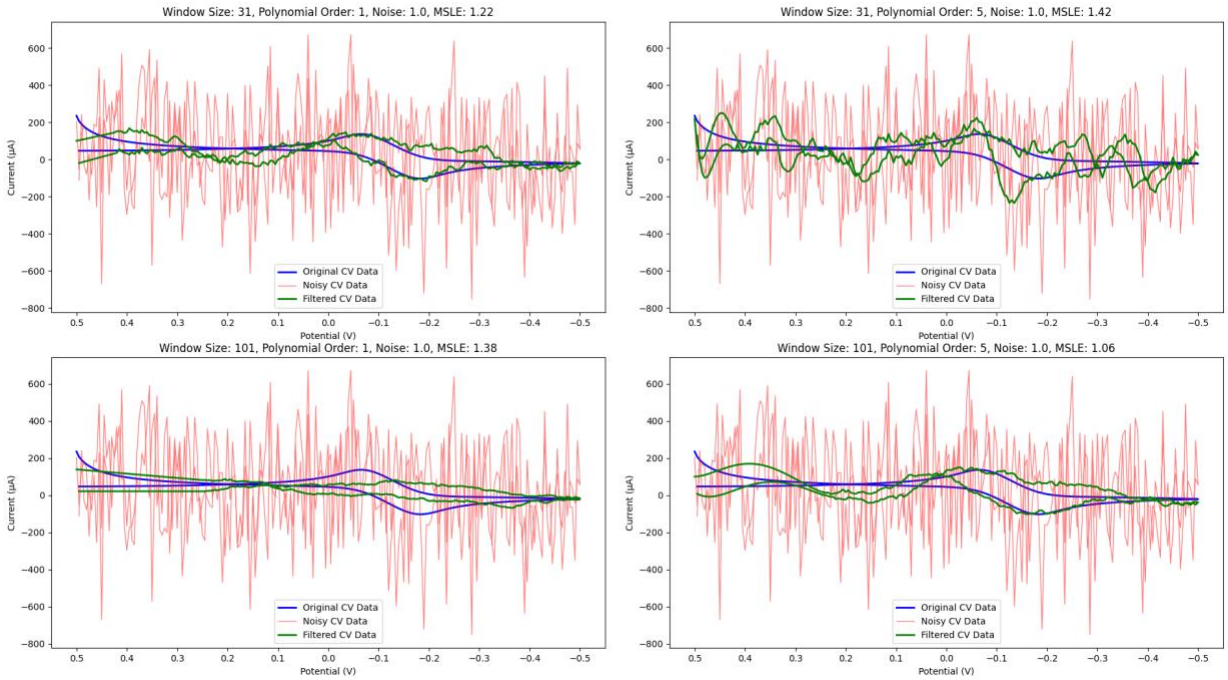

Figure 25. Application of Savitzky-Golay filter with 100% random noise, with four different models (window size, polynomial order).

Based on the results of Figures 19-25, table 17 shows the performance of the four different models at different noise levels, along with the MSLE values. Notice that, for each noise level, the best result is the one with the lowest MSLE.

Table 17. Noise levels, models, and MSLE values ($^{\perp}$ being the best for each noise level)

Noise	**Model**		**MSLE**
	Window Size	*Polynomial Order*	
0.0	31	1	0.16
	31	5	$0.00^{\perp}$
	101	1	0.95
	101	5	0.18
0.1	31	1	$0.16^{\perp}$
	31	5	0.21
	101	1	1.02
	101	5	0.19
0.3	31	1	0.35
	31	5	0.61
	101	1	1.00
	101	5	$0.31^{\perp}$
0.5	31	1	$0.09^{\perp}$
	31	5	1.28
	101	1	0.55
	101	5	0.14
0.7	31	1	0.62
	31	5	0.62
	101	1	$0.55^{\perp}$
	101	5	0.64
0.9	31	1	0.73
	31	5	0.95
	101	1	$0.61^{\perp}$
	101	5	1.05
1.0	31	1	1.22
	31	5	1.42
	101	1	1.38
	101	5	$1.06^{\perp}$

It is worth mentioning that best results at different noise levels varied, with different combinations of window sizes and polynomial orders displaying varying performances. In future works, it could be relevant to analyze more combinations of window sizes and polynomial orders, in order to determine whether other combinations of these variables could have yielded more consistent and improved results.

To further investigate the ability of each model to perform across noise levels, table 18 shows the four different models, along with the average MSLE values across noise levels of 10,

30, 50, 70, and 90%. Noise levels of 0 and 100% were not included in this analysis due to them being only useful as controls. Herein, resistance to noise is defined as yielding lower MSLE values, which are reported together with the corresponding standard deviations.

Table 18. Resistance to noise analysis was performed for each model.

Model (*Window Size, Polynomial Order*)	**Average MSLE**	**Standard Deviation**
31, 1	0.476	0.379
31, 5	0.727	0.486
101, 1	0.886	0.287
101, 5	0.510	0.378

To analyze whether differences in average MSLE values across models are statistically significant, average MSLE values along with the corresponding standard deviations (as error bars) were plotted in Figure 26.

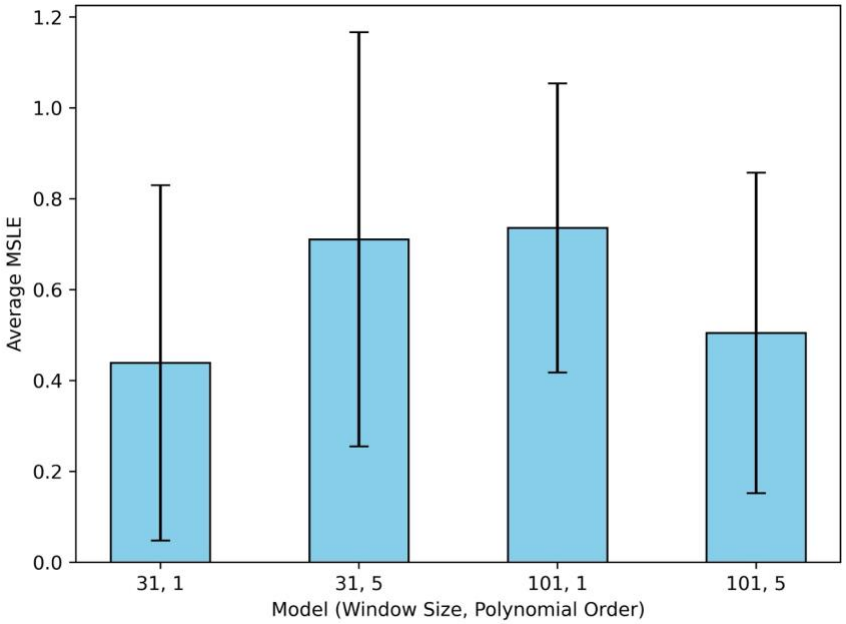

Figure 26. Bar chart of average MSLE values for each model, with error bars. Differences were not statistically significant. But some models were effective in reconstituting the CV signals.

Although models 31,1 and 101,5 seem to be the most resistance to noise in regard to performance towards filtering the noise, performing statistical analysis is import to determine whether the differences above are statistically significant. When ANOVA tests are performed

between the results, the *p*-value of 0.281 (>0.05) suggests that there is not enough evidence to suggest that the differences are statistically significant. Therefore, further studies including varying combinations of window sizes and polynomial orders could be relevant to find better suitable models. Additionally, further exploration of other statistical methods and theories could be relevant in aiding signal processing and analysis.

Python scripts below were used for plotting the bar charts in Figure 26 and for plotting the original CV data, noisy data, and filtered data across different noise levels and models, as displayed in figures 19-25, respectively.

```
"""
Author: Rodrigo Silva Ferreira
Project: MSc Thesis in Chemistry - University of Pittsburgh
Date: 17MAR2024
Purpose: Plot bar charts with error to compare resistance to noise of the four different models,
        based on the average MSLE values of each model.
"""

import matplotlib.pyplot as plt
import numpy as np
models = ['31, 1', '31, 5', '101, 1', '101, 5']
avg_msle = [0.439, 0.711, 0.736, 0.505]
std_dev = [0.391, 0.456, 0.318, 0.353]
positions = np.arange(len(models))
width = 0.5
fig, ax = plt.subplots()
bars = ax.bar(positions, avg_msle, width, yerr=std_dev, capsize=5, color='skyblue', edgecolor='black')
ax.set_xlabel('Model (Window Size, Polynomial Order)')
ax.set_ylabel('Average MSLE')
ax.set_xticks(positions)
ax.set_xticklabels(models)
plt.tight_layout()
plt.savefig('Resistance to Noise - Bar Charts with Errors.jpeg', dpi=300) # Save as .JPEG
```

```
"""
Author: Rodrigo Silva Ferreira
Project: MSc Thesis in Chemistry - University of Pittsburgh
Date: 21FEB2024
Purpose: Demonstrate how data analysis techniques can be helpful to optimize CV signals
Method:
Plot CV data: original data from Fc/Fc+, noisy data, and filtered data using Savitzky-Golay filter
Expectation: Four plots containing original CV data, noisy data, and filtered data
with four combinations of window sizes and polynomial orders.
"""

# Step 1: Import the libraries
import pandas as pd
import numpy as np
import matplotlib.pyplot as plt
from scipy.signal import savgol_filter
import math

# Step 2: Load data from Excel file and extract potentials and currents from columns
file_path = '/Users/ros114/Downloads/8269661/fc_fc+.xlsx'
data = pd.read_excel(file_path)
E = data['E /V']
I = data['I /uA']

# Step 3: Define four different combinations for window sizes and polynomial orders.
combinations = [
    (31, 1),
    (31, 5),
    (101, 1),
    (101, 5),
]

# Step 4: Add varying levels of random noise to the original data.
noise_levels = [0, 0.1, 0.3, 0.5, 0.7, 0.9, 1.0]
```

```
for noise in noise_levels:
    msle_results = []
    I_noisy = I + np.random.normal(0, max(I) * noise, size=I.shape)  # Calculate max_current within the loop

    # Step 5: Prepare plots 2x2 with the respective sizes.
    fig, axs = plt.subplots(2, 2, figsize=(16, 12))
    axs = axs.flatten()

    # Step 6: Apply the Savitzky-Golay filter and plot them.
    for i, (window_length, poly_order) in enumerate(combinations):
        I_restored = savgol_filter(I_noisy, window_length, poly_order)

        # Step 7: Define data within specified potential range of the observed peak (-0.5 to 0.2V)
        valid_indices = (E >= -0.5) & (E <= 0.2)
        E_valid = E[valid_indices]
        I_original_valid = I[valid_indices]
        I_restored_valid = I_restored[valid_indices]

        # Step 8: Calculate mean squared logarithmic error (MSLE) of the results
        msle = np.mean((np.log(I_original_valid + 1) - np.log(I_restored_valid + 1)) ** 2)
        msle_results.append((window_length, poly_order, msle))

        # Step 9: Define parameters for plots
        axs[i].plot(E, I, label='Original CV Data', color='blue', linewidth=2)
        axs[i].plot(E, I_noisy, label='Noisy CV Data', color='red', linewidth=1, alpha=0.5)
        axs[i].plot(E, I_restored, label='Filtered CV Data', color='green', linewidth=2)
        axs[i].set_title(f"Window Size: {window_length}, Polynomial Order: {poly_order}, Noise: {noise}, MSLE:
{msle:.2f}")
        axs[i].set_xlabel('Potential (V)')
        axs[i].set_ylabel('Current (μA)')
        axs[i].legend()
        axs[i].invert_xaxis()
        axs[i].set_xticks(np.arange(E.max(), E.min() - 0.1, -0.1))

    # Step 10: Print results in the terminal
    print(f"Results for Noise Level: {noise}")
    for window_length, poly_order, msle in msle_results:
```

```
    print(f"Window Size: {window_length}, Polynomial Order: {poly_order}, Noise: {noise}, MSLE: {msle:.2f}")

# Step 11: Create plots and display them
plt.tight_layout()
plt.show()
```

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